Journal of Experimental Botany, Vol. 63, No. 7, pp. 2411–2420, 2012
doi:10.1093/jxb/err359 Advance Access publication 16 January, 2012

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

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

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Received 22 May 2011; Revised 25 August 2011; Accepted 18 October 2011

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 Mn^{2+} and H_2O_2 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 Mn^{2+}/H_2O_2 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 Mn^{2+}, 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 Mn^{II} (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-Chiristoffers *et al.*, 2007). The brown spots, located primarily in the apoplast of epidermal cells, consist of oxidized Mn (Mn^{IV}) 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 H_2O_2-producing enzymes using NADH as electron donor (also termed NADH-oxidases), and also as H_2O_2-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 Mn^{2+} and phenols by H_2O_2-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 H_2O_2 (Halliwell, 1978) has recently been confirmed by the enhanced NADH-oxidase activity in the leaf apoplast of cowpea grown with excess Mn (Fecht-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 Bachi, 1995; Lindon and Teixeira, 2000). The exact mechanism of catalytic scavenging of the superoxide anion radical ($O_2^-\cdot$) and H_2O_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 $O_2^-\cdot$ and hydroxyl radicals (·OH) (Halliwell, 1977; Stadtman *et al.*, 1990; Sakihama *et al.*, 2002). Production of ·OH in the mixture H_2O_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.*, 2002*a*, *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.*, 2002*a*, *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_2SO_4, 0.1 KCl, 2.0 $Ca(NO_3)_2$, 0.5 $MgSO_4$, 0.1 KH_2PO_4, and (in μM): 10 H_3BO_3, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$. Iron was supplied as Fe^{III}EDDHA at 20 μM. After 7 d pre-culture at optimal Mn concentration (0.5 μM), plants were subjected to 0.5 μM and 100 μM Mn, respectively, for 2 weeks. Concomitantly, one half of the plants were supplied with 1.5 mM Si as $Si(OH)_4$ prepared by passing Na_2SiO_3 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 μmol 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 descibed 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 symplastic 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

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 Römheld, 2002). The pellet was subsequently washed three times with 1% (w/w) 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 Römheld (2003) and the CEC was determined by incubating fresh cell wall material (~1 g) in 10 ml of 50 mM $BaCl_2$ (pH 5.0) for 30 min with stirring at 4 °C. After centrifugation at 2000 *g*, the pellet was resuspended in a fresh $BaCl_2$ 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 HNO_3+2 ml of H_2O_2, 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 Ba^{2+}. No other cations (with the exception of Mn in high Mn treatment) were detected in the cell wall material after $BaCl_2$ extraction.

Determination of Mn

Collected apoplastic fluid and supernatants (water extracts and SDS extracts) were evaporated and digested to dryness in 5 M HNO_3. 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 HNO_3. 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 HNO_3. In all samples, Mn was determined by ICP-OES after dissolving the dry residues in 0.5 M HNO_3.

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 HNO_3+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) H_3BO_3. Si was determined by ICP-OES after a final dilution of the samples of either 1:100 (v/v) for cell wall material or 1:10 (v/v) for LAF.

Electron paramagnetic resonance (EPR) determination of Mn^{2+}

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) Mn^{2+} symmetrically coordinated by six water molecules $[Mn(H_2O)_6]$ (see Figs 2 and 3). This signal is superimposed on the broad signal of Mn^{II} 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 (Mn^{2+}) and bound Mn was performed by spectral simulations using the program WINEPR 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 H_2O_2. One unit of POD activity was defined as the amount of enzyme which catalyses the conversion of 1 µmol of H_2O_2 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 H_2O_2] was 0.1 µM. The order of adding 0.5 mM $Si(OH)_4$, as an unspecific 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 Multiphor 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 H_2O_2 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 results (Table 2) show that in the high Mn treatment (100 μM), the concentration of water-extractable (soluble) Mn expressed on a leaf dry weight basis increased ~10-fold compared with the control. Additionally, the relative proportion of the soluble Mn fraction decreased from ~50% (control) to up to 25% (high Mn) of the total leaf Mn. The concentration of free Mn in the LAF also increased dramatically at high Mn supply (Fig. 1A). The concentration of protein-bound Mn and its relative proportion was lower in comparison with the water-extractable Mn fraction in both treatments, but also increased in response to elevated Mn supply. While the concentration of Mn in the cell wall-bound fraction increased markedly in the high Mn treatment (~40-fold), the relative proportion of Mn in this fraction increased slightly, from 38% (control) to 65% (high Mn). Si application did not affect water-soluble, protein-, and wall-bound Mn fractions in the control treatment (0.5 μM), but significantly decreased free (LAF; Fig. 1A) and weakly bound (water-extractable) apoplastic and symplastic (protein-bound) Mn fractions in the high Mn treatment (Table 2). On the other hand, the cell wall-bound Mn significantly increased in the leaves of Si-treated plants grown on excess Mn (Table 2). For instance, in the high Mn treatment, the relative proportion of wall-bound Mn increased from 65% (–Si plants) to 88% (+Si plants). Supply of Si to roots did not change the CEC of the cell wall material obtained from cucumber leaves in either the adequate (0.5 μM) or the high (100 μM) Mn-treated plants (Table 3). On the other hand, the non-exchangeable Mn fraction (residue after $BaCl_2$ extraction) was significantly higher in the cell wall of Si-treated plants grown with a high Mn supply. With an Mn supply of 0.5 μM, however, regardless of Si treatment, no Mn residue was detected in the leaf cell wall material. Here it is important to note that the Mn treatments did not affect the concentration of Si determined in the freshly obtained LAF nor did it affect the cell wall Si concentration (Table 4).

The effect of Si nutrition on the compartmentation of leaf Mn was also verified by the less destructive EPR determination. The most prominent feature of the EPR

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).

Mn treatment	Si supply	Symptoms of Mn toxicity	Biomass (mg DW per plant)		Leaf Mn concentration (μmol g DW^{-1})
			Root	Shoot	
0.5 μM	–	0	62±10 ^{b}	220±10 ^{b}	0.8±0.2 ^{a}
	+	0	79±5 ^{c}	240±13 ^{c}	1.0±0.2 ^{a}
100 μM	–	1	40±2 ^{a}	73±12 ^{a}	20.5±0.8 ^{b}
	+	0	64±7 ^{b}	200±15 ^{b}	24.5±1.9 ^{c}

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

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.

Mn treatment	Si supply	Water extractable		Protein bound		Cell wall bound	
		(μmol g DW^{-1})	(%)	(μmol g DW^{-1})	(%)	(μmol g DW^{-1})	(%)
0.5 μM	–	0.38±0.01 ^{a}	56	0.04±0.00 ^{a}	5	0.26±0.01 ^{a}	38
	+	0.35±0.02 ^{a}	43	0.05±0.00 ^{a}	6	0.39±0.02 ^{b}	49
100 μM	–	4.98±0.08 ^{c}	23	2.20±0.04 ^{c}	10	13.65±0.25 ^{c}	65
	+	2.20±0.04 ^{b}	9	0.49±0.01 ^{b}	2	21.78±0.48 ^{d}	88

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

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 Ba^{2+} (50 mM). Non-exchangeable Mn (strongly wall-bound fraction) represents Mn remaining in the cell wall material after $BaCl_2$ extraction.

Mn supply	Si supply	CEC (μeq g DW^{-1})	Non-exchangeable Mn (μmol g DW^{-1})
0.5 μM	–	467±21 [a]	ND
	+	468±10 [a]	ND
100 μM	–	468±11 [a]	619±12 [a]
	+	469±15 [a]	968±24 [b]

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.

Mn treatment	Si supply	Si concentration LAF (mM)	Cell wall (mmol g DW^{-1})
0.5 μM	–	0.03±0.01 [a]	0.39±0.04 [a]
	+	0.51±0.13 [b]	3.97±0.17 [b]
100 μM	–	0.04±0.02 [a]	0.47±0.08 [a]
	+	0.48±0.26 [b]	3.54±0.54 [b]

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

spectrum shown in Fig. 2 is a very sharp signal centred at g=2.003, which is frequently found in dry plant material and can be attributed to the delocalized electron in polycondensed benzene rings within cell walls (Todorović *et al.*, 2008). In the intact leaves, this signal may also arise from stable free radicals associated with photosystem II (Jücker *et al.*, 1999). However, this central signal is less prominent in the LAF samples (Fig. 3). In both cases, its amplitude was unchanged irrespective of the Mn treatments. Pertinent to this study is the EPR spectrum of a high spin Mn^{2+} ($3d^5$, S=5/2) which shows the characteristic six hyperfine lines (Figs 1, 2); the intensity of this signal was found to correlate with Mn treatments and total leaf and LAF concentrations of Mn (Table 1; Fig. 1A). The EPR signal arising from a high spin Mn^{2+} ion does not interfere with the inconsequential central signal (Jücker *et al.*, 1999). Application of Si slightly decreased the EPR signal of free Mn^{2+} from the apoplastic fluid and bulk leaves of plants subjected to high Mn (Figs 2, 3). Indeed, the results of the relative proportions of free (Mn^{2+}) and bound Mn (Table 5), obtained by simulation of the EPR spectra from Fig. 2, are in agreement with the results of fractionated extraction of Mn (Table 2).

Compared with the control (0.5 μM), excess Mn treatment (100 μM) significantly increased the Mn concentration in the LAF (Fig. 1A). Although application of Si reduced

Table 5. Effect of Si supply to roots on the relative proportion of free (Mn^{2+}) and bound forms of Mn in the bulk cucumber leaves obtained by simulation of the EPR spectra from Fig. 2.

Mn treatments	Si supply	Relative proportion of Mn (%)	
		Free	Bound
0.5 μM	–	31	69
	+	30	70
100 μM	–	15	85
	+	8	92

the LAF Mn concentrations under both treatments, a significant decrease was found only in the high Mn treatment. The concentration of H_2O_2 in the LAF showed the same pattern but was significantly increased when the supply of external Mn was excessive, while the H_2O_2 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). IEF confirmed that the higher overall POD activity with excess Mn resulted from the accumulation of the same isoforms present with the adequate Mn supply (0.5 μM), rather than from the synthesis of the new PODs. However, among cationic (basic) POD isoenzymes identified at pIs 8.2, 8.6, 8.8, and 9.3, the last two were not present with Si treatment (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 $O_2^{\cdot-}$ 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

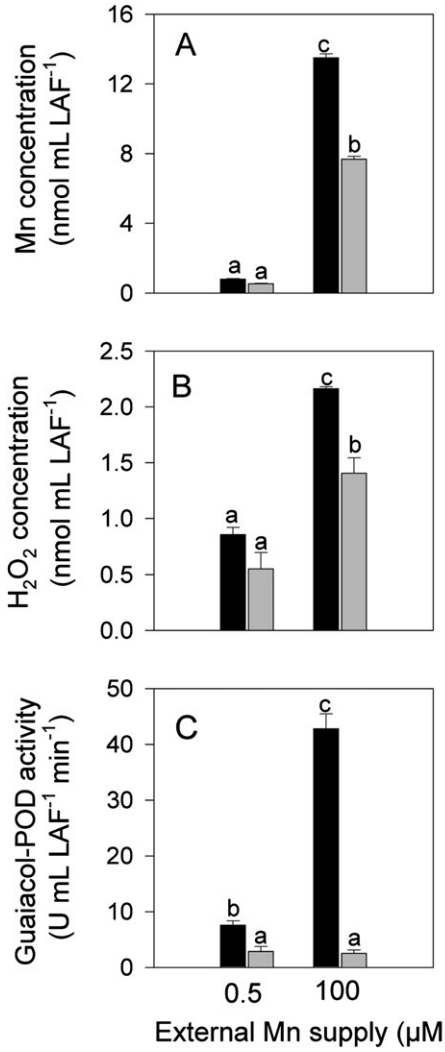

Fig. 1. Effect of Si treatments (–Si, black bars; +Si, grey bars) on the concentrations of Mn (A) and H_2O_2 (B), and the total activity of guaicaol-POD (C) in the leaf apoplastic fluid (LAF) from cucumber plants subjected to adequate (0.5 μM) and excessive (100 μM) external concentrations of Mn. If 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.

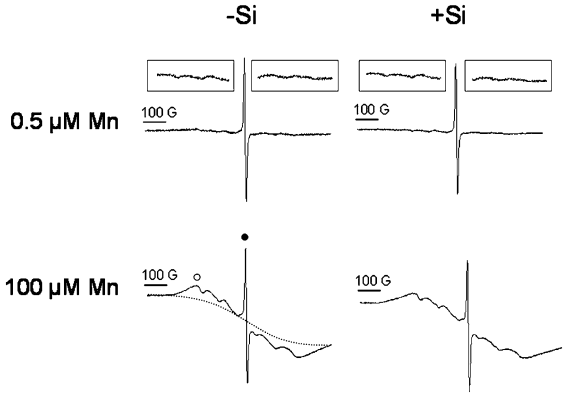

Fig. 2. EPR spectra of Mn^{2+} from the leaves of cucumber plants grown in nutrient solutions with adequate (0.5 μM) and high (100 μM) Mn concentrations, without (–Si) or with (+Si) supply of 1.5 mM Si. Inset: magnified regions of EPR spectra indicating the low amplitude Mn^{2+} signal at an Mn supply 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 Mn^{2+}; the dashed line represents the broad signal from Mn^{II} bound to the proteins and cell wall macromolecules.

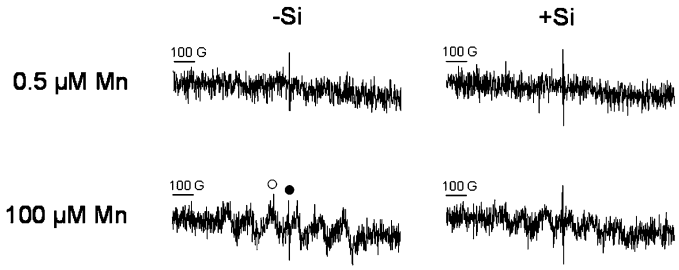

Fig. 3. EPR spectra of Mn^{2+} from the LAF of cucumber plants. The experimental conditions are indicated in the legend of Fig. 2. The filled circle indicates the inconsequential central signal; the open circle indicates the first of the characteristic six hyperfine lines of aqueous Mn^{2+}.

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 Mn^{2+} and monomeric Si in the LAF (see Fig. 1A; Table 4). As shown in Fig. 7, increasing the concentration of Mn^{2+} 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 Mn^{2+} 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 promising tool for rapid and relatively non-destructive determination of free Mn^{2+} in biological materials including plant

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_2O_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_2O_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

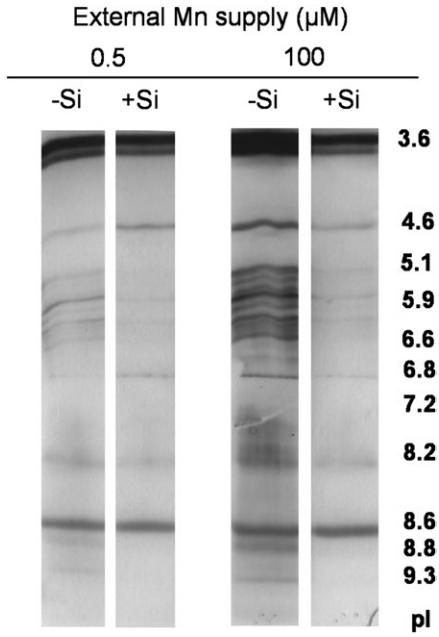

Fig. 4. Separation of the POD isoforms from the LAF by IEF. PODs were detected by staining with 9.2 mM guiacol and 5 mM H_2O_2. The cucumber plants were grown in nutrient solutions with normal (0.5 μM) and high (100 μM) supply of Mn, with or without 1.5 mM $Si(OH)_4$.

(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_2O_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^{II} (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_2O_2 in the leaf apoplast (Fig. 1B; see also Führs *et al.*, 2009). However, compartmentation and the accurate quantification of the apoplastic H_2O_2 concentration is still a matter of debate, because of the rapid diffusion of H_2O_2 across membranes. It has been demonstrated that Si application prevents the accumulation of free phenols in cucumber apoplast (Dragišić 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.*, 2002*b*) 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_2O_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_2O_2 (Fig. 1A, B). Hence, the Fenton reaction can be a source of ·OH not only generated from the *in vitro* H_2O_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:

$$Mn^{2+} + H_2O_2 \rightarrow Mn^{3+} + OH^- + \cdot OH$$

Since POD may be involved in the transformation of H_2O_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^{3+}, 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_2O_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^-) 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 ($H_4SiO_4^0$), 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 $(SiO_2)_n \times nH_2O$, have been experimentally confirmed (Inanaga *et al.*, 1995; Iwasaki *et al.* 2002*b*; 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^{II} (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

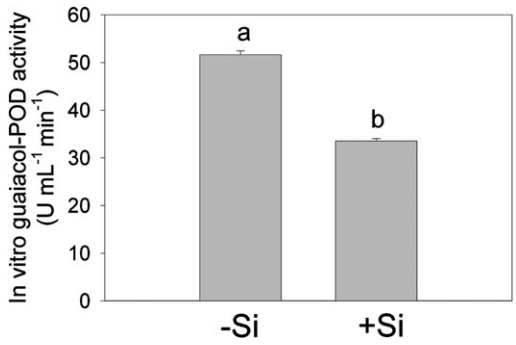

Fig. 5. Effect of Si on the activity of guaiacol-POD *in vitro*. If added, the final concentration of $Si(OH)_4$ in the reaction mixture was 0.5 mM. Data are means (n=4) ±SD of a representative experiment. Different superscript letters denote significant differences at $P < 0.05$.

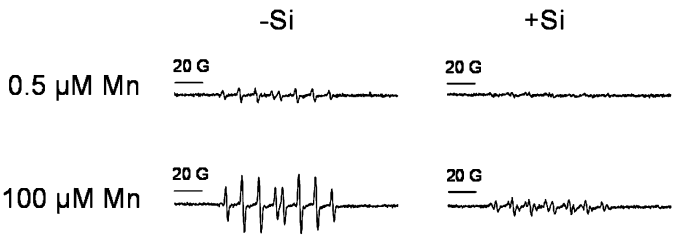

Fig. 6. EPR spectra of DEPMPO/·OH adducts from the LAF of cucumber plants. The cucumber plants were grown in nutrient solutions with normal (0.5 µM) and high (100 µM) supply of Mn, without or with 1.5 mM $Si(OH)_4$.

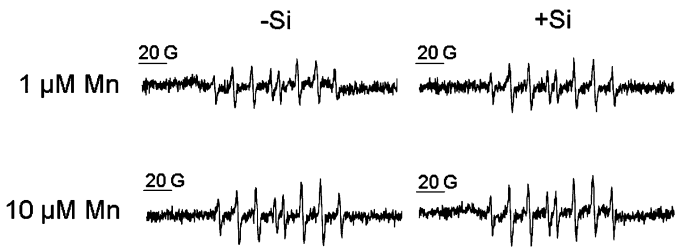

Fig. 7. EPR spectra of DEPMPO/·OH adducts generated by *in vitro* Fenton reaction. The incubation media contained, in addition to spin-trapping reagent, either 1 µM or 10 µM $MnSO_4$ and 1 mM H_2O_2 with or without 0.5 mM $Si(OH)_4$. The concentrations of Mn in the incubation media are in the same physiological range as measured in the LAF (see Fig. 1A); the 0.5 mM Si concentration in the incubation media represents the physiological range determined in the LAF of Si-fed cucumber plants.

factor inducing Mn toxicity symptoms, and Si has a protective effect in controlling ·OH production under excess Mn.

Acknowledgements

The paper is dedicated to the memory of Rade Konjevic. This work was supported by the Serbian Ministry of Education and Science (grants ON 173028 to MN and ON 173040). We thank Leon V. Kochian (Cornell University, Ithaca, NY) for critical reading of the manuscript, and Ernest A. Kirkby (University of Leeds, UK) for a final improvement of the English.

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