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Contrasting Performance and Different Tolerance of Chestnut Rose and Grape to Excess Manganese

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Abstract Grape (cultivar Jinshou) and chestnut rose (cultivar Gui 4) were exposed to excess manganese (Mn) treatments to characterize the physiological basis for Mn tolerance in woody plants. Chestnut rose exhibited a high sensitivity to this environmental constraint whereas grape appeared rather tolerant to Mn excess. Stomatal density and closure rate were affected by excess Mn in chestnut rose and brittleness of the leaf vein was reported as a novel Mn toxicity symptom in this species. Linear reductions in biomass accumulation and photosynthetic pigment concentrations with increasing Mn level were observed in chestnut rose but not in grape, except under the extremely high Mn concentration (118 mM). Our results showed that the contrasting performances between the two species were related to the differences in ion transfer and homeostasis. Mn was readily allocated to the photosynthetic organ in chestnut rose but was mainly restricted to the roots in grape. Excess Mn caused iron (Fe) and nitrogen (N) deficiencies in chestnut rose but not in grape. The synthesis of antioxidant phenylpropanoid compounds and chelating phytochelatins were activated in Mn-treated grape but strongly repressed in chestnut rose. The importance of these parameters in the overall strategy of Mn tolerance in grape is discussed.

Keywords *Vitis vinifera* · *Rosa roxburghii* · Mn toxicity · Ion homeostasis · Mn translocation · Leaf vein

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Introduction

Manganese (Mn) at adequate concentrations is an essential element for normal plant metabolism (Rengel 2004). Plant species, however, differ considerably in terms of Mn requirements, and optimal concentrations ranging from 30 to 500 μg g^{-1} dry weight (DW) have been reported (Clarkson 1988). In contrast, high leaf Mn concentrations (1,000–12,000 μg g^{-1} DW) are rather toxic in plants (Reeves 2006). In field conditions, numerous factors may induce high Mn concentrations in plants, including Mn mining deposits or the use of poor-quality fertilizers. Manganese bioavailability in soils is related to the pH of the substrate, and it is usually assumed that a decrease of 1 pH unit leads to a 100-fold increase in Mn availability (Kabata-Pendias and Pendias 2001). As a consequence, acidic soil commonly found in some subtropical and tropical regions with a pH below 5.0 is especially prone to Mn toxicity, which constitutes the most important growth-limiting factor after aluminum toxicity for plants in these regions (Foy 1984).

Toxic Mn levels may inhibit plant growth and disturb plant metabolism as a consequence of oxidative stress resulting from the accumulation of reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide ions ($O_2^{\cdot-}$), or the hydroxyl radical ($OH^{\cdot}$). They also lead to specific deficiencies in other essential cation elements, that is, calcium (Ca), iron (Fe), and magnesium (Mg), by interfering with

their uptake, translocation, and utilization (Chaney 1993; Kabata-Pendias and Pendias 2001). Plant resistance to high Mn concentrations may rely on Mn sequestration into vacuoles and/or endoplasmic reticulum, chelation in the cytosol, binding to some specific proteins such as metallothioneins, and induction of specific enzymes' activities such as those involved in the management of oxidative stress (Pittman 2005; Polle 2005). Lidon and others (2000, 2004) reported Mn accumulation in rice chloroplasts where a manganese protein mimicking superoxide dismutase was synthesized in the thylakoid. Different plant species exhibit different levels of tolerance to excess Mn (Paschke and others 2005). Some Mn-hypertolerant species, such as *Schima superba* (Theaceae), showed no Mn toxicity symptoms with Mn supply below 60 mM and could even tolerate Mn concentrations up to 150 mM (Yang and others 2008). To the best of our knowledge, the physiological-biochemical response of hypertolerant plants to the extremely high Mn treatment was only marginally studied until now.

Chestnut rose (*Rosa roxburghii* Tratt) is a typical fruit shrub of promising economical importance in South China (Wen and Deng 2005). Grape (*Vitis vinifera* L.) has been extended to the subtropical and tropical regions in South China to fulfill the increasing need of the fruit market. We observed that the grape species exhibited good growth performance in the acid soil of those areas. Moreover, wild relatives of the cultivated grape vine may be found growing in the topsoil of some Mn deposits, whereas local trees such as chestnut rose are not able to cope with this harsh environment. It may thus be hypothesized that high interspecific differences in plant Mn tolerance exist between these species. In this study we examined the effects of high Mn concentrations on chestnut rose and grape widely cultivated in South China. The specific aims of the study were (1) to compare their tolerance to excess Mn and (2) to explore the mechanisms mediating the Mn accumulation and detoxification processes in those two species.

Materials and Methods

Materials and Experiment Design

Grape cuttings (cultivar Jinshou, ca. 30 cm high and 0.8 cm basal diameter), and chestnut rose cuttings (cultivar Gui 4; ca. 20 cm high and 0.5 cm basal diameter) were planted in 8-L plastic pots filled with homogenized garden soil, with one plant in each pot. The soil had 4.21% organic matter, 132.0 μg g^{-1} available N, 107.3 μg g^{-1} available P, and 123.0 μg g^{-1} available K, with pH 7.23. The plants were grown under semicontrolled environmental conditions in a naturally lit greenhouse with a day/night photoperiod ranging from 13/11 to 14/10 h, a light intensity of 700–1,500 μmol m^2 s^{-1} at the top of the canopy, a temperature range of 10.7–33.0°C, and a relative humidity range of 62–83%. The plants were supplied with 800 ml Hoagland's solution each day. After culturing for 1 month, the cuttings were exposed to Mn stress, using $MnSO_4 \cdot H_2O$ as the source of Mn and a pH adjusted to 5.5 ± 0.2. There were four Mn treatments for each species and designed separately as follows: (1) chestnut rose treated with basic Hoagland's solution was supplemented with 0 (control), 12.0, 25.0, and 36.0 mM Mn to keep the available Mn concentrations in the soil at 30.0 (control), 170.0, 340.0, and 520.0 μg g^{-1} levels; (2) grape treated with basic Hoagland's solution was supplemented with 0 (control), 38.0, 77.0, and 118.0 mM Mn to keep the available Mn concentrations in the soil at 30.0 (control), 570.0, 1,020.0, and 1,620.0 μg g^{-1} levels. The Mn treatments were supplied every 3–5 days to maintain constant Mn concentration in the soil, and the soil pH ranged from about 5.8 to 6.4 during the whole treatment period. These treatments simulated the Mn levels from acidic soils to Mn mining soil conditions (85.0–1,700.0 μg g^{-1} Mn in soil). Eighty cuttings of each species were randomly allocated to four Mn treatments applied over 3 months, and each treatment included five replications and four cuttings per replication.

Biomass, Root/Shoot Ratio Measurements, and Element Analysis

Cuttings were harvested, dried, and weighed before Mn treatment. At the end of the experiment, all remaining cuttings were harvested and divided into leaves, stems (including branches), and roots. The different organs were dried at 70°C for 48 h and biomass accumulation was quantified by subtracting the initial biomass from the final one at harvest time. The root/shoot ratio in biomass was also determined.

For measurement of element (K, Mg, Cu, Mn, Fe, Ca, and Zn) concentrations, the sampled organ (leaf, stem, and root) was washed thoroughly with distilled and demineralized water. The dried tissue was digested in a HNO_3/HCl (4:1 v/v) solution and the elements were analyzed using a PerkinElmer Analyst 800 atomic absorption spectrometer (PerkinElmer, Waltham, MA, USA). The concentrations of nitrogen (N) and phosphorus (P) in the plant leaves were determined by the micro-Kjeldahl and vanadomolybdate methods, respectively (Page 1982).

Observation of Main Leaf Vein, Element Secretion, and Stomatal Behavior

Four young fully expanded leaves (third or fourth leaves that emerged after the beginning of Mn stress) were collected from the main branch of four plants per treatment for

the two studied plant species and thoroughly washed. Discs of 5 × 5 mm were air-dried and coated with a 100–150-Å layer of high-purity carbon for observation of the leaf surface and for element characterization using a scanning electron microscope (JSM-6490 LV) equipped with energy-dispersive spectroscopy (EDS, type INCA-350). Representative samples were chosen for photographic documentation. The stomatal density and stomatal closure rate were also counted. The SEM-EDS system was operated as described by Mou and others (2011).

Quantification of Leaf Membrane Permeability and Antioxidant Enzymes Activity

Leaf cell membrane permeability (MP) was determined according to the method of Zheng and others (2008) and expressed as the absorbance at 264 nm. For antioxidant enzymes activity analysis, young fully expanded leaves were sampled and homogenized in 50 mmol l^{-1} sodium phosphate buffer (pH 7.8) containing 1.0 mmol l^{-1} EDTA and 2% (w/v) polyvinylpyrrolidone. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. Superoxide dismutase (SOD) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). Catalase (CAT) activity was measured according to the method of Beer and Sizer (1952). Peroxidase (POD) activity was determined in a 4-ml reaction mixture containing 1 ml of enzyme extract, 3.35 mM H_2O_2, 0.05% (v/v) guaiacol, and 100 mM sodium phosphate buffer (pH 6.0) according to the method of Nickel and Cunningham (1969).

Analysis of Photosynthetic Pigments and Phenylpropanoid Compounds

Chlorophyll and carotenoids were extracted by *N,N*-dimethylformamide (DMF) and determined using the method of Moran and Porath (1980). The total phenolic compound was determined following the method of Lei and others (2007). The mean value was expressed as milligrams of gallic acid equivalents (GAEs) per gram of extract. The different phenylpropanoid compounds were extracted and hydrolyzed by acidified methanol containing 1% HCl (v/v) and 0.5 mg ml^{-1} tertiary butylhydroquinone (TBHQ) (Tokusoglu and others 2003). The final extract was analyzed using a Shimadzu HPLC system (LC-10AT) with a Shim-pack CLC-ODS column at 28°C. Phenylpropanoid compounds were eluted using a formic acid/acetonitrile solvent (75/25 v/v) at the rate of 0.8 ml/min and detected with a Shimadzu diode array. The phenylpropanoid compounds such as ferulic acid, resveratrol, myricetin, and quercetin were determined by calibration with commercially available standards. As an important heavy-metal chelating compound, phytochelatins (PCs) were determined according to the method of May and others (1998).

Statistical Analysis

Statistical analysis was performed with Statistical Package for the Social Science (SPSS) ver. 13.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) for Mn treatment was applied for each species, and the least significant difference (LSD) test was employed to detect possible differences between the treatments.

Results

Biomass Accumulation and Mn Distribution Under Excess Mn Treatment

In our study, biomass accumulation of chestnut rose linearly decreased with Mn concentration (Fig. 1a, $P < 0.001$) and growth was completely inhibited at the 36-mM Mn level over the entire duration of the experiment. In contrast, biomass accumulation in grape was decreased only by the highest Mn level (118 mM) and remained unaffected at lower Mn concentrations. Biomass partitioning was significantly affected by Mn treatment (Fig. 1b). Indeed, the root/shoot ratio decreased in response to all Mn treatments in grape, whereas it was decreased only in response to 25–36 mM Mn in chestnut rose.

As expected, the Mn concentration in different plant parts increased as a consequence of exogenous Mn application (Fig. 2). As far as grape is concerned, most Mn remained in the roots and only a minor part was translocated to the shoot (Fig. 2a). Chestnut rose displayed an opposite behavior as absorbed Mn was efficiently translocated to the shoot part and mainly accumulated in leaves (Fig. 2b). On a whole-plant basis, the mean Mn concentrations at 25 and 36 mM in chestnut rose were similar to those recorded in grape exposed to 38 mM and 77 mM, respectively (Fig. 2c).

Nutrient Balance Under Excess Mn Levels

In the present study, N, P, and K concentrations were determined in leaf tissues only. As shown in Fig. 3, the leaf N and K concentrations remained unaffected by Mn treatment in grape. Manganese decreased N concentration in chestnut rose but it linearly increased the leaf K concentration (Fig. 3a, b). The leaf P concentration increased in response to Mn treatments in both species, but to a higher extent in chestnut rose (Fig. 3b).

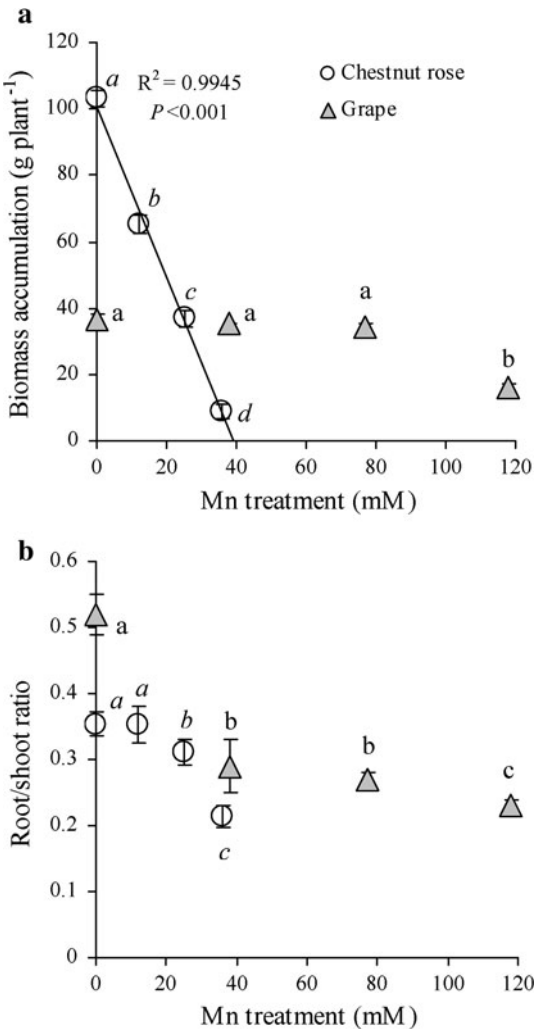

Fig. 1 The effects of Mn stress on **a** biomass accumulation and **b** root/shoot ratio in grape and chestnut rose. *Different letters* beside the spots indicate significant differences for Mn treatments at $P < 0.05$, and *regular* and *italic letters* represent different Mn treatments for grape and chestnut rose, respectively. Values shown are mean ± SE

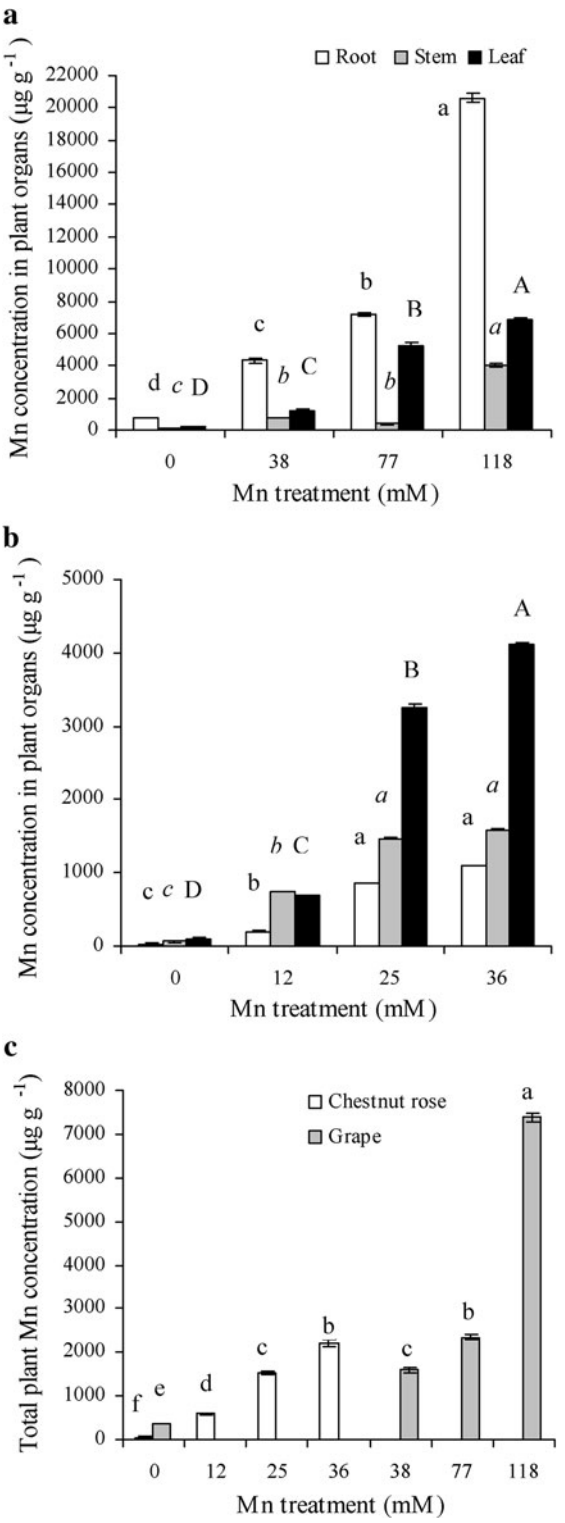

Fig. 2 Mn concentration under excess Mn conditions in different plant parts of **a** grape and **b** chestnut rose and **c** in the whole plants for both species. *Different letters* beside the columns indicate significant differences at $P < 0.05$. The *regular*, *italic* and *capital letters* in **a** and **b** represent the Mn treatment differences in roots, stems, and leaves, respectively, and the regular letters in **c** represent the Mn concentration differences in the whole plants of the two species. Values shown are mean ± SE

The calcium (Ca) concentration in roots, leaves, and stems of both species declined with increasing Mn levels except in the leaves of chestnut rose (Table 1). On the other hand, the Mg concentration marginally increased in response to all treatments in both species, except in the roots of chestnut rose (Table 1). Calcium and Mg concentrations were higher in chestnut rose than in grape, with the noticeable exception of Ca concentration in roots. The mean iron (Fe) concentration was decreased by over 50% in all parts of chestnut rose, whereas it slightly increased in the shoots of grape. In contrast, zinc concentration remained almost unaffected in chestnut rose but significantly decreased at the moderate Mn level (38 and 77 mM) and then markedly increased at the highest Mn level in all grape parts. Copper (Cu) concentration was significantly

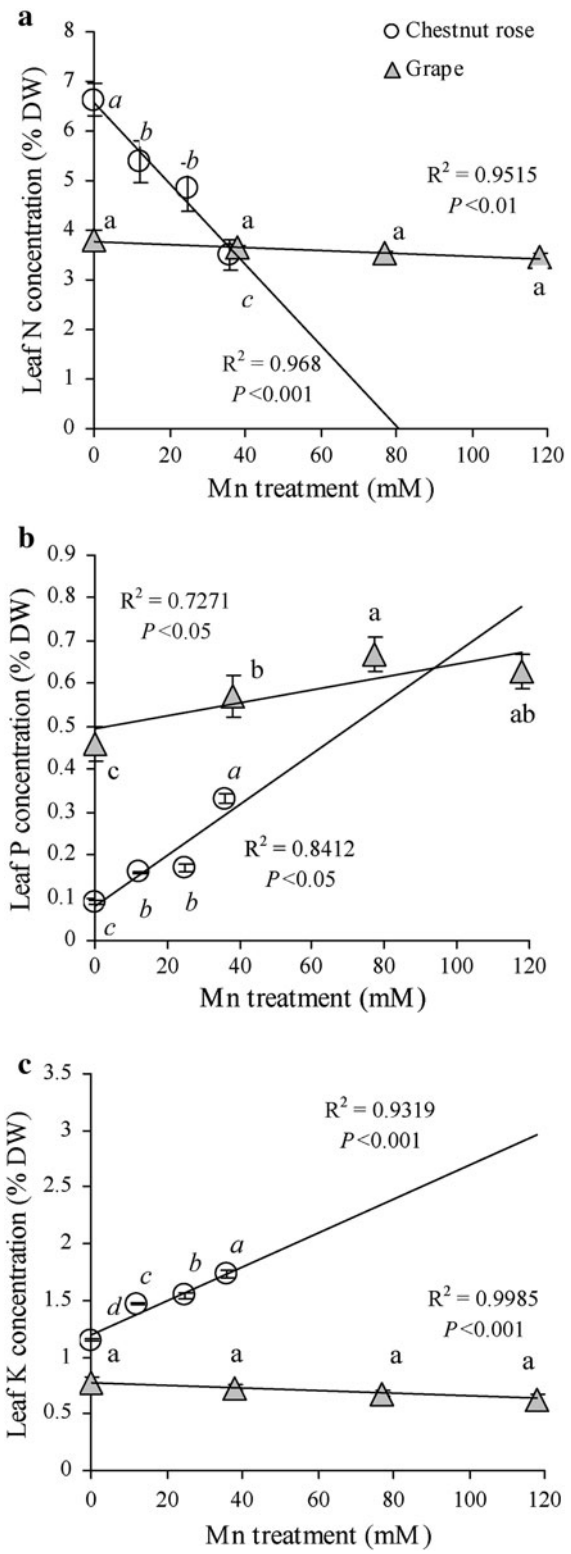

Fig. 3 The effects of Mn treatments on **a** leaf N concentration, **b** leaf P concentration, and **c** leaf K concentration of grape and chestnut rose. *Different letters* beside the spots indicate significant differences for Mn treatments at $P < 0.05$. The *regular* and *italic letters* represent the treatment differences in grape and chestnut rose, respectively. Values shown are mean ± SE

increased by Mn treatment in all parts of chestnut rose but was significantly reduced in grape roots and remained unaffected in leaves and stems.

Photosynthetic Pigments and Stomatal Status in Response to Mn Excess

Leaf photosynthetic pigment (chlorophyll and carotenoid) concentrations were significantly decreased with increasing Mn level in chestnut rose. In contrast, such concentrations increased in grape exposed to 36–77-mM Mn levels and were markedly reduced at the highest Mn dose (118 mM) (Fig. 4a, b). Stomatal density was decreased by more than 20% in all Mn treatments in chestnut rose but was reduced only at the highest Mn level in grape (Fig. 4c). Furthermore, stomatal closure rate increased with increasing Mn level in chestnut rose but only at the highest Mn level in grape (Fig. 4d).

Effect of Excess Mn on Main Leaf Vein and Element Secretion

The leaf vein in chestnut rose was frequently broken as observed with SEM (Fig. 5b, d, f). Over 80% of the examined leaf veins exhibited the brittleness phenomenon after 25-mM Mn treatment (leaves after 36.0-mM treatment were not analyzed because the leaf veins seemed to be broken and damaged during sample preparation), whereas the grape leaf veins remained unaffected (Fig. 5a, c). Also, crystallized material was secreted into the leaf surface under excess Mn. As detected by EDS spectra, Ca, S, Si, Mg, and Na were involved in the crystallized compounds (Fig. 5e).

Effects of Excess Mn on Antioxidant Enzyme Activity and Membrane Permeability

As presented in Table 2, the activities of the various antioxidant enzymes were affected by Mn treatments in chestnut rose and grape. Compared with the control, SOD activity in chestnut rose was increased by about 30% in response to 12–25 mM Mn, but it was not affected in grape, except for a significant reduction recorded at 118 mM. In both plant species, CAT activity was strongly increased by 2–18 fold except for the highest Mn treatment. POD activity was significantly decreased by the highest Mn level and remained unaffected at other Mn levels in grape. The POD activities in chestnut rose increased as a consequence of Mn treatment and peaked at 25 mM Mn. On the other hand, membrane permeability values were increased with increasing Mn levels in both species (Table 2).

Table 1 Effects of Mn treatment on some element concentrations in the leaf, stem, and root of grape and chestnut rose

Element	Grape				Chestnut rose			
	Mn^{2+} mM	Leaf	Stem	Root	Mn^{2+} (mM)	Leaf	Stem	Root
Ca (mg g^{-1})	0.0	4.55 ± 0.04a	1.24 ± 0.05a	3.10 ± 0.04a	0.00	9.21 ± 0.36a	5.07 ± 0.16a	1.56 ± 0.05a
	38.0	4.66 ± 0.05a	1.08 ± 0.09b	2.33 ± 0.07b	12.0	9.52 ± 0.37a	3.95 ± 0.24b	1.32 ± 0.08b
	77.0	3.10 ± 0.10b	0.74 ± 0.01c	2.20 ± 0.09b	25.0	9.36 ± 0.98a	3.76 ± 0.17b	1.33 ± 0.09b
	118.0	2.33 ± 0.07c	0.63 ± 0.01d	2.15 ± 0.12b	36.0	7.20 ± 0.66b	3.30 ± 0.26c	1.07 ± 0.07c
Mg (mg g^{-1})	0.0	0.60 ± 0.01d	0.22 ± 0.01c	0.38 ± 0.01c	0.00	6.27 ± 0.07d	3.06 ± 0.03c	1.57 ± 0.01a
	38.0	0.88 ± 0.02c	0.23 ± 0.01c	0.62 ± 0.01b	12.0	6.64 ± 0.07c	3.61 ± 0.06b	1.53 ± 0.01a
	77.0	0.97 ± 0.02b	0.42 ± 0.02b	1.11 ± 0.11a	25.0	7.88 ± 0.03b	3.65 ± 0.03b	1.48 ± 0.02b
	118.0	1.18 ± 0.11a	0.62 ± 0.01a	0.38 ± 0.01c	36.0	8.37 ± 0.11a	4.72 ± 0.11a	1.49 ± 0.02b
Fe (μg g^{-1})	0.0	111.4 ± 2.6b	24.6 ± 0.6c	167.3 ± 5.6b	0.00	47.4 ± 0.52a	142.8 ± 8.11a	302.3 ± 4.6a
	38.0	126.6 ± 2.5a	23.7 ± 1.1c	184.2 ± 5.5a	12.0	26.3 ± 0.40b	45.5 ± 0.78b	163.9 ± 1.2b
	77.0	123.2 ± 3.6a	29.5 ± 2.2b	156.6 ± 4.3b	25.0	27.4 ± 1.01b	35.9 ± 1.46b	161.8 ± 4.4b
	118.0	131.5 ± 2.6a	34.8 ± 0.8a	139.0 ± 3.6c	36.0	24.7 ± 0.87b	35.1 ± 1.94b	135.9 ± 5.6c
Zn (μg g^{-1})	0.0	60.3 ± 3.5b	29.5 ± 0.6b	56.8 ± 0.2b	0.00	34.7 ± 0.8b	19.3 ± 0.3a	7.8 ± 0.2a
	38.0	39.4 ± 1.7c	22.9 ± 0.3c	43.0 ± 0.2d	12.0	35.5 ± 1.1b	16.5 ± 0.3b	7.9 ± 0.4a
	77.0	22.8 ± 1.3d	21.9 ± 0.2c	45.9 ± 0.26c	25.0	34.7 ± 0.6b	16.7 ± 0.3b	7.9 ± 0.07a
	118.0	85.7 ± 1.2a	34.5 ± 0.5a	71.5 ± 0.3a	36.0	36.5 ± 1.7b	16.2 ± 0.3b	7.2 ± 0.05b
Cu (μg g^{-1})	0.0	10.8 ± 0.4b	8.81 ± 0.1a	31.4 ± 1.0a	0.00	7.8 ± 0.7b	7.5 ± 0.8b	6.2 ± 0.4c
	38.0	13.1 ± 0.1a	8.48 ± 0.13a	19.2 ± 0.2b	12.0	9.9 ± 0.5a	10.3 ± 1.0a	8.8 ± 0.5a
	77.0	11.5 ± 0.02b	7.8 ± 0.5a	20.5 ± 0.7b	25.0	10.3 ± 0.4a	8.2 ± 0.3b	7.2 ± 0.2b
	118.0	11.8 ± 0.2b	8.2 ± 0.1a	19.0 ± 0.2b	36.0	10.9 ± 0.5a	8.3 ± 0.3b	6.6 ± 0.6bc

Values shown are mean ± SE. Values within a column in each species followed by the *same letter* do not differ significantly at $P < 0.05$ level by LSD pairwise comparisons

Effect of Excess Mn on Phenylpropanoid Metabolism and Phytochelatins (PCs)

Total phenolic compounds were significantly increased in grape, whereas their mean concentration obviously decreased in chestnut rose under Mn stress (Table 3). In addition, the different compounds involved in the phenylpropanoid pathway were affected differently by Mn stress in the two species (Table 3). Ferulic acid was significantly increased in chestnut rose under Mn stress but remained constant in grape except at the highest Mn treatment. On the other hand, resveratrol was markedly enhanced in grape by excess Mn but was significantly decreased in chestnut rose. The flavonoid compounds such as myricetin and quercetin were markedly decreased by Mn stress in chestnut rose but only slightly affected in grape. Concentrations of phytochelatins (PCs) significantly increased in grape with increasing Mn levels, whereas they were significantly decreased in chestnut rose (Table 3).

Discussion

Biomass accumulation and photosynthetic pigment concentrations are commonly known as sensitive markers for heavy-metal toxicity in plants (MacFarlane and Burchett 2001; Paschke and others 2005). According to these criteria, the two studied plant species exhibited contrasting behaviors in response to Mn excess (Figs. 1, 4) and chestnut clearly appeared to be more sensitive than grape to this ion toxicity. Lei and others (2007) observed the collapse and splitting of the meristematic tissue in the central vein of *Populus cathayana* under excess Mn but did not report symptoms at the whole-leaf organ. In the present study, brittleness of the leaf vein was observed only in chestnut rose (Fig. 5) and could be considered a novel Mn toxicity symptom not reported before. A possible explanation for the brittleness could be that the Mn concentration in the xylem tissue of the central vein could be very high in this species due to slow movement of Mn out of vascular bundles. Such a high Mn concentration could then damage the leaf vein and cause the brittleness. On the other hand, the brittleness of the leaf vein may block transpiration flow and thus compromise water movement to the leaf and nutrition in chestnut rose. This may lead to partial stomatal closure (Fig. 4) and to marked reductions in the leaf N content (Fig. 3a), which further reduces growth and photosynthetic pigment concentrations in this species (Figs. 1, 4). However, similar physiological modifications did not occur in grape, even at the 77-mM Mn level

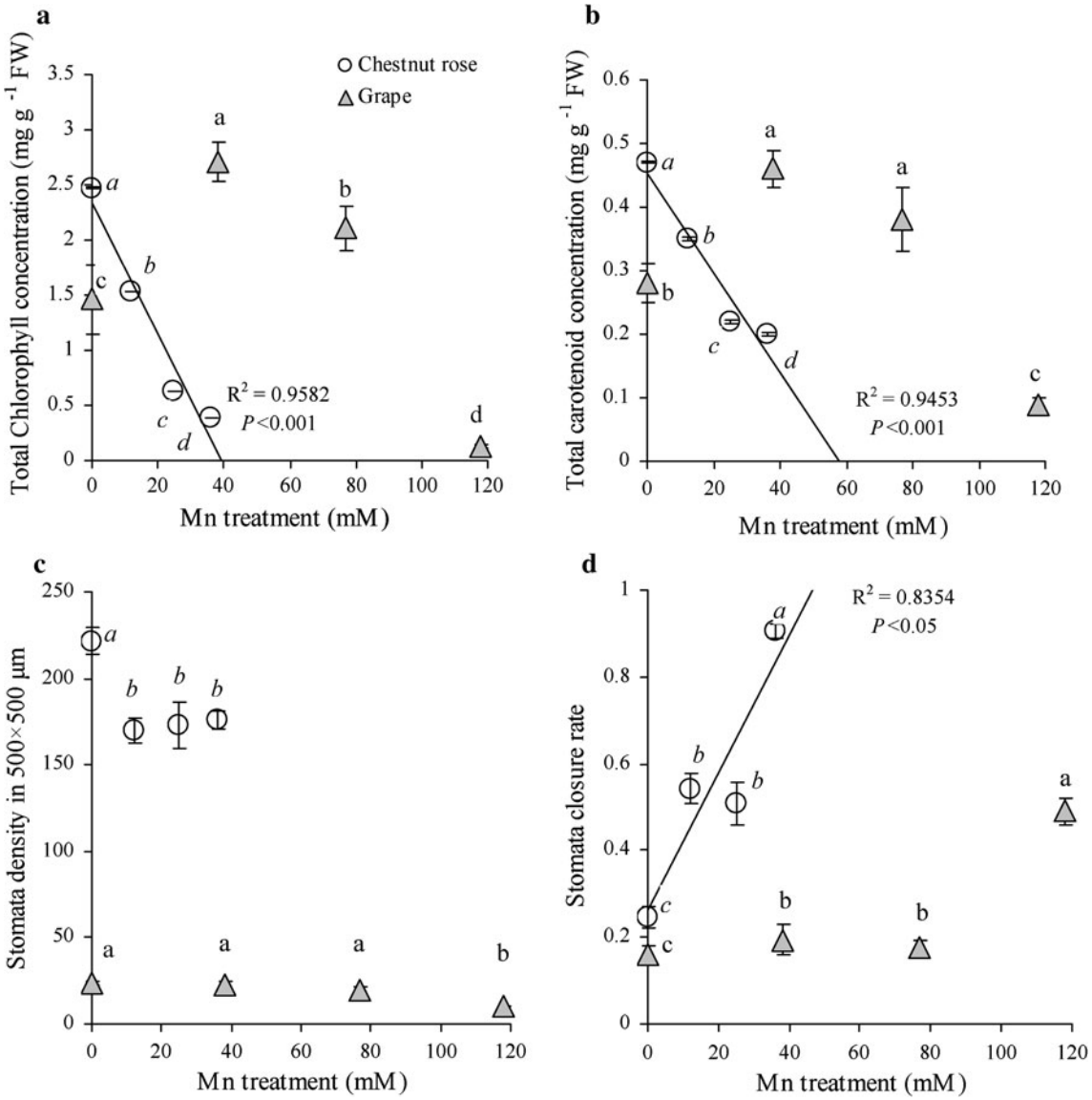

Fig. 4 The effects of Mn stress on **a** leaf chlorophyll concentration, **b** leaf carotenoid concentration, **c** leaf stomatal density, and **d** leaf stomatal closure rate in grape and chestnut rose. *Different letters* beside the spots indicate significant differences for Mn treatments at $P < 0.05$. The *regular* and *italic letters* represent the treatment differences in grape and chestnut rose, respectively. Values shown are mean ± SE

(Figs. 1, 4). We previously observed that grape growth was even stimulated by Mn concentration as high as 15–30 mM Mn in sand-cultured conditions (Mou and others 2011). These observations prompted us to suggest that grape might be regarded as a Mn-tolerant species according to the definition of Ernst and others (2008).

Earlier studies on temperate legume, corn, alfalfa, and Douglas fir demonstrated that the high Mn tolerance in these crops depends on the retention of excess Mn within the root system and subsequent low translocation efficiency of Mn from the root to the shoot (El-Jaoual and Cox 1998; Dučić and others 2006). The grape cultivar analyzed in our study displayed a similar behavior. Assuming that Mn is freely mobile in the xylem as are other metal species and that over 6,000 μg g^{-1} Mn was stored in the grape roots, the restriction of root-to-shoot translocation of Mn in grape could at least in part be due to restriction of xylem loading. Recent studies showed that the xylem-loading process is the major limiting process for root-to-shoot translocation of cadmium in *Solanum torvum* and the japonica rice cultivar 'Sasanishiki' (Moria and others 2009; Uraguchi and others 2010). Due to the high Mn concentration in its roots, the grape cultivar Jinshou could also be recommended as a pioneer plant for Mn phytostabilization in Mn-contaminated mine tailing areas (Padmavathiamma and Li 2009).

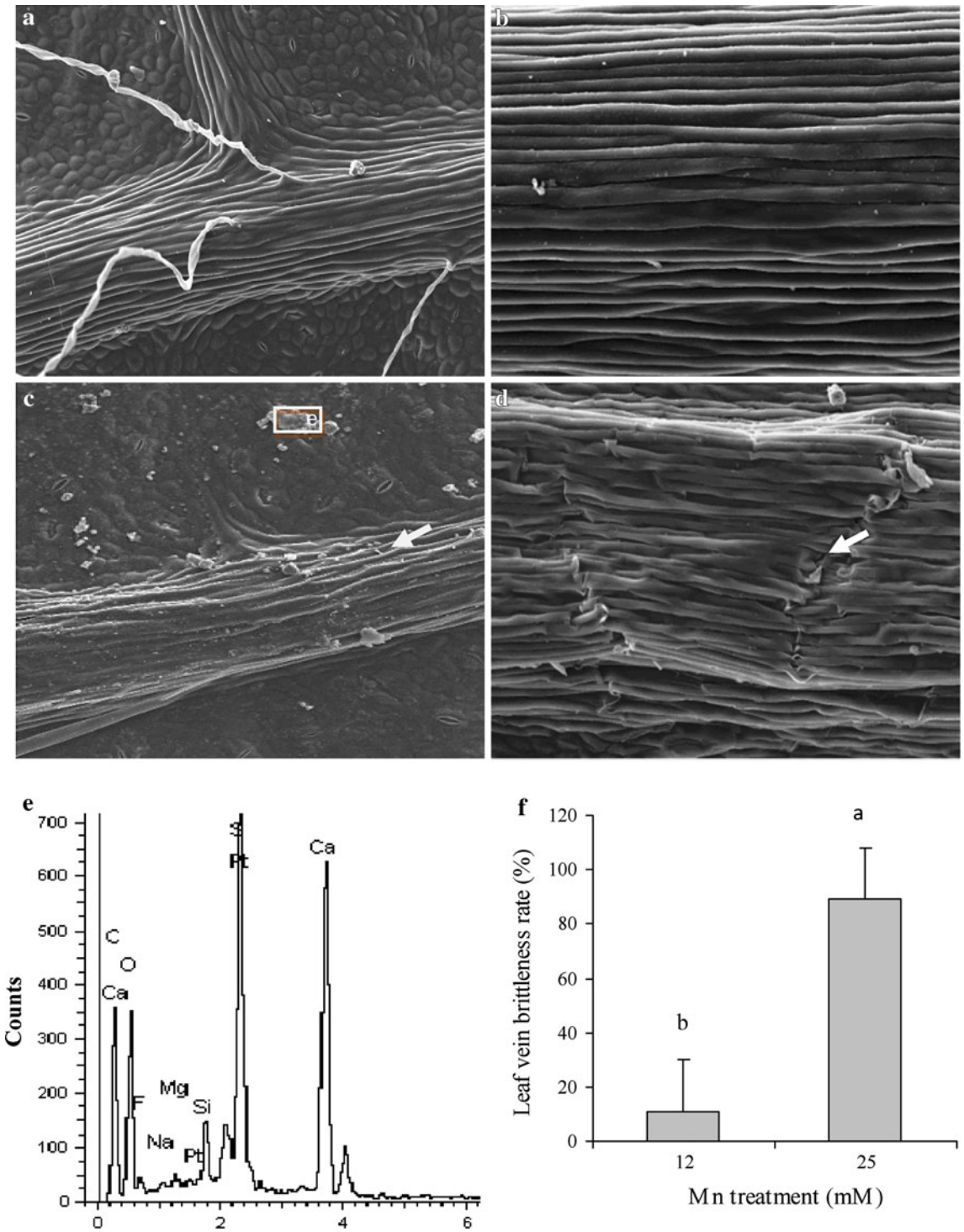

Fig. 5 The main leaf vein of grape and chestnut rose under Mn treatment investigated by scanning electron microscopy (SEM) and element secretion pattern in the leaf surface detected by SEM–EDS. **a**, **c** Grape leaf vein treated with 0 and 77 mM Mn, separately. **b**, **d** Chestnut rose leaf vein treated with 0 and 25 mM Mn. **e** EDS spectra of crystallized compounds on grape leaf surface in **c**. **f** Leaf vein brittleness rate (%) in chestnut rose under 12 and 25 mM. *Different letters* beside the columns indicate significant differences at $P < 0.01$

High Mn concentration had a strong impact on the uptake and distribution of the other elements in the two tested species (Table 1; Figs. 2, 3). Some transporters for Mn, such as IRT1, Nramp, and OPT3, have been identified in plants so far (Pittman 2005; Cailliatte and others 2010; Ishimaru and others 2010). These transporters are also

Table 2 Effects of Mn treatment on the antioxidant enzyme activity and membrane permeability of grape and chestnut rose

Plant species	Mn^{2+} (mM)	SOD (U mg^{-1} protein)	CAT (μmol mg^{-1} protein min^{-1})	POD (U mg^{-1} protein min^{-1})	Membrane permeability ($OD_{264\ nm}$ g^{-1} FW h^{-1})
Grape	0.0	296.0 ± 6.24a	1.68 ± 0.085c	0.36 ± 0.08a	5.32 ± 0.41c
	38.0	307.0 ± 6.51a	5.98 ± 0.509b	0.26 ± 0.08ab	5.19 ± 0.85c
	77.0	297.2 ± 5.01a	22.50 ± 5.27a	0.23 ± 0.03ab	6.18 ± 0.38b
	118.0	226.6 ± 11.02b	1.38 ± 0.28c	0.13 ± 0.05b	7.33 ± 0.51a
Chestnut rose	0.0	292.3 ± 17.64b	2.37 ± 0.54c	0.57 ± 0.01d	3.66 ± 0.88c
	12.0	370.2 ± 10.86a	7.73 ± 0.62a	1.00 ± 0.01b	4.01 ± 0.26bc
	25.0	352.6 ± 12.80a	5.86 ± 0.60a	1.77 ± 0.02a	4.29 ± 0.29b
	36.0	330.2 ± 3.90ab	3.97 ± 0.55b	0.80 ± 0.01c	5.39 ± 0.50a

Values shown are mean ± SE. Values within a column followed by the *same letter* do not differ significantly at $P < 0.05$ level by LSD pairwise comparisons

Table 3 Effects of Mn stress on leaf phenolic and flavonoid concentrations (mg g^{-1} dry weight) in grape and chestnut rose

Plant species	Mn^{2+} (mM)	Total phenolic compounds (mg g^{-1} DW)	Ferulic acid (mg g^{-1} DW)	Myricetin (mg g^{-1} DW)	Quercetin (mg g^{-1} DW)	Resveratrol (mg g^{-1} DW)	PCs (mg g^{-1} DW)
Grape	0.0	24.4 ± 1.82a	0.61 ± 0.05b	1.21 ± 0.06a	1.08 ± 0.04a	1.57 ± 0.2c	1.11 ± 0.11c
	38.0	37.2 ± 4.53b	0.64 ± 0.04b	0.76 ± 0.05b	1.20 ± 0.10a	10.7 ± 1.0a	2.20 ± 0.28bc
	77.0	42.2 ± 4.96b	0.63 ± 0.04b	0.81 ± 0.06b	1.00 ± 0.06a	5.2 ± 0.6b	3.23 ± 0.31b
	118.0	55.7 ± 4.67b	0.87 ± 0.05a	1.37 ± 0.07a	1.11 ± 0.06a	6.8 ± 0.7b	7.90 ± 1.44a
Chestnut rose	0.0	121.7 ± 8.7a	0.15 ± 0.02b	1.03 ± 0.05a	0.14 ± 0.02a	9.9 ± 0.7a	5.04 ± 0.81a
	12.0	100.2 ± 6.1b	0.25 ± 0.03a	0.88 ± 0.03a	0.13 ± 0.02a	7.6 ± 0.6b	4.21 ± 0.76b
	25.0	77.4 ± 3.0c	0.23 ± 0.03a	0.42 ± 0.03b	0.04 ± 0.01b	6.8 ± 0.5b	2.89 ± 0.45c
	36.0	72.2 ± 5.9c	0.23 ± 0.03a	0.55 ± 0.04b	0.04 ± 0.01b	6.8 ± 0.6b	2.26 ± 0.37c

Values shown are mean ± SE. Values within a column followed by the *same letter* do not differ significantly at $P < 0.05$ level by LSD pairwise comparisons

involved in the absorption and transport of other elements like Fe and Zn. Under excess Mn conditions, the competition between Mn and other divalent metals for the binding sites of those transporters probably resulted in a decrease in Ca (in both species), Zn (in grape), and Fe (in chestnut rose) concentrations. Whereas the modest reductions of Ca and Zn did not affect plant metabolism in the present study, the leaf chlorosis of chestnut rose could be due to marked Fe deficiencies (Table 1), which is consistent with the findings of previous studies (El-Jaoual and Cox 1998). Recently, Ishimaru and others (2010) demonstrated that a rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of both iron and manganese, and our data indeed demonstrate that Mn excess interferes with iron distribution to all plant parts (Table 1).

The recorded Mn-induced increase in Mg and P in leaves of both species may partly help the plant to cope with Mn toxicity (Table 1; Fig. 2b). This hypothesis is supported by previous studies on other plant species (Bot and others 1990; Goss and Carvalho 1992). Our data, however, suggest that such a process remained inefficient in the case of chestnut rose. In our study, calcium (Ca), sulfur (S), potassium (K), silicon (Si), and some other elements were also detected in the excreted or crystallized compounds on the grape leaf surface (Fig. 5c, e), suggesting their important roles in metal homeostasis and in the alleviation of cellular Mn toxicity in grape. Recently, Doncheva and others (2009) observed that a silicon supplement could alleviate Mn toxicity in maize. Many previous studies also demonstrated that S metabolism plays an important role in the reduction of heavy-metal toxicity through the synthesis of endogenous antioxidants such as glutathione (GSH) or the synthesis of chelating compounds like phytochelatins (Cobbett and others 1998; Ernst and others 2008).

Chelation of heavy metals by a range of ligands constitutes an important strategy to avoid metal toxicity in the plant cytosol (Grill and others 1987; Blum and others 2007). This is especially the case for phytochelatins (PCs) (Rauser 1995). In our study, PC content increased in grape but decreased in chestnut rose with the increasing Mn treatment, which may also contribute to the difference in

Mn tolerance recorded between the species. An alternative strategy against cytosolic Mn toxicity would be the activation of antioxidant systems. The accumulation of cellular heavy metals indeed stimulates the formation of free radicals leading to oxidative stress through damage to numerous cellular structures and macromolecules (Hall 2002), as suggested here by the Mn-induced increase in cell membrane permeability (Table 2). In the present study, CAT activity was clearly enhanced by excess Mn and its increase was larger than that of POD, especially at 77 mM Mn, which implies that CAT played a pivotal role for H_2O_2 reduction (Table 2). Additionally, plant antioxidant capacity of grape leaves was further increased by the enhancement of antioxidant compounds such as phenolic compounds and resveratrol (Rice-Evans and others 1997), whereas increased PCs may, at the same time, help to limit oxidative stress through Mn sequestration (Table 3). This, however, was not sufficient to fully avoid oxidative stress, recorded as a Mn-induced increase in membrane permeability. In fact, the major flavonoid compounds with strong antioxidant capacity, quercetin and myricetin, remained at constant levels in grape but were pronouncedly decreased in chestnut rose under Mn stress, and this could also explain the different cellular tolerances to Mn toxicity in those two species. Furthermore, hydroxycinnamic compounds such as ferulic chemicals are expected to increase lignification processes under high oxidative pressure (Ghanati and Ishka 2006), which is consistent with our observation in chestnut rose (under all Mn treatments) and grape (under extremely highest Mn level).

In conclusion, our results demonstrated the contrasting performances of two plant species: chestnut rose displayed a high sensitivity to Mn toxicity, whereas grape behaved as a tolerant plant species. Biomass accumulation and photosynthetic pigment concentrations were linearly reduced by increasing Mn stress in chestnut rose but were only marginally affected by Mn concentrations as high as 77 mM Mn in grape. Although the underlying mechanism needs to be further explored, the following reasons could account for this. First, the leaf vein in chestnut rose was sensitive and brittle under excess Mn, which led to a linear increase of the stomata closure rate and a reduction of stomata density, whereas those symptoms did not occur in grape. Second, Mn was readily transferred to photosynthetic organs in chestnut rose, whereas it was mainly stored in the roots of grape. Third, a negative nutrient imbalance occurred in chestnut rose, as shown by linear reductions of N and Fe concentrations inducing leaf chlorosis in relation to a decrease in the leaf photosynthetic pigment concentration. Fourth, the cytosol detoxification capacity in grape was higher than that in chestnut rose, as suggested by enhanced PC synthesis in grape leaf which contrasts with the PC decrease recorded in chestnut rose. The antioxidant compounds such as total phenolics (flavonoid, ferulic compounds, and so on) and resveratrol were decreased in chestnut rose but increased or maintained at high levels in grape and may thus help alleviate Mn toxicity.

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