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Article

Effects of Exogenous Manganese (Mn) on Mineral Elements, Polyamines and Antioxidants in Apple Rootstock *Malus robusta* Rehd.

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Abstract: Manganese (Mn) is one of the essential microelements in all organisms. However, high level of Mn is deleterious to plants. In this study, the effects of exogenous manganese application on mineral element, polyamine (PA) and antioxidant accumulation, as well as polyamine metabolic and antioxidant enzyme activities, were investigated in *Malus robusta* Rehd., a widely grown apple rootstock. High level of Mn treatments decreased endogenous Mg, Na, K and Ca contents, but increased Zn content, in a Mn-concentration-dependent manner. Polyamine metabolic assays revealed that, except the content of perchloric acid insoluble bound (PIS-bound) spermine, which increased significantly, the contents of putrescine (Put), spermidine (Spd) and spermine (Spm) all decreased progressively, accompanied with the decreased activities of arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17), and the increased activities of diamine oxidase (DAO, EC 1.4.3.6) and polyamine oxidase (PAO, EC 1.5.3.3). Further antioxidant capacity analyses demonstrated that contents of anthocyanin, non-protein thiols (NPT) and soluble sugar, and the activities of guaiacol peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1), also increased upon different concentrations of Mn treatments. Our results suggest that endogenous ion homeostasis is affected by high level of Mn application, and polyamine and antioxidant metabolism is involved in the responses of *M. robusta* Rehd. plants to high level of Mn stress.

Keywords: Manganese; *Malus robusta* Rehd; mineral element; polyamine; antioxidant

1 Introduction

Manganese (Mn) is the second most prevalent metal that is essential for all organisms [1]. In plants, high level of Mn will diminish the contents of carotenoid and chlorophyll, affect CO_2 assimilation in leaves,

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change the structure of antioxidant enzymes, and lead to increased production of reactive oxygen species, which, in return, damage cell membrane [2–4]. Since Mn at reduced state and low pH is more available for plants, Mn toxicity has become a widespread phenomenon observed on acid and waterlogged lands [5].

The protection of cells from oxidative injury under abiotic stress condition largely depends on the competence of plants managing its resistant systems. Antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and antioxidants such as non-protein thiol (NPT), anthocyanin and soluble sugar, play a crucial role in protecting plant cells from oxidative damage [6–8]. Due to the chemical similarity and common transporters of heavy metal, considerable attention has been paid to the involvement of plant nutrient elements in the acquisition of tolerance to various environmental stresses [9]. Polyamines (PAs) are a class of low molecular weight aliphatic cations widely found in diverse organisms including higher plants [10]. Variations in PA contents were associated with several types of stresses, such as salinity [11,12] and heavy metals [13,14], and plant dormancy [15,16]. It has been also reported that PAs function in the regulation of a large number of physiological processes including embryogenesis, cell division, morphogenesis, development, ethylene production, fruit ripening, flower development and dormancy [17–19] in many woody species [20–23].

To date, unprecedented pollution caused by heavy metals has become a global environmental problem that is likely to override the adaptive potential of plants, especially of the tree species with long reproductive cycles. As one of the most important fruit tree species grown worldwide, the multiplication of apple trees is largely relied on the availability of grafting materials. The cultivar *M. robusta* Rehd. has been used as main rootstock for apple grafting due to its high grafting affinity, seed germination, shoot extraction and survival rate [24]. However, studies on the physiological effects of exogenous Mn application on *M. robusta* Rehd. plants are scarce. Here, we demonstrate that ion homeostasis and antioxidant capacity in *M. robusta* Rehd. plants are involvement in response to Mn stress. The accumulations of endogenous mineral nutrient, polyamine and antioxidant, and the activities of polyamine metabolic and antioxidative enzymes were altered in *M. robusta* Rehd. plants upon different Mn stress treatments.

2 Materials and Methods

2.1 Plant Materials and Mn Treatments

Seeds of *M. robusta* Rehd. were sterilized with 5.25% germicidal bleach (Clorox, Oakland, California) for 25 min, then 70% (v/v) ethanol for 20 min, and rinsed three times with sterile water. Seeds were germinated on sterilized filter paper soaked with 1/10 Hoagland's solution in dark for 1 week. One-week-old seedlings were then transplanted to 0.5 liter plastic pots and grown in greenhouse under a 16 h light/8 h dark photoperiod with 80% humidity at 25 ± 2°C. The seedlings were watered every three days with 1/10 Hoagland's solution (EC = 0.21 ms cm^{-1}; pH = 6.0). After two weeks, young plants in the same size were selected and hydroponically cultured in 4 L of 1:10 Hoagland's nutrient solution supplemented with 0, 0.1, 0.2, 0.4 or 0.8 mM $MnSO_4.H_2O$ for two weeks. Each treatment was repeated on three occasions. All the experiments were conducted with at least three replicates.

2.2 Mineral Element Measurements

Plants at the end of two-week-treatment were washed thoroughly with 10 mM EDTA, dried at 70°C for 2 days, and digested in the solution of $HNO_3/HClO_4$ (3:1) at 95°C until the digesting solution became clear. Digested residue was dissolved in a minimal volume of 7% HCl, then diluted with distilled water. The solution samples were analyzed for nutrient concentration using inductively coupled plasma atomic emission spectroscopy (Prodigy, Leemanlabsinc, Hudson City, USA). A total volume of 10 ml was unified in all solution. Treated with the conditions described above, 0.4 g of tea leaves (GBW08513) were weighed as QA/QC samples, aiming to check the accuracy of the Mn analysis method. A standard curve was calculated by inherent levels of Mn solution in concentrations of 0, 1, 10, 20, 50 μg/ml, and the correlation coefficient was above 0.9999. The content of Mn was quantified using the standard curve and expressed as μg g^{-1} FW. Estimation for Mg, Na, K, Ca and Zn were similar to the determination seen for Mn.

2.3 Polyamine Determinations

For polyamine content analyses, plant material (2 g) was homogenized in 4 ml of 6% (v/v) cold perchloric acid (PCA), kept on ice for 1 h, and centrifuged at 21000 g for 30 min. The pellet was extracted twice with 2 ml 5% (v/v) PCA and centrifuged again. The supernatants were used to determine the contents of free and PS-conjugated PAs, whereas the pellet was used to determine the contents of PIS-bound PAs. For the analysis of PIS-bound PAs, the pellets were resuspended in 5% (v/v) PCA, mixed with 12 N/HCl (1:1, v/v), hydrolyzed at 110°C for 24 h in flame-sealed glass ampoules, and the hydrolyzates were filtered, dried at 70°C and re-suspended in 1 ml of 5% (v/v) PCA. For PS-conjugated PA assays, 2 ml of the supernatant were mixed with 2 ml of 12 N HCl, and hydrolyzed at 110°C for 24 h in flame-sealed glass ampoules. The supernatant, hydrolyzed supernatant and the pellet were then benzoylated [25].

The benzoyl derivatives were separated and analyzed using a HPLC system (Agilent 1100, USA) equipped with an UV detector under the following conditions: 200 mm × 4.6 mm C_{18} reverse-phase column (Kromasil, Sweden); particle size, 5 μm; column temperature, 30°C; mobile phase, 64% (v/v) methanol; flow rate: 0.8 ml min^{-1}, detecting wavelength: 254 nm. The internal standard was 1, 6-hexanediamine.

2.4 Polyamine Metabolic Enzyme Activity Assays

The ADC and ODC activities were determined as described previously with some modifications [26]. Basically, a total amount of 1.5 g fresh plant material was homogenized in 50 mM phosphate buffer (pH 6.3) consisting of 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 40 μM pyridoxal phosphate (PLP), 5 mM dithiothreitol (DTT), 5 mM ethylene diamine tetra acetic acid (EDTA), 20 mM ascorbic acid (Vc) and 40 μM polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 12000 g for 40 min and the supernatants were used for enzyme activity assays. The reaction mixture (1.5 mL) consisted of 1 mL of the assay buffer with 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 40μM pyridoxal phosphate and 5 mM DTT, 0.3 mL of either the ADC or ODC enzyme extract and 0.2 mL of 25 mM L-arginine (L-ornithine) was incubated at 37°C for 60 min, then centrifuged (4°C) at 3000 *g* for 10 min, after which 0.5 mL of the supernatant was mixed with 1 mL of 2 mM NaOH, and then 10 μL benzoyl chloride was added to the mixture and stirred continuously for 20 s. After the reaction proceeded at 25°C for 60 min, 2 mL of saturated NaCl and 2 mL of ether were added to the reaction mixture and stirred thoroughly, then centrifuged (4°C) at 1500 *g* for 5 min, 1 mL of ether phase was collected and evaporated at 50°C. The remainder was dissolved in 0.5 mL of methanol (HPLC grade), and its absorption value at 254 nm was measured with a spectrophotometer (Thermo GENESYS 10, USA) for ADC (the solution was diluted into 20 mL fords before measuring) and an HPLC system (Agilent 1100, USA) for ODC, respectively. A standard curve with Agmatine (Agm) or Put was used to calculate the activities of ADC and ODC (expressed as μmol Agm g^{-1} FW h^{-1} (U) and μmol Put g^{-1} FW h^{-1}(U), respectively).

DAO and PAO activities were determined as described previously [27]. Briefly, fresh samples were homogenized in 100 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10000 *g* for 20 min at 4°C. The supernatant was used for enzyme assay. The reaction mixture contained 2.5 mL of potassium phosphate buffer (100 mM, pH 6.5), 0.2 mL 4-aminoantipyrine/N, N-dimethylaniline reaction solution, 0.1 mL of horseradish peroxidase (250 U mL^{-1}) and 0.2 mL of the enzyme extract. The reaction was initiated by the addition of 0.1 mL of Put (final concentration of 20 mM) for DAO determination and 0.1 mL of Spd (final concentration of 20 mM) for PAO determination. One 0.001 absorbance unit of the change in the optical density at 550 nm min^{-1} was considered as one unit of enzyme activity.

2.5 Antioxidant Analyses

POD activity was determined with guaiacol as substrate in a total volume of 3 ml [28]. One unit of POD activity was calculated by the change in absorbance at 470 nm $min^{-1}g^{-1}$ fresh weights at 25°C. CAT activity was measured at 405 nm by an assay of hydrogen peroxide based on the formation of its stable complex with ammonium molybdate [29]. One unit of CAT activity was defined as decomposition of 1 μmol hydrogen

peroxide in 1 min at 25°C. SOD activity was estimated according to the method reported by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) [30]. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the initial reduction of NBT under light. Anthocyanin and soluble sugar were examined using the reagents purchased from Nanjing Jiancheng Bioengineering Institute, China. NPT content was measured according to the method described previously [31]. Plant material (0.5 g) was ground in 3 mL of 5% (w/v) sulfosalicylic acid. After centrifugation at 10000 g for 15 min at 4°C, the supernatants were collected and immediately mixed with Ellman's reaction mixture (5 mM EDTA and 0.6 mM DTNB in 120 mM of phosphate buffer, pH 7.5), and then the mixture was incubated at 30°C for 5 min. The absorbance was recorded at 412 nm, and NPT content was expressed as micromoles per gram of fresh weight.

2.6 Statistical Analysis

All values are expressed as mean ± standard deviation (SD) from three individual experiments. The data were subjected to an analysis of variance in SPSS Statistics 17.0. The correlation coefficients were expressed using *r* values. Different letters in the same column indicate a significant difference at the 5% level.

3 Results

3.1 Altered Mineral Element Content in M. Robusta Rehd. Plants

As a first step to understand the effects of exogenous Mn application on ion homeostasis in *M. robusta* Rehd., the content of Mn in the five-week-old plants treated with different concentrations of Mn (0, 0.1, 0.2, 0.4 and 0.8 mM $MnSO_4.H_2O$) were investigated (Fig. 1A). Mn treatments did not caused any phenotype change in the plants (data not shown). However, endogenous Mn content increased gradually with the increase of Mn concentration applied (Fig. 1B). Compared with that in the control plants, an 11 folds increase of endogenous Mn concentration (1214.25 μg g^{-1} fresh weight) was observed in the plants treated with 0.8 mM Mn.

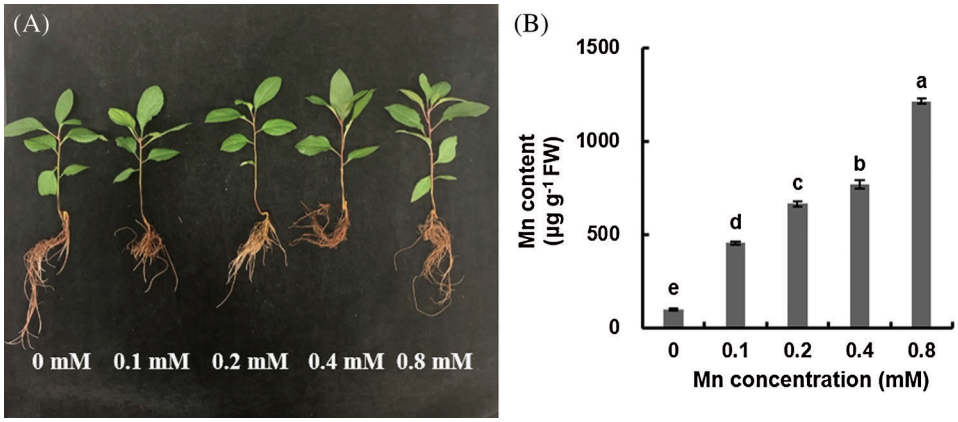

Figure 1: Endogenous Mn accumulation. (A) Phenotype of a five-week-old *M. robusta* Rehd. plant. (B) Mn content. Data were expressed as mean ± SD of three replicates. Value designated over the bars in different letter are significant different at $p < 0.05$

We then examined the contents of other mineral elements, and observed that except the content of Zn, which also increased, the contents of all the other elements tested, such as Mg, Na, K and Ca, decreased gradually, with a respective 31.03%, 40.59%, 48.69% and 37.00% decrease in the plants treated with 0.8 mM Mn (Fig. 2).

3.2 Decreased Polyamine Contents in M. Robusta Rehd. Plants

Polyamines play an important role in plant response to heavy metal stress [11,12]. We first evaluated the contents of total, free, PS-conjugated and PIS-bound Put in the five-week-old plants treated with different concentrations of Mn. In the presence of high level of Mn, the content of total, free, PS-conjugated and

PIS-bound Put decreased, with a maximum 73.38%, 82.93%, 66.27% and 72.68% decrease, respectively, in the plants treated with 0.8 mM Mn (Fig. 3A). Similar decreases were also observed in the contents of total, free, PS-conjugated and PIS-bound Spd, with a maximum 15.55%, 15.30%, 8.76% and 46.33% decrease, respectively, in the plants treated with 0.8 mM Mn (Fig. 3B). Different from Put and Spd, total, free and PS-conjugated Spm decreased, but PIS-bound Spm increased significantly, with a 2.07-folds increase in the plants treated with 0.8 mM Mn (Fig. 3C). Consequently, the total and PIS-bound (Spd + Spm)/Put ratios increased, whereas the free (Spd + Spm)/Put ratio decreased, with the increasing Mn concentrations applied (Fig. 3D). Unlike the total, free and PIS-bound (Spd + Spm)/Put ratios, the PS-conjugated (Spd + Spm)/Put ratio increased first, then decreased in the plants treated with different concentrations of Mn (Fig. 3D).

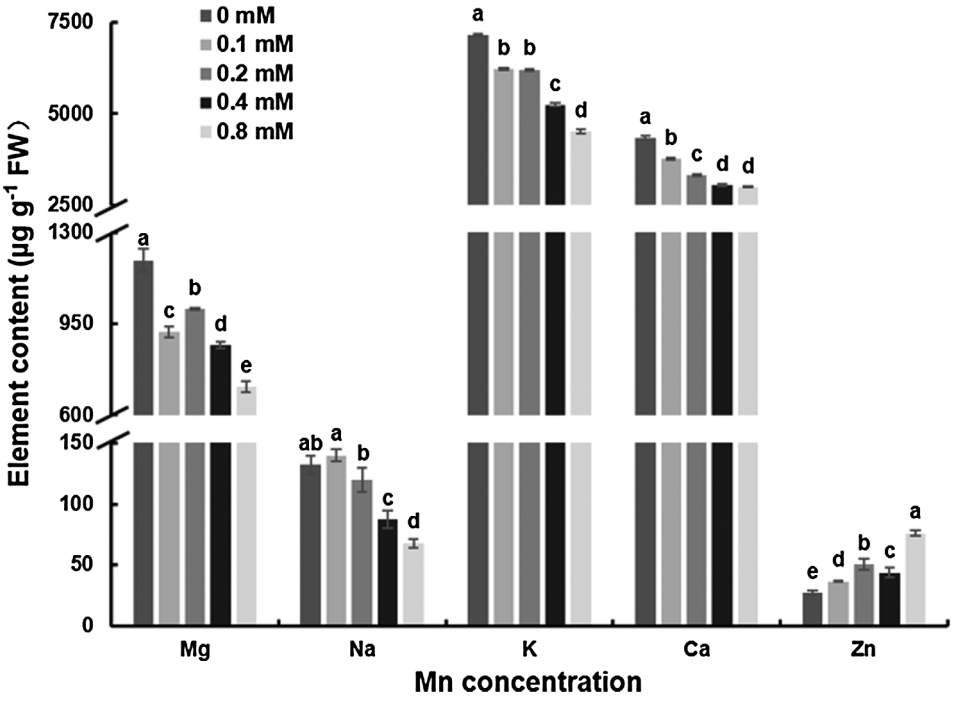

Figure 2: Mineral element analyses. Data were expressed as mean ± SD of three replicates. Value designated over the bars in different letter are significant different at $p < 0.05$

In order to understand how exogenous Mn application altered the endogenous accumulation of polyamines, the activities of polyamine metabolic enzymes were compared in the five-week-old plants treated with different concentrations of Mn. Accompanied with the increased Mn concentrations applied, the activities of ADC and ODC decreased progressively, with a maximum 84.09% and 91.84% decrease, respectively, in the plants treated with 0.8 mM Mn (Figs. 4A, 4B). Oppositely, the activities of DAO and PAO increased progressively, reaching its highest levels in the plants treated with 0.4 mM Mn (Figs. 4C, 4D).

3.3 Increased Antioxidant Capacity in M. Robusta Rehd. Plants

Heavy metal stress can adversely affect the normal growth of plants and lead to oxidative damage to plant cells. Although the activities of POD, CAT and SOD decreased in the plants treated with high concentration of Mn, the activities of these antioxidant enzyme increased significantly in the plants treated with low concentration of Mn (Figs. 5A–5C). When plants were treated with 0.8 mM Mn, the activities of POD and CAT reduced 95.30% and 76.40%, respectively, compared to that in the control plants (Figs. 5A, 5B). The effects of Mn application on anthocyanin, NPT and soluble sugar

accumulation were also examined. Similar to the antioxidant enzyme activities, although the contents of anthocyanin and NPT decreased in the plants treated with high concentration of Mn, the contents of them increased significantly in the plants treated with low concentration of Mn (Figs. 5D, 5E). The contents of soluble sugar increased significantly in all the plants treated with different concentrations of Mn (Fig. 5F).

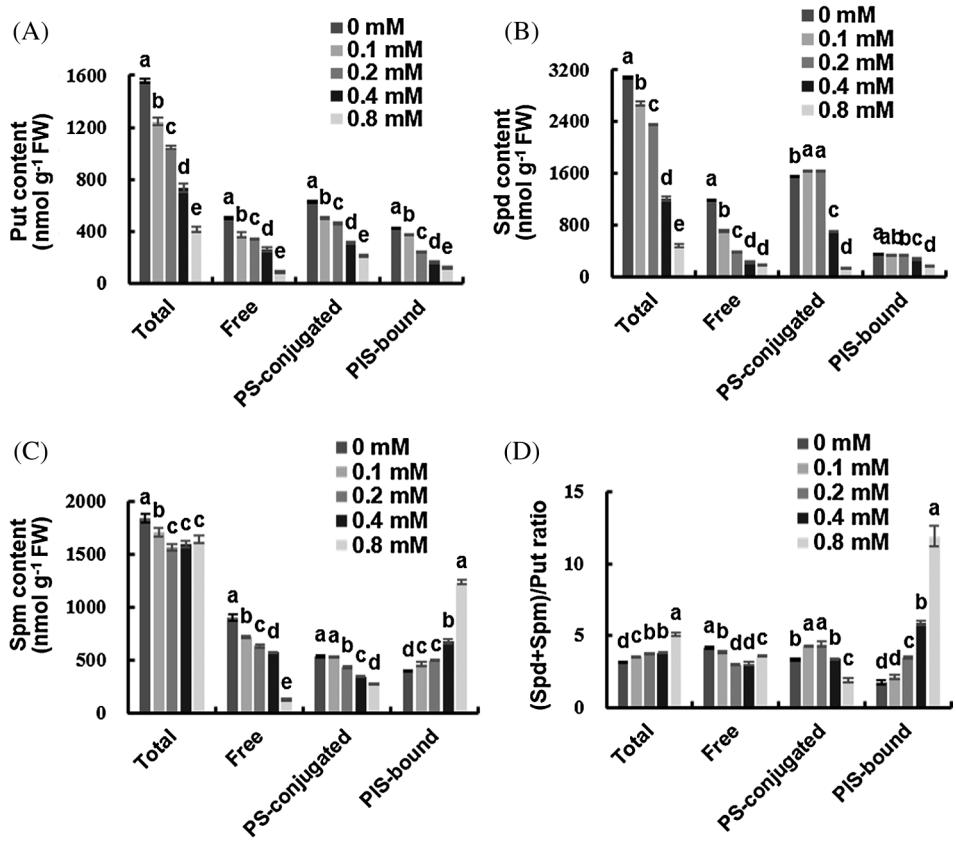

Figure 3: Determination of endogenous polyamine (PA). (A) Put content. (B) Spd content. (C) Spm content. (D) (Spd+Spm)/Put ratio. Data were expressed as mean ± SD of three replicates. Value designated over the bars in different letter are significant different at $p < 0.05$

4 Discussion

In higher plants, heavy metals can indirectly affect ion transport [32]. Mn required for the optimal growth of plants is relatively low. However, the capacity of Mn uptake far exceeds the demand of plants. In tape grass, high level of mercury declined the uptake of various nutrients, leading to deleterious effects on plant growth [33]. We found that in *M. robusta* Rehd. plants treated with different concentrations of Mn, Mn accumulation progressively increased in a concentration-dependent manner (Fig. 1B). Therefore, the dramatically increased Mn accumulation may disrupt the homostasis of other mineral nutrients required for the normal growth of plants. Indeed, negative correlations between the concentrations of Mn and other macroelements, such as Mg, Na and K, were observed in *M. robusta* Rehd. plants treated with different concentrations of Mn (Fig. 2). Mn is usually transported across the plasma membrane nonspecifically through Ca^{2+}-permeable channels [33–35]. Therefore, the altered concentrations of mineral elements caused by Mn application could be a result of the competition and specific interaction of these mineral elements due to their similarity in ionic radius or binding strength for ligands during

their absorption and translocation [36]. The negative correlation between Mn and Ca concentrations was also observed in *Arabidopsis*, tobacco and poplar [37–41]. Due to the direct competition between Mn and other essential nutrients for the same binding site, cation absorption has been used as a general indicator of metal tolerance [42]. In wheat, Ca ion plays a crucial function in the signaling network of plant response to both biotic and abiotic stresses [43]. Alteration in Ca content was closely associated with Cd, Zn, Cu, and Al toxicity [44]. In *Arabidopsis* and ryegrass, heavy metals such as Cd, Ni and Mn led to elevated Zn accumulation [45,46]. Consistently, an elevated Zn content was also observed in *M. robusta* Rehd. plants treated with different concentrations of Mn (Fig. 2), indicating that Zn ion could lead to enhancement of apple plants resistance capacity to Mn-induced stress.

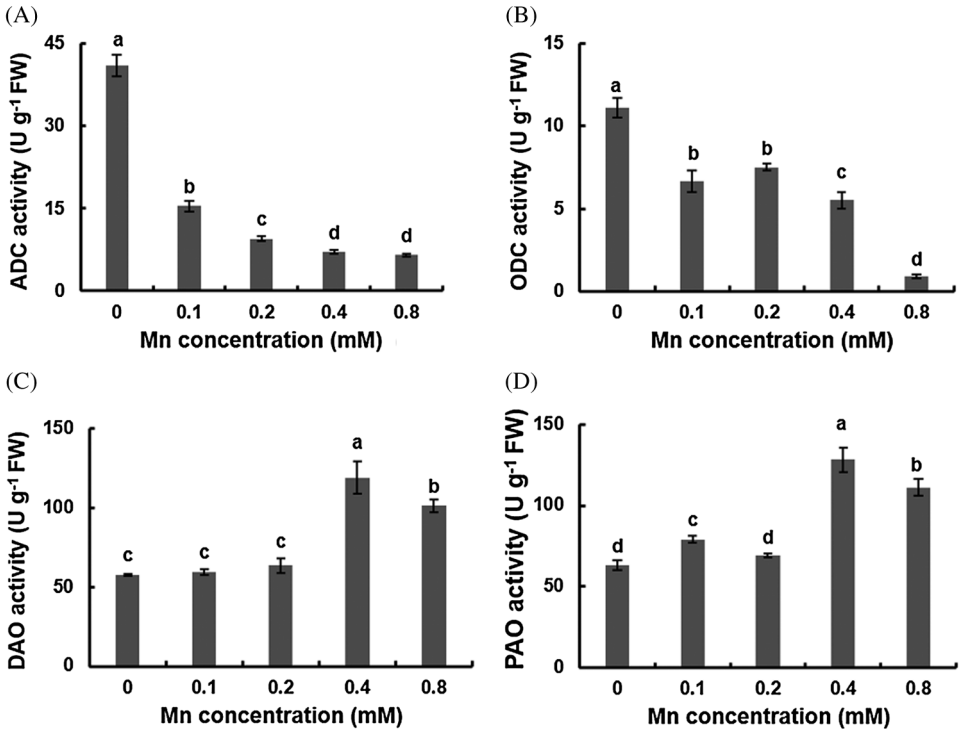

Figure 4: Measurement of polyamine metabolic enzyme activities. (A) ADC activity. (B) ODC activity. (C) DAO activity. (D) PAO activity. Data were expressed as mean ± SD of three replicates. Value designated over the bars in different letter are significant different at $p < 0.05$

It has been well documented that PAs undertake complex functions in plant adaptation to various abiotic and biotic stresses [47–49]. Putrescine, spermidine and spermine are the major polyamines which exist in free, soluble conjugated and insoluble bound forms [10]. We found that polyamine homeostasis was also disturbed in *M. robusta* Rehd. plants treated with different concentrations of Mn, as indicated by a huge decrease of total Put and Spd contents, along with a slight drop of total Spm content (Figs. 3A–3C). Put is synthesized from arginine or ornithine by ADC and ODC, respectively, and its degradation was catalyzed by DAO [17]. High level of Put in plant tissues can eventually lead to apoptotic cell death [12]. The decreased total Put content could be mainly attributed to the reduced activities of ODC and ADC, and the elevated activity of DAO (Figs. 4A–4C), possibly duing to that the precursor were cut down by Mn. Under adverse condition, Spd functions as a protectant to protect plasma membrane from damage by helping to maintain the membrane integrity, preventing the activation of superoxide-generating NADPH oxidases, and inhibiting the activities of protease and RNase [50,51]. Upon high level of Mn treatments,

total Spd content in *M. robusta* Rehd. plants decreased gradually, possibly due to the accelerated degradation caused by the increased PAO activity (Figs. 3B and 4D), perhaps because flavin adenine dinucleotide (FAD) linked by non-covalent bonds were added in Mn toxicity. By contrast, total Spm content decreased slightly compared to total Put and Spd contents (Fig. 3C). Spm interacts with many negatively charged molecules to modulate their surface charge, and consequently regulate membrane permeability [51]. Hence, the reduced total Spd and Spm contents may cut down the resistance of *M. robusta* Rehd. plants to Mn toxicity. Consistent with the previous observation in other plants, a continuous increase of PIS-bound Spm content was also observed, indicating its important role in the protection of *M. robusta* Rehd. plants from Mn stress [52,53]. The elevated (Spd + Spm)/Put ratio has been taken as a criterion of tolerance to salt, osmotic, heat, chilling and heavy metal stresses in plants [54]. We found that the total, PS-conjugated and PIS-bound (Spd + Spm)/put ratios were dramatically elevated and PA metabolic enzyme activities were altered, suggesting a protective role of PAs in plant resistant to Mn stress (Figs. 3D and 4A–4D).

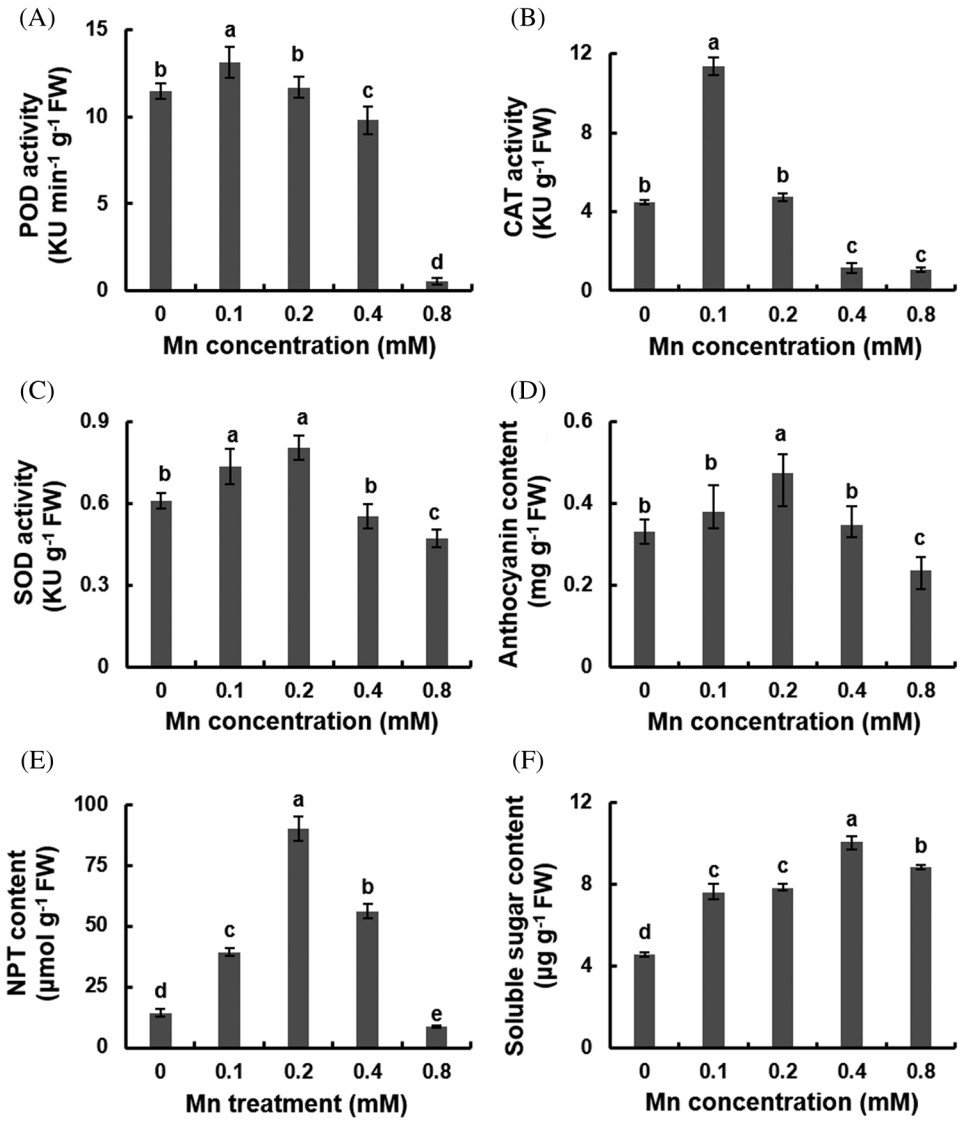

Figure 5: Antioxidant system analyses. (A) POD activity. (B) CAT activity. (C) SOD activity. (D) anthocyanin content. (E) NPT content. (F) soluble sugar content. Data were expressed as mean ± SD of three replicates. Value designated over the bars in different letter are significant different at $p < 0.05$

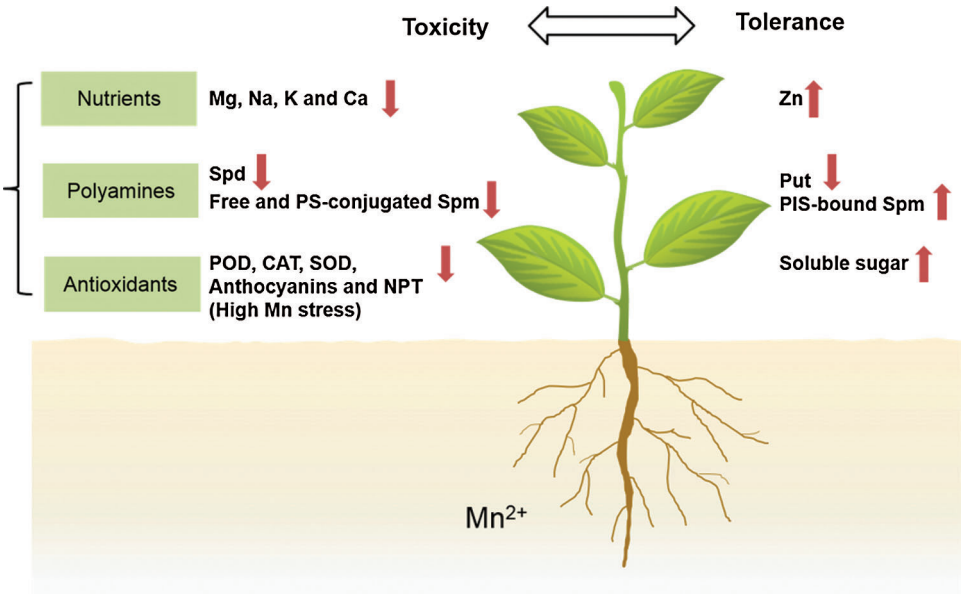

Figure 6: A schematic map to show the effects of exogenous Mn application on mineral elements, polyamines (PAs) and antioxidants in *M. robusta* Rehd. plants

Heavy metals may cause oxidative damage to plant cells either directly or indirectly through the formation of reactive oxygen species (ROS) [55]. Plants have evolved several antioxidant mechanisms, such as ROS-scavenging enzymes (superoxide dismutase, catalase, etc.) and non-enzymatic systems like phenolic acids, to control the production of ROS [56]. Increase in the activities of these antioxidant enzymes significantly ameliorated the heavy metal stress-stimulated oxidative stress, generally associated with enhanced Mn tolerance, in common bean, cucumber (*Cucumis sativus*), tomato, *Tanacetum parthenium* and perennial ryegrass [46,57–59]. In *M. robusta* Rehd. plants treated with different concentrations of Mn, the activities of POD, CAT and SOD increased at low concentrations of Mn treatments, then declined at high concentrations of Mn treatments (Figs. 5A–5C). Anthocyanins act as metal chelators, reducing agents and radical scavengers to detoxify the reactive oxygen species generated by oxidative stress [60]. As an important component of the detoxification system, NPT also plays a crucial role in plants resistance to heavy metal stress [61]. They both are potent antioxidants that protect plants from damage caused by abiotic stresses [62,63]. The increased anthocyanins and NPT contents could have minimized the oxidative damage caused by Mn stress in *M. robusta* Rehd. plants (Figs. 5D, 5E). The accumulation of soluble sugar has been considered as an adaptive trait of plants to heavy metal stress [64]. A consistent increase of soluble sugar content was observed in *M. robusta* Rehd. plants treated with different concentrations of Mn (Fig. 5F). Similar results were also observed in maize, *Citrus grandis* and *water mint* plants after high level of Mn treatments [2,60,65]. Therefore, the increased soluble sugar content in *M. robusta* Rehd. plants could be a result of the increased assimilate production of the affected photosynthesis and carbohydrates metabolism caused by heavy metal stress.

5 Conclusion

Taken together, our findings demonstrate that although Mn is an essential micronutrient, high level of Mn can be toxic to *M. robusta* Rehd. plants. Upon high level of Mn treatments, the contents of mineral elements (Mg, Na, K and Ca) and polyamines (Spd and Spm) decreased, whereas the contents of antioxidants (anthocyanin, NPT and soluble sugar) increased (Fig. 6). The reduced content of Put, the elevated contents of Zn, PIS-bound Spm, anthocyanin and soluble sugar involved in plant defense

mechanism are closely associated with Mn stress. The results of this study offer a clear understanding of the external applied Mn effects on the biochemical and physiological responses in apple rootstock (*M. robusta* Rehd.), which prove helpful to explore resistance strategies adopted by fruit trees under Mn stress, especially apples. Further transcriptomic studies on the expressions of genes involved in the uptake and transport of these mineral elements will provide more detailed information on their influence with each other under heavy metal stress condition.

Author Contributions: Dazhuang Qi, Meixia, Liang, Jianzhao Li and Xuqiang Qiao carried out the experiments, analyzed the data. Fudong Jiang conducted some of the Mn stress experiments. Xuqiang Qiao and Hongxia Zhang wrote the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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