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Response of Organic Acids to Zinc Homeostasis in Zinc-Deficient and Zinc-Toxic Apple Rootstock Roots*[1]

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ABSTRACT

To elucidate the mechanisms of tolerance to zinc (Zn) deficiency and Zn toxicity in the root of apple trees, the apple rootstock *Malus hupehensis* (Pamp.) Rehd seedlings were selected to study the responses of organic acids to Zn homeostasis in roots under low Zn (0 μmol L^{-1}), adequate Zn (as control, 4 μmol L^{-1}) and toxic Zn (100 μmol L^{-1}) treatments. The differences of Zn concentrations and accumulations in the roots were highest, compared with those in the stems and leaves, when apple seedlings were subjected to low and toxic Zn treatments for 1 d. The concentrations and accumulations of oxalic and malic acids in the roots in the low and toxic Zn treatments increased by 20% to 60% compared with those of the control treatment. Significantly negative correlations were found between the total Zn concentrations and the concentrations of oxalic and malic acids in the roots under 1 d of low Zn treatment. However, contrary correlations were found for the toxic Zn treatment. Meanwhile, the maximum influx rates of Zn^{2+} under low and toxic Zn treatments increased by 30% and 20%, respectively, compared with the rate of the control treatment. Both Zn deficiency and Zn toxicity increased the concentrations of organic acids in root after short-time Zn treatment, which could resist Zn stress through balanding Zn homeostasis in *M. hupehensis* Rehd.

Key Words: *Malus hupehensis* (Pamp.) Rehd, Zn compartmentation, Zn deficiency, Zn toxicity, Zn uptake

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Zinc (Zn) is the only metal which is present in the six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Auld, 2001). Zinc, an essential micronutrient, is required for various physiological and metabolic processes in plants. But, it is toxic when present in excess amounts (Fukao *et al.*, 2009). Approximately 30% of the world's cultivated soil is deficient in Zn. This is a serious problem. On the other hand, one could find excessive Zn in soil owing to fertilizers and soil pollution (Kawachi *et al.*, 2009). This causes acute Zn deficiency and phytotoxicity in higher plants (Alloway, 2004; Broadley *et al.*, 2007). Apple, as one of the four main fruits (orange, banana, apple and grape) in the world, is beneficial to human health as it is rich in mineral ions (Boyer and Liu, 2004; Veeriah *et al.*, 2006). However, as apple tree is more sensitive to Zn deficiency, Zn homeostasis can be destroyed in it. This not only leads to chlorosis of rosette leaves and young leaves but also blocks the growth of roots (Alloway, 2004). In order to improve Zn concentration in Zn-deficient orchards, substantial amounts of Zn fertilizer was applied to the soil. However, excessive Zn can also destroy Zn homeostasis, affecting the carbohydrate metabolism in plants (Foy *et al.*, 1978). So the unsuitable Zn concentration adversely affects the yield and nutritional quality of apples, indirectly affecting human health through food chain.

Proper Zn homeostasis is critical for the growth and development of plants. Under conditions of Zn deficiency and toxicity, plants must balance uptake, utilization, and storage of Zn through absorption, translocation, compartmentation, and secretion. This helps in maintaining proper ion homeostasis.

In higher plants, Zn deficiency and toxicity lead to a production in Zn chelators, such as phytate, metallothionein, phytochelatin, low-molecular-weight amino acids and organic acids (Lane *et al.*, 1987; Sarret *et al.*, 2002; Hoffland *et al.*, 2006; Tennstedt *et al.*, 2009; Monsant *et al.*, 2011). It also causes a production

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in Zn transporters, such as zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like protein, cation diffusion facilitator and metal tolerance proteins (MTP) (Grotz *et al.*, 1998; Guerinot and Eide, 1999; Kawachi *et al.*, 2009). Apart from this, both Zn deficiency and toxicity lead to changes in subcellular Zn compartmentation (Hacisalihoglu and Kochian, 2004; Ma *et al.*, 2005). In order to adapt to Zn stress, plants often use different strategies, such as chelation-based strategy, metal compartmentation-based strategy, and Zn transporter-based strategy (Clemens *et al.*, 2002; Hall, 2002; Haydon and Cobbett, 2007).

Under the conditions of Zn deficiency and toxicity, the concentrations of the exudates of organic acids from tissues change to increase the bioavailability and tolerance of Zn in soil and plants (Hoffland *et al.*, 2006; Wójcik *et al.*, 2006; Xu *et al.*, 2007). Organic acids are usually present in plants and rhizosphere. Organic acids are low-molecular-weight organic compounds, containing predominantly carbon, hydrogen, and oxygen and possessing one or more carboxyl groups (Jones, 1998). Owing to their special structure, they prove to be indispensable in resisting Zn stress. A very small amount of cellular metal ion content exists as free ions (Outten and O'Halloran, 2001) in higher plants. So, organic acids can function as chelators that sequester Zn ions in the cytosol or subcellular compartments (Ernst, 1975; Mathys, 1977; Haydon and Cobbett, 2007; Monsant *et al.*, 2011). Moreover, they could not only be useful for translocation within the plant but also for mobilization from the soil (White *et al.*, 1981; Hoffland *et al.*, 2006; Haydon and Cobbett, 2007).

After being transported to the proper tissue, metals must be properly distributed at the subcellular level to ensure that they are present in sufficient concentration in the necessary compartments (Palmer and Guerinot, 2009). Subcellular compartmentation involves the accumulation of excess Zn in the vacuoles and cell wall. It also maintains more Zn in the cytoplasm. In other words, subcellular compartmentation is an important way of improving Zn efficiency (Hacisalihoglu *et al.*, 2003; Hacisalihoglu and Kochian, 2004). During this process, the form of Zn chelators and the system of Zn transporters play an important role in balancing the distribution of Zn in plants. As organic acids are primarily located within the vacuole (Ryan and Walker-Simmons, 1983; Guerinot and Eide, 1999), an association between metals and organic acids indicates that metal-organic acid complexes participate in subcellular compartmentation (Sun *et al.*, 2006). In addition, some transport mechanisms are involved in the secretion or uptake of Zn ion ligands by cells and in the movement of Zn-binding ligands transported from one cell to another by specific transporter between subcellular compartments (Mathys, 1977; Haydon and Cobbett, 2007). Therefore, organic acid can be associated with both Zn chelators and Zn transporters, which are required for mobilization into the roots, translocation, accumulation, storage, and detoxification within the plant. Thus, an efficient distribution of Zn is ensured in plants.

In recent years, several research studies have only investigated how organic acids play a pivotal role in Zn tolerance to Zn deficiency and Zn toxicity of gramineous plants. Very little information is available about the effect of organic acids on Zn tolerance to Zn deficiency and Zn toxicity encountered in perennial woody apple trees. According to an earlier study, nutritional storage is an important characteristic of apple trees (Alongi *et al.*, 2003). So, the mechanism of Zn tolerance is more complex in apple trees compared with that in gramineous plants.

In this study, the rootstock of apomictic apple, *Malus hupehensis* (Pamp.) Rehd, was selected to study the role of organic acids in Zn homeostasis in the roots of apple tree, to deduce the relationships among organic acid, latent Zn transporter, Zn compartmentation, and Zn homeostasis in the root of apple trees, and to provide the scientific rationale of Zn fertilization in the apple orchard.

MATERIALS AND METHODS

Plant growth conditions

In the period extending from 2008 to 2009, a climate-control chamber was used to perform the experiment at the College of Life Sciences, State Key Laboratory of Crop Biology, Shandong Agricultural University, China. Apple (*M. hupehensis*) seeds were surface-sterilized in 30 g L^{-1} $KMnO_4$ for 15 min. Then, till the emergence of the third leaf, they were cultured with distilled water at 4 °C for about 45 d in a phytotron. Subsequently, the seedlings were grown in the 1/2 and 1/1 strength nutrient solutions, respectively, for 7 d (preculture). Then, they were transferred into the white plastic pots containing 800 cm^3 nutrient solution. The pH of nutrient solution was adjusted daily to 6.5 with 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} HCl. The nutrient solution was aerated continuously and renewed every 3 d. During the entire experimental period, plants were grown in a climate-controlled chamber having a light intensity of 400–500 μmol m^{-2}

s^{-1}, a light/dark period of 16 h/8 h, a day/night temperature of 28 °C/22 °C, and a day/night relative humidity of 80%/85%.

Zn deficiency and toxicity treatments

The basic nutrient solution was prepared according to Han *et al.* (1994), however Zn was removed. This basic nutrient solution contained many different ions in different concentrations, namely 73 mg L^{-1} N, 31 mg L^{-1} P, 78.2 mg L^{-1} K, 70.47 mg L^{-1} Ca, 20.8 mg L^{-1} Mg, 16.7 mg L^{-1} S, 0.11 mg L^{-1} B, 0.5 mg L^{-1} Mn, 0.25 mg L^{-1} Cu, 0.54 mg L^{-1} Na, 0.5 mg L^{-1} Cl, and 1.31 mg L^{-1} Fe (the source of iron being EDTAFeNa). The chelator-buffered nutrient solution designed by Yang *et al.* (1994) was used to control Zn^{2+} activity. After being precultured for 14 d (see above), the seedlings were exposed to different Zn treatments, including Zn deficiency (low Zn, 0 μmol L^{-1}), the control (adequate Zn, 4 μmol L^{-1}), Zn toxicity (toxic Zn, 100 μmol L^{-1}). The Zn^{2+} was supplied in sulfate form. All these Zn treatments were added with 57.2 μmol L^{-1} Na_2-EDTA. After subjecting the seedlings to different Zn treatments, the root morphology parameters were measured at 1, 5, 13, and 20 d by Epson expression 10000XL scanner (WinRHIZO, Epson, Japan). Three replicates of each treatment were performed.

Correlation between Zn and organic acid concentrations in roots

After the seedlings were precultured for 14 d, basal nutrients containing low levels of Zn (0, 0.5, 1, 1.5, and 2 μmol L^{-1}) were added. Zn^{2+} was supplied in sulfate form. The high levels of Zn (40, 60, 80, and 100 μmol L^{-1}) were applied in solutions. All the treatments were added with 57.2 μmol L^{-1} Na_2-EDTA. After being treated with Zn for 1 d, the seedlings were harvested and soaked in 20 mmol L^{-1} Na_2-EDTA solution for 15 min to release the Zn^{2+} adsorbed on root surfaces. The harvested plants were washed thrice with distilled water. Then, the roots, stems, and leaves of plants were collected and rapidly frozen in liquid N_2. After being lyophilized in freeze-dry apparatus (4 °C), the samples were stored immediately at −20 °C till the analyses of organic acid and Zn concentrations. We calculated the Zn transfer factor using the following equation: Zn transfer factor = the accumulation of Zn in shoots/the accumulation of Zn in roots. Three replicates of each treatment were performed. For measurement of Zn concentration in the plants, the experimental materials were washed thrice with distilled water. Then, they were dried in an oven at 105 °C for 15 min. Dry mass of the seedlings was determined after drying them to constant weight at 80 °C. Atomic absorption spectrophotometry (AAS, SP9-400 type, England PYE Co., Cambridge, UK) and HNO_3-$HClO_4$ hot digestion method were used for measuring the concentration of Zn in the plants.

Extraction and analyses of organic acids from roots

The method of Harmens *et al.* (1994) for extraction of organic acids was modified and applied as follows. The lyophilized seedling root was homogenized in liquid nitrogen. Then, about 20 mg of powder was re-suspended in 5 mL 80% (v/v) alcohol for 12 h. It was then heated and stirred at 75 °C in a centrifuge tube for 30 min. The resulting solution was centrifuged at 5 000 × *g* for 10 min (Eppendof 5804, Hamburg, Germany). The supernatant was collected and 2.5 mL 80% (v/v) alcohol was added to the residue for two cycles. Then, 10 mL supernatant was collected and concentrated. Thereafter, the solution was dried by rotary evaporator at 40±2 °C. The supernatant was redissolved in 2 mL distilled water, and the solution was filter-sterilized (0.45 μm). These sterile samples were stored immediately at −20 °C for later analysis. Before measuring the sample, the organic acid solution was filtered twice with a 0.45 μm filter film. High performance liquid chromatography (HPLC, Shimadzu, Japan) was used to isolate the organic acids from roots and determine the contents of organic acids. The chromatographic conditions are described as follows: column, Aglient C_{18} (5 mm, 4.6 mm × 150 mm); mobile phase, 0.018 mol L^{-1} KH_2PO_4 (pH 2.85):methanol = 98:2 (v/v); injection volume, 10 μL; flow rate, 0.8 mL min^{-1}; column temperature, 30 °C; detector, Shimadzu SPD-10A spectrophotometer, ultraviolet at 214 nm. Determination of organic acids was performed using the standard reagent method, and the contents of these organic acids were determined through the peak area method.

Concentration-dependent kinetics of Zn uptake

The tests to determine the effects of external Zn concentrations on Zn uptake were performed according to the methods of Claassen and Barber (1974). After being precultured for 14 d, the seedlings were exposed to Zn deficiency (low Zn), the control (adequate Zn), and Zn toxicity (toxic Zn) solution for 1 d, respectively. The desorption procedures were conducted for 24 h in distilled water to remove apoplastic Zn. Then, the seedlings were incubated in fresh nutrient solutions

containing different concentrations of Zn (0, 2, 4, 10, 20, 40, 60, 80, and 100 μmol L^{-1}). After being incubated for 4 h, the solutions were complemented to their former volume using distilled water. The Zn uptake rate (R) was calculated using the following equation:

$$R = (V_0 \cdot C_0 - V_1 \cdot C_1)/w \cdot t \quad (1)$$

where V_0 is the fresh nutrient solution volume, C_0 is the fresh nutrient solution concentration, V_1 is the solution volume after 4 h absorption, C_1 is the solution concentration after 4 h absorption, w is the root dry weight, and t is the absorption time.

Zn concentrations of the nutrient solution were subsequently determined after acid digestion. Three parallel experiments were performed.

Preparation of protoplast and vacuoles

According to the method of Ma *et al.* (2005), the preparation of protoplast was modified as follows: roots were sliced into 1 to 2 mm pieces with a sharp razor and floated on protoplast isolation medium. The protoplast isolation medium consisted of 0.6 mol L^{-1} mannitol, 20 mmol L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES), 0.5 mmol L^{-1} $CaCl_2$, 10 g L^{-1} Cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan), and 1 g L^{-1} Pectolyase Y-23 (Kikkoman, Tokyo, Japan; pH 6.0 adjusted with 1 mol L^{-1} KOH). Usually, 0.5 g roots were incubated in 10 mL medium in a Petri dish (9 cm in diameter) at room temperature (20 °C). After incubating for 5 h and gently shaking in a shaker under shading, the medium was filtered through 10 layers of cheesecloth. The filtered medium was centrifuged in a swinging-bucket rotor at 120 × g for 5 min. The residue was suspended again in the medium containing 0.6 mol L^{-1} sucrose, 20 mmol L^{-1} MES, and 0.5 mmol L^{-1} $CaCl_2$. A discontinuous gradient was formed by successively layering the solutions containing 0.48 mol L^{-1} sucrose, 0.12 mol L^{-1} mannitol, 20 mmol L^{-1} MES, 0.5 mmol L^{-1} $CaCl_2$, and the protoplast isolation medium without enzymes. After centrifugation at 900 × g for 20 min, we collected the fraction at the interphase of the 0.48 mol L^{-1} sucrose and protoplast isolation medium without enzymes layers. The sucrose was removed by washing with protoplast isolation medium without enzymes. The purity of protoplasts was examined under a light microscope. The purified protoplasts (approximately 1 mL) were used in the determination of Zn.

The isolated and purified protoplast suspension was mixed with 10 mL of vacuole isolation medium, which consisted of 0.5 mol L^{-1} mannitol, 1 mmol L^{-1} ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA), 0.5 mmol L^{-1} 3-[(3-cholamidoproyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 20 mmol L^{-1} MES (pH 8.0, adjusted with 2 mol L^{-1} *Tris* solution). Then, the mixture was gently inverted by hand for 3 min. After centrifugation at 900 × g for 3 min, the residue was suspended again in 10 mL of 0.5 mol L^{-1} mannitol. The solution of 20 mmol L^{-1} MES (pH 8.0) was used for removing EGTA and CHAPS. After centrifugation at 900 × g for 3 min, the residue was suspended again in a solution containing 0.5 mol L^{-1} mannitol, 20 mmol L^{-1} MES (pH 8.0), and 100 g L^{-1} ficoll. A discontinuous gradient was formed by layering a solution containing 0.5 mol L^{-1} mannitol and 20 mmol L^{-1} MES (pH 8.0) without ficoll. The fraction between the 0% and 10% layers was collected after centrifugation at 900 × g for 15 min, and ficoll was removed by washing with 0.5 mol L^{-1} mannitol and 20 mmol L^{-1} MES (pH 8.0). The purified vacuoles were then sub-sampled, and the Zn concentration was measured through different statistical analyses.

Statistical analyses

Variance of analysis (ANOVA) was performed on the experimental data. Differences between means were evaluated for significance using the Duncan's multiple range test at $P \leq 0.05$. Pearson correlation coefficients were calculated between the different plant parameters.

RESULTS

Growth and root morphology of M. hupehensis seedlings

At different treatment days, *M. hupehensis* seedlings showed different variation patterns in Zn concentrations (Fig. 1). No obvious difference was found among the control, low Zn, and toxic Zn plant seedlings after 1 d treatment (Fig. 1a). However, Zn deficiency became apparent in rosette of leaves after 13 d treatment. Shoot elongation was reduced and smaller leaves in apex just occurred (Fig. 1c, d). Zn toxicity resulted in chlorosis of leaves at 5 d (Fig. 1b). After 13 d of Zn treatments, smaller leaves were reported for the low Zn treated seedlings, while chlorosis occurred more severely in the toxic Zn treated seedlings (Fig. 1c, d, e).

There was no difference in the roots of the control and low Zn seedlings. The length, surface area, diameters, and tip numbers of roots were initially identical in the control and low Zn seedlings (Fig. 2). Compared with the seedlings in the control treatment, the length and surface area of the roots of low Zn seedlings both increased by 40% at 5 d. However, the length, surface area, diameter, and tip number of roots decreased by 25.1%, 15.4%, 32.6% and 44.0%, respectively, at 20 d

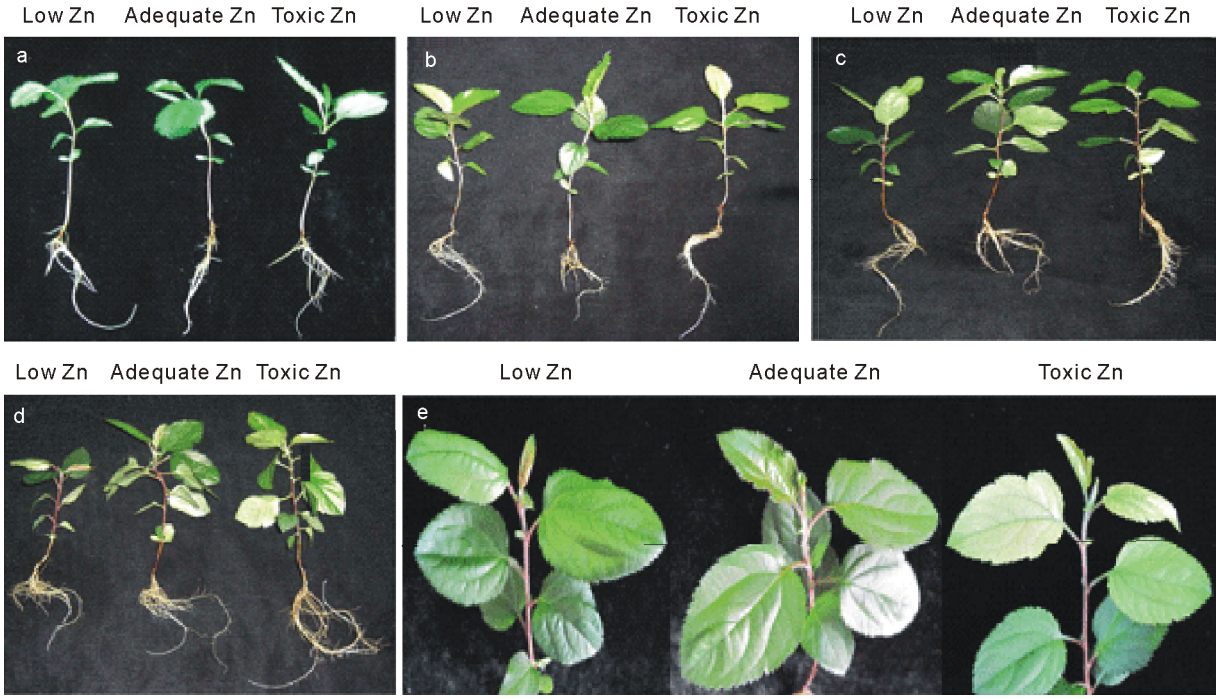

Fig. 1 Symptoms of *Malus hupehensis* seedlings at 1, 5, 13 and 20 d (a–d, respectively) and the symptoms of leaves at 15 d (e) as affected by low, adequate and toxic Zn treatments.

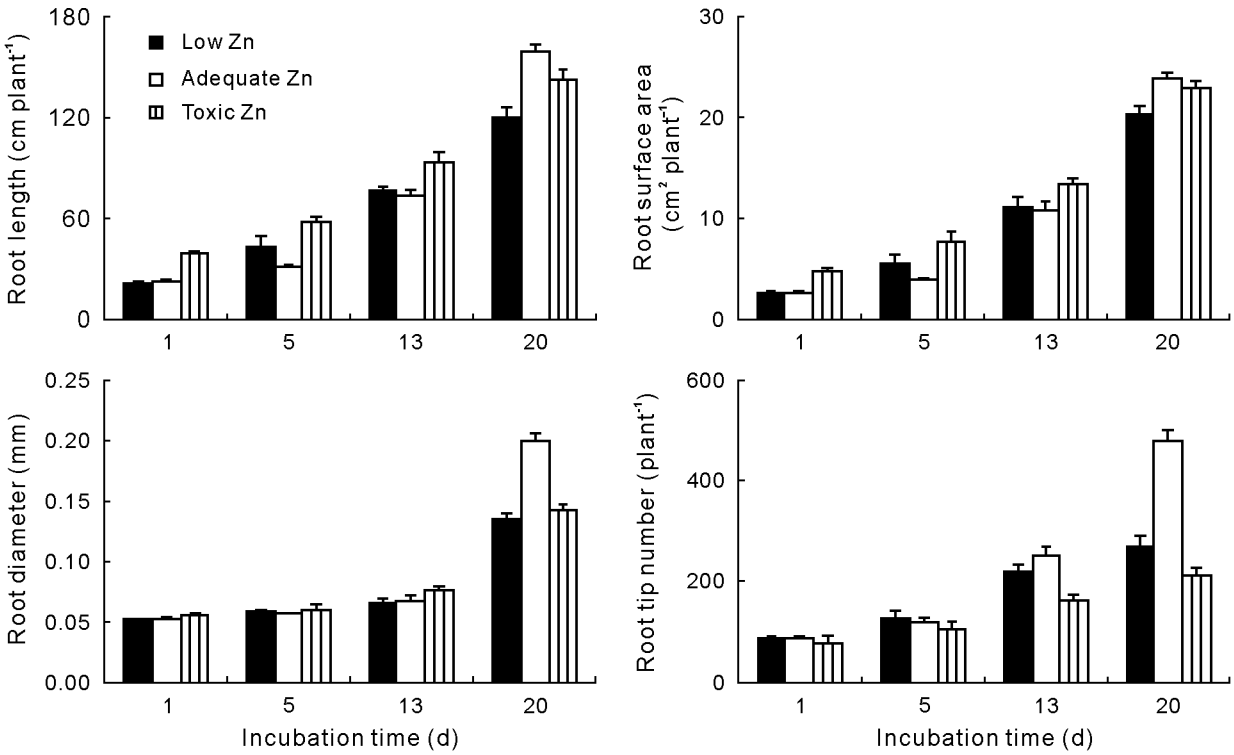

Fig. 2 Root length, surface area, diameter and tip numbers of the *Malus hupehensis* seedlings as affected by low, adequate and toxic Zn treatments. Vertical bars indicate standard errors of the means ($n = 3$).

in the low Zn treated seedlings, compared with the control.

When seedlings grew under toxic Zn treatment, the length and surface area of roots increased significantly compared with those of the control. Compared to the length of root in the control, a 30% to 90% increase occurred in the length of the roots of seedlings treated with toxic Zn for about 1 to 13 d. On the other hand,

compared with the control treatment, 20% to 100% increase was reported in the surface area of roots of seedlings subjected to toxic Zn treatment for about 1 to 13 d. The length, surface area, diameter, and tip number of roots decreased by 10.4%, 4.0%, 29.3% and 55.8%, respectively, through 20 d toxic Zn treatment, compared with the control treatment. These results suggest that root morphology, especially the length and surface area of roots, can be changed by Zn deficiency and Zn toxicity.

Zn concentration and accumulation in various tissues of seedlings

Fig. 3 shows the concentrations and accumulations of Zn in roots, stems, and leaves of *M. hupehensis* seedlings treated with low, adequate, and toxic Zn during different incubation times ranging from 1 to 20 d. Under low Zn treatment, Zn concentrations and accumulations in the tissues were in the orders of root > stem ≈ leaf and leaf > stem > root, respectively. In the time period ranging from 1 to 20 d, Zn concentrations showed a decreasing trend in various tissues. The concentrations of Zn ranged from 15.4 to 10.4 mg kg^{-1} in the roots, 9.5 to 6.3 mg kg^{-1} in the stems, and 13.9 to 8.9 mg kg^{-1} in the leaves (Fig. 3). On the other hand, Zn accumulations showed an increasing trend in various tissues. The accumulations of Zn ranged from 0.07 to 0.2 mg kg^{-1} in the roots, 0.2 to 0.4 mg kg^{-1} in the stems, and 0.8 to 1.5 mg kg^{-1} in the leaves (Fig. 3). However, compared with the control, the concentrations of Zn decreased by 34% to 76% in the roots, 7% to 72% in the stems, and 0% to 57% in the leaves with the increasing treatment time. While the accumulations of Zn in the roots, stems and leaves decreased by 40% to 72%, 9% to 58% and 8% to 74%, respectively. The concentration and accumulation of Zn in the roots treated by low Zn for 1 d were both decreased by 41%. But, there was no significant difference in both the concentration and accumulation of Zn in the stems and leaves, as compared with that of the control. However, the concentration and accumulation of Zn in the seedlings treated with low Zn for 20 d decreased by 57% to 76% and 58% to 74%, respectively, when compared with those of the control. The biggest decrease difference in the concentration and accumulation of Zn was found in the roots under Zn deficiency.

Under toxic Zn treatment, Zn concentrations and accumulations in the plant tissues of *M. hupehensis* seedlings were in the order of root > stem > leaf and root ≈ leaf > stem, respectively (Fig. 3). Both Zn concentration and accumulation showed decreasing trends

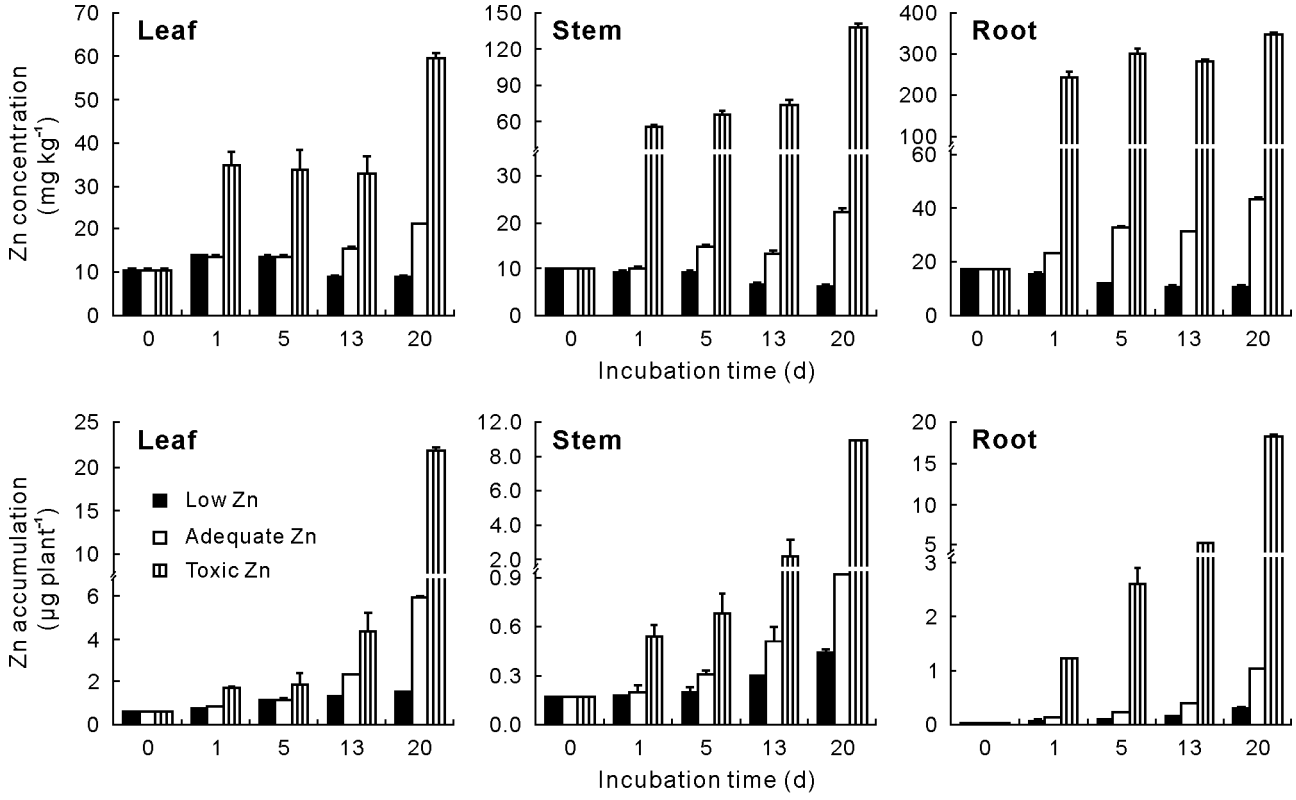

Fig. 3 Zn concentrations and accumulations in the leaves, stems and roots of *Malus hupehensis* seedlings as affected by low, adequate and toxic Zn treatments. Vertical bars indicate standard errors of the means ($n = 3$).

in various tissues with increasing time. The concentrations of Zn ranged from 243.4 to 346.2 mg kg^{-1} in the roots, 55.5 to 136.7 mg kg^{-1} in the stems, and 34.7 to 59.5 mg kg^{-1} in the leaves (Fig. 3). While the accumulation of Zn ranged from 1.2 to 18.4 mg kg^{-1} in the roots, 0.5 to 8.9 mg kg^{-1} in the stems, and 1.7 to 21.7 mg kg^{-1} in the leaves (Fig. 3). Compared with the control, with the increasing treatment time, Zn concentrations in the roots, stems and leaves increased by 700% to 940%, 330% to 510%, and 110% to 180%, respectively. Zn accumulations in the roots, stems, and leaves increased by 830% to 1 680%, 180% to 740%, and 100% to 260%, respectively. After treatment with toxic Zn for 1 d, the concentration and accumulation of Zn significantly increased ($P \leq 0.05$) by 800%, 210%, and 100% in the roots, stems, and leaves, respectively, compared with those of the control treatment. However, the concentration and accumulation of Zn of the seedlings treated by toxic Zn for 20 d increased by 180% to 700% and 260% to 1 680%, respectively, compared with the control. The biggest increase difference in concentration and accumulation of Zn was found in the roots treated with toxic Zn. The results indicate that the roots are the first and the most important tissue reacting to the Zn deficiency and toxicity.

Organic acids concentration and accumulation in roots of seedlings

To determine the effects of Zn deficiency and toxicity on the organic acids in the roots of apple tree, the concentrations and accumulations of oxalic, malic, and tartaric acids in the roots were measured after subjecting to different Zn treatments (Fig. 4). When subjected with low and toxic Zn treatments, the concentrations of all the three organic acids in the roots increased at 1 d, but these concentrations decreased at 20 d. However, the accumulations of organic acids in the roots increased with the treatment time (Fig. 4).

Compared with the control, low Zn and toxic Zn increased the concentrations of oxalic and malic acids in the roots by 30% to 50% ($P \leq 0.05$) after 1 d of treatment, but there was no significant difference in the concentration of tartaric acid. However, the concentrations of the three organic acids significantly decreased ($P \leq 0.05$) by 45% to 64% when treated with Zn for 20 d (Fig. 4). On the other hand, compared with the control, the accumulations of oxalic and malic acids significantly increased in the roots after being exposed to low and toxic Zn solutions for 1 d (Fig. 4). These results indicate that both Zn deficiency and toxicity

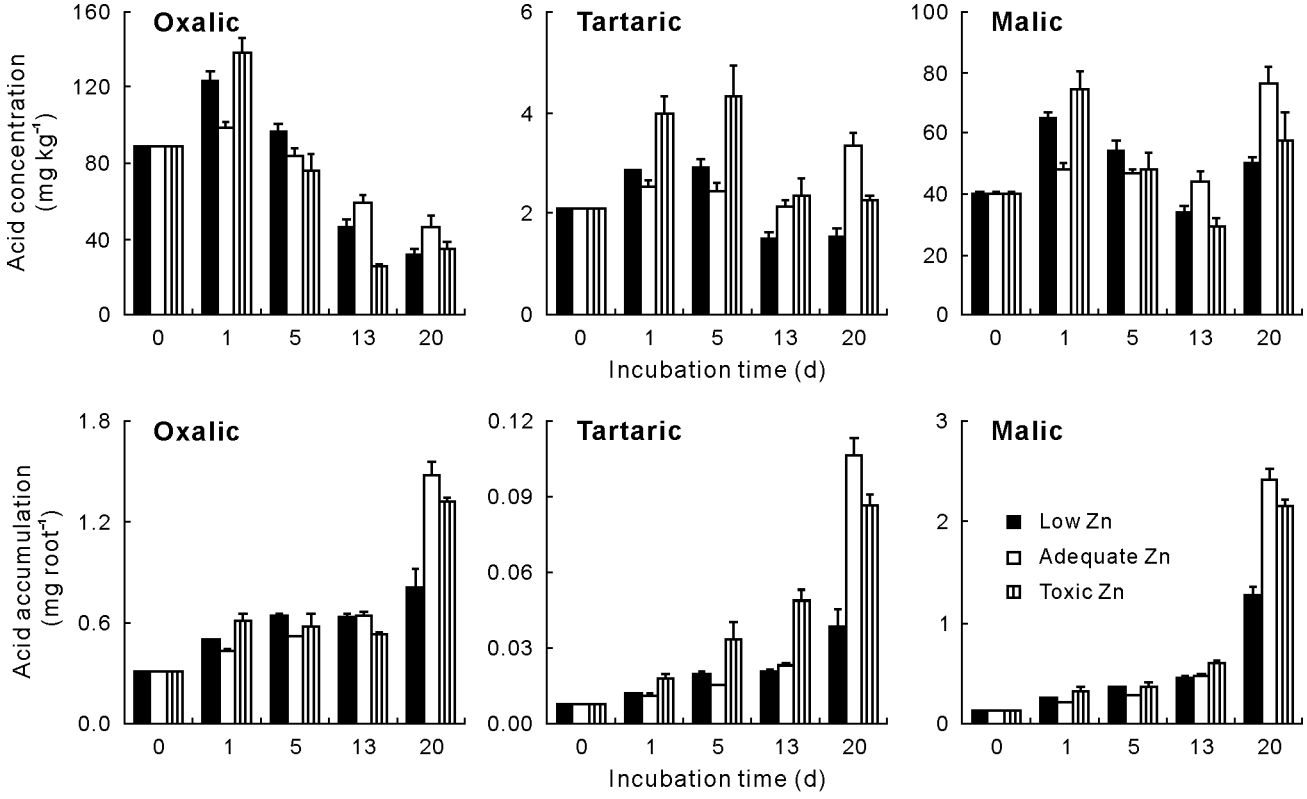

Fig. 4 Concentrations and accumulations of oxalic, tartaric and malic acids in the roots of *Malus hupehensis* seedlings as affected by low, adequate and toxic Zn treatments. Vertical bars indicate standard errors of the means ($n = 3$).

can change the metabolism or transportation of organic acids and promote the distribution of organic acid in the roots.

Correlations between organic acid concentrations in roots and Zn concentrations in solution and roots

Significant correlations were found among total Zn concentrations in the roots and in the solution and the concentrations of oxalic and malic acids in the roots (Table I). The results showed that at low Zn levels (0 to 2 μmol L^{-1}), there were significantly negative correlations between the concentrations of oxalic and malic acids and Zn concentrations in the solution ($r = -0.660^{**}$ and $r = -0.877^{**}$, respectively) and in the roots ($r = -0.772^{**}$ and $r = -0.951^{**}$, respectively). At high Zn levels (40 to 100 μmol L^{-1}), positive correlations were found between the concentrations of oxalic and malic acids and the concentrations of Zn in the solution ($r = 0.708^{**}$ and $r = 0.695^{*}$, respectively) and in the roots ($r = 0.930^{**}$ and $r = 0.970^{**}$, respectively). These indicated that organic acids might regulate the Zn concentration in the roots under the conditions of Zn deficiency and toxicity.

TABLE I

Correlations between organic acid concentrations and Zn concentrations in the solution and roots of *Malus hupehensis* seedlings under low (0, 0.5, 1, 1.5 and 2 μmol L^{-1}) and high Zn levels (40, 60, 80 and 100 μmol L^{-1}) for 1 d

Zn concentration	Under low Zn levels		Under high Zn levels	
	Oxalic acid	Malic acid	Oxalic acid	Malic acid
Solution	−0.660**	−0.877**	0.708**	0.695*
Root	−0.772**	−0.951**	0.930**	0.970**

*, **Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Concentration-dependent dynamics of Zn uptake in roots

Fig. 5 shows the concentration-dependent absorption of Zn in the roots of seedlings after low Zn and toxic Zn treatments for 1 d. Both types of absorption were described well with a modified Michaelis-Menten type equation at low (2 to 40 μmol L^{-1}) and high Zn levels (20 to 100 μmol L^{-1}). Compared with the control treatment, low Zn and toxic Zn treatments increased Zn uptake over the entire range of applied Zn in the uptake solutions.

The concentration-dependent dynamics of Zn uptake is expressed as $V = V_{max} \cdot C/(K_m + C)$, where V is the uptake rate of Zn in roots, V_{max} is the maximum uptake rate of Zn in roots, K_m is the Michaelis-Menten rate constant of the saturated (second) component,

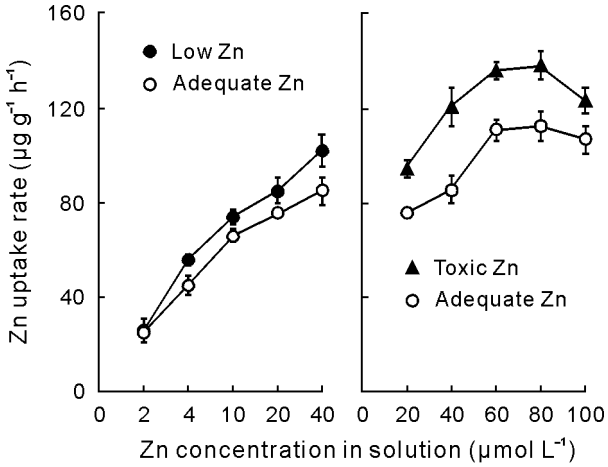

Fig. 5 Kinetics of Zn uptake of *Malus hupehensis* seedlings, treated by low, adequate, and toxic Zn for 1 d, in the solutions containing low (2 to 40 μmol L^{-1}) and high Zn levels (20 to 100 μmol L^{-1}) for 4 h. Vertical bars indicate standard errors of the means ($n = 3$).

and C is the Zn concentration in the nutrient solution. The saturated component represents true uptake across the plasma membrane and not the cell-wall-bound Zn remaining. Values of V_{max} for the low Zn and toxic Zn treatments were 1.3 and 1.2 times, respectively, that for the control treatment (Table II). The corresponding K_m values did not change significantly. These results indicate that Zn deficiency and Zn toxicity can significantly enhance Zn influx into the root symplast and have little influence on the affinity of Zn transporter.

TABLE II

Kinetic parameters[a)] of Zn uptake in roots of *Malus hupehensis* seedlings as affected by Zn deficiency and toxicity after 1 d exposure

Treatment	V_{max}	K_m	r
	mg kg^{-1} h^{-1}	μmol L^{-1}	
		Zn deficiency	
Low Zn	253.7a[b)]	7.54a	0.995**
Adequate Zn	197.4b	6.44a	0.997**
		Zn toxicity	
Toxic Zn	149.6a	0.53a	0.882*
Adequate Zn	128.7b	0.75a	0.917**

*, **Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

[a)] V_{max} is the maximum uptake rate of Zn in roots and K_m is Michaelis-Menten rate constant of the saturated (second) component.

[b)] Means followed by the same letter within each column are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Zn transfer factor in seedlings

Compared with the control, Zn concentration in the roots significantly decreased after 1 d of low Zn

treatment, but no differences were reported in the concentrations of Zn in stems and leaves, indicating that there was no more Zn transport to the shoots at this time. Zn transfer factor in the seedlings under the toxic and adequate Zn treatments after 1 d exposure were 1.96 and 4.53, respectively, decreasing by 57%.

There were high correlations ($P \leq 0.01$) among Zn transfer factor, Zn concentration in roots, and oxalic acid in roots under high Zn levels (40 to 100 μmol L^{-1}) for 1 d; the correlation coefficient between Zn transfer factor and Zn concentration in roots was −0.996 and that between Zn transfer factor and oxalic acid in the roots was −0.943, indicating that oxalic acid can regulate Zn^{2+} transportation from roots to shoots under Zn toxicity. The correlation between Zn transfer factor and malic acid in roots was low, with the correlation coefficient of 0.289.

Zn subcellular distribution in seedlings

Under low and toxic Zn treatments for 1 d, subcellular Zn concentrations decreased in the following order of toxic Zn > adequate Zn > low Zn in both protoplast and vacuole of the roots of *M. hupehensis* (Table III). No significant differences were found between the low Zn and adequate Zn treatments both in the protoplast and vacuole of roots. But, Zn concentration increased by 233% and 70%, respectively, in the protoplast and vacuole of roots under toxic Zn treatment, compared with the control treatment. It is suggested that toxic Zn increased subcellular Zn concentrations in the roots of *M. hupehensis*.

TABLE III

Subcellular distribution of Zn in roots of *Malus hupehensis* as affected by low, adequate and toxic Zn treatments for 1 d

Treatment	Vacuole	Protoplast	Total Zn
	mg kg^{-1}		
Low Zn	6.33b[a)]	10.83b	34.50c
Adequate Zn	7.83b	13.33b	58.33b
Toxic Zn	13.33a	44.33a	215.58a

[a)] Means followed by the same letter within each column are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

DISCUSSION

In this study, we firstly confirmed that organic acids play a role in the tolerance to Zn deficiency and Zn toxicity in apple trees. This observation is similar to the previous findings in gramineous plants (Hoffland *et al.*, 2006; Xu *et al.*, 2007). In the time period ranging from 1 to 20 d, there were decrease and increase of Zn concentration in all tissues under low Zn and toxic Zn treatments, respectively (Fig. 3). During this period, we found that Zn deficiency became apparent in rosette leaf while Zn toxicity triggered more severe symptoms of chlorosis in *M. hupehensis* seedlings (Fig. 1e). The root morphology was also changed visibly (Fig. 2). Under low and toxic Zn treatments for 1 d, although there was no difference in symptoms (Fig. 1a) in seedlings, Zn concentrations and accumulations significantly changed in tissues (Fig. 3). The changes in roots were quite significant, when compared with those in the stems and leaves. This indicates that there is an exclusion mechanism preventing Zn transport to more sensitive root cells and leaves to react to initial Zn deficiency and Zn toxicity. Interestingly, compared with the control, the concentrations and accumulations of oxalic and malic acids in roots significantly increased under Zn treatment for 1 d (Fig. 4). Meanwhile, the correlations between total Zn and the concentrations of oxalic and malic acids in the roots were significantly negative under Zn deficiency, but were significantly positive under Zn toxicity (Table I). This was attributed to the changes in several enzymatic activities related to organic acid metabolism (Sagardoy *et al.*, 2011). Santa-María and Cogliatti (1998) propounded that root was a Zn pool regulating Zn metabolism in apple trees. Moreover, Zn deficiency and Zn toxicity induced the changes in carbohydrate metabolism of root, which further affected Zn homeostasis in roots after short-time Zn treatment. So, the 1st d of different Zn treatments was a key time in assessing the role played by organic acid in the tolerance of Zn deficiency and Zn toxicity.

Possible ways of organic acid tolerance to Zn deficiency in roots

Early studies showed that organic acid was involved in the bioavailability of Zn uptake in the roots under Zn deficiency (Hoffland *et al.*, 2006). In this study, we investigated the correlation between total Zn concentrations and organic acid concentrations in roots at low Zn levels. The results suggested that there were significantly negative correlations between the concentrations of oxalic and malic acids and the concentrations of Zn in the solution ($r = -0.660^{**}$ and $r = -0.877^{**}$, respectively) and in the roots ($r = -0.772^{**}$ and $r = -0.951^{**}$, respectively) (Table I). This suggests that oxalic and malic acids probably regulate Zn concentrations in the roots of *M. hupehensis* seedlings under Zn deficiency. Firstly, with an increasing treatment time, we found that both the root length and root surface area increased by 40% at 5 d of low Zn treatment (Fig. 2). Meanwhile, the accumulations of oxalic, tar-

taric, and malic acids also increased. As a substance of plant carbon skeleton, organic acid may promote the root growth in order to obtain more Zn to resist long-time Zn deficiency (Genc *et al.*, 2007).

Secondly, in our study, the concentrations of oxalic and malic acids in root exudation increased after 1 d treatment in response to Zn deficiency (data not shown). On one hand, the organic acids in the root can be released into the rhizosphere to maintain electrical neutrality (Jones, 1998; Raghothama, 2000). Thus, the decreasing pH of rhizosphere can be used to increase the capacity of Zn mobility (Cieslinski *et al.*, 1998). On the other hand, organic acids may chelate Zn to increase Zn bioavailability (Hoffland *et al.*, 2006). The increase in the concentrations of oxalic and malic acids may adjust Zn homeostasis in the root and rhizosphere, thereby preventing Zn deficiency in *M. hupehensis*.

In this work, we have reported the results of concentration-dependent Zn uptake study in the *M. hupehensis* seedlings under low Zn treatment for 1 d. It was found that the $V_{\max}$ of Zn under Zn deficiency was 1.3 times that under the control (Fig. 5, Table II). This indicates that there is a higher density of Zn transporters on the plasma membranes in the seedlings root cells under Zn deficiency. Zn-organic acid complex and some Zn transporters identified under Zn deficiency can be transported by special Zn transporters from one region to another in plants (Ramesh *et al.*, 2003; Haydon and Cobbett, 2007). In addition, different organic acids together with Zn can form complexes with different molecular weight (Zhang *et al.*, 2009). The steady high-molecular-weight Zn-organic complexes can be fixed in special cell (Ernst, 1975; Mathys, 1977). Our study illustrated that Zn deficiency kept Zn concentration intact in the protoplasts after 1 d treatment (Table III), suggesting that Zn^{2+} or Zn-organic acid complex may be transported to protoplasts by special Zn transporters. Moreover, Zn coupled with organic acid may form steady high-molecular-weight complex to fix in protoplasts, which helps to maintain more Zn in the protoplasts and improve Zn efficiency in the roots of *M. hupehensis*. However, the mechanisms of the relationships among organic acid, Zn uptake, and translocation by Zn transporters in the roots of apple tree need further investigation.

Possible ways of organic acid tolerance to Zn toxicity in roots

In the present study, positive correlations were found between the concentrations of oxalic and malic acids and the concentrations of Zn in the solution ($r = 0.708^{**}$ and $r = 0.695^{*}$, respectively) and in the roots ($r = 0.930^{**}$ and $r = 0.970^{**}$, respectively) at high Zn levels (Table I). These results indicated that oxalic and malic acids could regulate the concentration of Zn in the roots of *M. hupehensis* seedlings under Zn toxicity.

Firstly, we found that the root morphology changed after 5 to 13 d of toxic Zn treatment (Fig. 2), as similar study in duckweed (Radić *et al.*, 2010). This may be attributed to the fact that the organic acid acts as a substance of plant carbon skeleton to promote the root growth and dilute excessive Zn in the roots of *M. hupehensis* seedlings to tolerate Zn toxicity.

Secondly, significantly positive correlations were found between the concentration of Zn and the concentrations of malic and oxalic acid in the roots of *M. hupehensis* seedlings. Zn^{2+} ions largely accumulated in the roots under Zn toxicity. This indicated that the accumulation of organic acids helped in maintaining the ion balance required for alleviating Zn toxicity. A similar finding was reported by Freeman *et al.* (2004).

Thirdly, the concentrations of organic acids in root exudation increased significantly after being treated with toxic Zn for 1 d (data not shown). Organic acids of root exudates are one of the low-molecular-weight ligands of metals, which can also enhance the tolerance to nutrient stress by promoting or inhibiting metal ion uptake in roots (Kamh *et al.*, 2001; Dong *et al.*, 2008). Under the condition of Zn toxicity, organic acids and Zn may form Zn-organic acid complexes in the root and rhizosphere for facilitating Zn detoxification in the roots of *M. hupehensis*. A similar finding was reported by several authors in other plants (Clemens, 2001; Sarret *et al.*, 2002; Wójcik *et al.*, 2006; Haydon and Cobbett, 2007; Straczek *et al.*, 2008).

When Zn is transported from roots to shoots, organic ligands can help to facilitate the long-distance Zn transportation (White *et al.*, 1981). Zn-citrate and Zn-malate were also detected in xylem and shoots (Salt *et al.*, 1999; Monsant *et al.*, 2011). These Zn complexes are involved in xylem transport and Zn storage in shoots. Zn transfer factor decreased under Zn toxicity for 1 d, affecting the transport capacity from root to shoot. After high level of Zn treatment for 1 d, we investigated the relationships between organic acids and Zn in the roots of *M. hupehensis*. We found that there was a higher negative correlation between the concentration of oxalic acid and Zn transfer factor in the roots ($r = -0.943^{**}$) at high Zn levels of 40 to 100 μmol L^{-1}. On the other hand, there was no significant correlation in malic acid. This indicated that under Zn toxicity, organic acid may regulate the transportation of Zn in *M. hupehensis* seedlings, and oxalic acid may chelate

excessive Zn^{2+} to form a high-molecular-weight Zn-oxalic acid complex. This complex inhibited the transportation of Zn from roots to shoots.

In this work, we conducted the concentration-dependent Zn uptake study in the *M. hupehensis* seedlings under toxic Zn treatment for 1 d (20 to 100 μmol L^{-1}). The results showed that the V_{max} of Zn was 1.2 times that in the control (Fig. 5, Table II). This indicated there was a higher density of Zn transporters on the plasma membranes in the seedlings root cells under Zn toxicity. Meanwhile, Zn toxicity significantly increased Zn concentrations in protoplasts and vacuole, when compared with the control (Table III). Recently, it is reported that a metal tolerance protein MTP3 in *Arabidopsis thaliana* plays an important role in Zn tolerance and Zn compartmentation. MTP3 can transport Zn to symplast of root under higher Zn stress (Arrivault *et al.*, 2006). It is also reported that T-DNA mutants in *AttDT* contain lower malate content in bulk leaf tissue. These mutants have decreased malate transport across the tonoplast and accumulate less malate in isolated intact vacuoles, as compared with the wild type (Emmerlich *et al.*, 2003; Hurth *et al.*, 2005). It seems that organic acids can form Zn-organic acid complex. These complexes can be transported together with Zn by specific Zn transporters into non-physiological activity regions to detoxify excessive Zn and maintain Zn homeostasis in roots of *M. hupehensis* seedlings. Further study is required to understand more comprehensive mechanisms of the tolerance to Zn toxicity existing in apple trees.

CONCLUSIONS

Both Zn deficiency and Zn toxicity increased the concentrations of organic acids and the rate of Zn uptake, thereby changing the Zn transfer factor and subcellular Zn distribution. Significant correlations occurred between total Zn concentration and the concentrations of oxalic and malic acids in the roots of *M. hupehensis* seedlings after short-time Zn treatment. This indicated that organic acid could play a role in maintaining Zn homeostasis to resist Zn stress in apple trees.

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