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EFFECTS OF MANGANESE TOXICITY ON THE GROWTH OF SOYBEAN (*GLYCINE MAX* L.) AT THE SEEDLING STAGE

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Key words: Soybean, Manganese toxicity, Iron, Magnesium

Abstract

Effects of manganese (Mn) toxicity stress on the growth of soybean, the number of Mn spots on leaves and the absorption of iron and magnesium were studied by nutrient solution hydroponics. The results showed that the presence of Mn spots on leaves was the main symptom of Mn toxicity in soybean. When the concentration of exogenous Mn was 25 µmol/l, the leaf generated obvious Mn oxidation spots; when the concentration of exogenous Mn exceeded 50 µmol/l, the growth of soybean was inhibited, and the number of Mn spots increased significantly. With the increase in exogenous Mn concentration, the Mn concentration in the roots, young leaves and old leaves of soybean increased significantly. When the concentration of exogenous Mn reached 200 µmol/l, the number of Mn spots on primary leaves, old leaves and young leaves increased significantly. Although the iron concentration in the roots remained the same, the iron content in the old and young leaves decreased significantly. On the other hand, although Mn toxicity significantly reduced the concentration of magnesium in soybean roots, it increased the concentration of magnesium in old and young leaves.

Introduction

Manganese (Mn) is one of the important trace elements for plant's growth and is absorbed by plants in the form of Mn^{2+} and is involved in chloroplast composition and can maintain the normal structure of the chloroplast membrane (Li *et al.* 2019, Zhang *et al.* 2020). Mn is also involved in the photosynthetic electron transport system and photolysis of water in the photosystem. Therefore, Mn is closely related to plant photosynthesis (Fecht-Christoffers *et al.* 2003). However, when the concentration of Mn in plants exceeds the critical value, chlorophyll synthesis is blocked, photosynthesis is inhibited, and leaf yellowing and shrinkage are typical symptoms of Mn toxicity, such as brown Mn oxidation spots (Führs *et al.* 2010, Liu *et al.* 2019). The excessive absorption of Mn also affects the absorption, transport and making use of other nutrient components, for example, iron, magnesium, calcium, phosphorus and so on, leading to an imbalance in mineral nutrition and thus Mn toxicity (Davis 1996).

The mechanism of plant adaptation to Mn toxicity mainly includes internal tolerance and efflux mechanisms, while the internal tolerance mechanism of Mn mainly includes the improvement of antioxidant enzyme activity, separation of Mn in the extracellular body and vacuoles of leaves (Bueno and Piqueras 2002). A study on cowpea (*Vigna sinensis*) reported that under Mn toxicity stress, cowpea TVu91 can improve the activity of exophytic peroxidase (PODs) in leaves, remove hydrogen peroxide (H_2O_2) produced by Mn toxicity stress in the body, and improve the internal tolerance of plants to Mn toxicity (Fecht-Christoffers *et al.* 2003). Excessive expression of metal tolerance proteins ShMTP, AtMTP and PtMTP regulates the Mn transporter so

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that Mn enters vacuoles, promotes the accumulation of Mn in vacuoles, and enhances the ability of plants to tolerate Mn toxicity (Peiter *et al.* 2007). The efflux mechanism mainly alleviates the toxic effect of Mn by secreting organic acids to chelate rhizospheric Mn ions (Shen *et al.* 1998). For example, under the condition of Mn toxicity, perennial ryegrass cultivars mainly remove the toxic effect of Mn by accumulating Mn in the root, reducing transport overground, and secreting organic acid-chelating rhizosphere ions (Mora *et al.* 2009). In addition, increasing the availability of exogenous elements such as silicon, iron, calcium and phosphorus can also significantly improve the tolerance of plants to Mn toxicity (Dou *et al.* 2009).

Soybeans (*Glycine max*) are native to China and are an annual herb of the genus *Glycine*. As an important economic crop, soybeans are an important source of vegetable oil and vegetable protein for humans (Thapa *et al.* 2016). Soybeans occupy an irreplaceable position in the national economy and are closely related to national food security and the sustainable development of the national economy of People's Republic of China. At present, the food processing industry and the edible oil processing industry, which use soybeans and soybean products as raw materials, are developing rapidly, daily increasing the demand for soybeans. In acidic soils, Mn toxicity is second only to aluminum toxicity as an obstacle to crop growth (Foy and Adams 1984). However, there are few reports on the influence of Mn toxicity on soybean growth and its Mn tolerance mechanism. Thus, in the present study the influence of exogenous Mn concentration on the growth of soybeans and the absorption and movement of nutrient elements, such as Mn, iron and magnesium and the mechanism of soybean Mn tolerance was investigated.

Materials and Methods

The soybean variety used was Yuechun 03-3 (YC03-3), which was obtained from the Root Biology Center of South China Agricultural University (Xue *et al.* 2018). Seed of uniform size were selected, sterilized with 10% NaClO, sown in quartz sand, and germinated in a greenhouse at 25°C. The seedlings were transplanted into 14 L plastic pots for nutrient solution cultivation in the greenhouse. The culture temperature was 25°C/28°C (night/day), the humidity of the greenhouse was 75%, and the illumination intensity was 800 μmol/m^2/s. When the first ternately compound leaves were unfolded, seedlings of the same growth were selected and transplanted into 14 L plastic pots for hydroponic culture. The plastic pot was 30 cm wide × 40 cm long ×12 cm high. The components of the hydroponic nutrient solution were 1500 μM KNO_3, 400 μM NH_4NO_3, 25 μM $MgCl_2$, 1200 μM Ca $(NO_3)_2.4H_2O$, 40 μM Fe-EDTA (Na), 500 μM $MgSO_4.7H_2O$, 300 μM K_2SO_4, 300 μM $(NH_4)_2SO_4$, 0.5 μM $CuSO_4.5H_2O$, 1.5 μM $ZnSO_4.7H_2O$, 500 μM KH_2PO_4, 0.16 μM $(NH_4)_5MoO_{24}.4H_2O$, and 2.5 μM $NaB_4O_7.10H_2O$. The nutrient solution was changed every 7 d, and KOH or H_2SO_4 was used every 2 d to adjust the pH to 5.8 (Chen *et al.* 2016).

After the seedlings were cultivated for 5 d, when the second ternate compound leaves were fully expanded, the soybean seedlings were treated with Mn sulfate ($MnSO_4$), and 5 Mn concentration gradients were set: 5 (normal Mn), 25, 50, 100 and 200 μmol/l. The pH value of the culture solution was adjusted to 5.0 with KOH or H_2SO_4 every day. After 16 d of Mn treatment, the soybean plants were harvested, and various indexes were measured.

After harvesting the soybean plants, the height of the plant and the fresh weight of the aboveground and underground parts were immediately measured. Plant materials were transferred to a drying oven at the temperature of 105°C for 30 min, and after drying until constant weight at the temperature of 75°C, their dry weight was determined. Each index was repeated 4 times.

The amount of Mn oxidation spots on the leaves was determined by the square method (Horst *et al.* 1999), and the number of spots on the middle, back, and front sections of the leaves were measured with a 1 cm^2 transparent plastic film. The average value was determined, and the test was repeated 4 times (Chen *et al.* 2016).

The contents of Mn, magnesium and iron in the shoots and roots of the plants were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Tüzen 2003). Each index was repeated 4 times.

After the soybean plants were harvested, the roots were cut and placed in the root configuration scanning and image analysis system. The computer and special software WinRhizo were used to scan and photograph the plants to obtain the total root length, root tip length, lateral root length and root length (Pornaro *et al.* 2017). Data such as the surface area of root, root volume and average diameter of root were determined in quadruplicate (Pornaro *et al.* 2017).

All data were processed with Microsoft office Excel 2010, and data analysis of the data was accomplished via the statistics software of statistical product and service solutions (SPSS) (13.0 for MS Windows, USA) analysis system for Duncan's multiple comparison and significant difference analysis ($p \leq 5\%$). Different letters indicate significant differences.

Results and Discussion

Mn toxicity caused Mn spots on soybean leaves, and the symptoms were aggravated with the increase in the availability of exogenous Mn. When the concentration of exogenous Mn reached 25 µmol/l, obvious Mn spots appeared on the primary and old leaves of soybeans. Obvious Mn spots appeared at 100 µmol/l Mn on the young leaves. When the concentration of exogenous Mn reached 200 µmol/l, the number of Mn spots on primary leaves, old leaves and young leaves increased significantly. The number of Mn spots on primary leaves (146.50) was observably higher than on old leaves (95.00), and the number of Mn spots on old leaves was significantly higher than on young leaves (13.25). At the same time, obvious signs of shrinkage appeared at the leaf margins of the primary, old, and young leaves (Fig. 1A-C). The increasing number of Mn spots and shrinkage in leaves are the main symptoms of Mn poisoning in soybeans.

Results of this study demonstrated that Mn toxicity could cause Mn spots and shrinkage of soybean leaves. This finding is similar to the results reported in rice (*Oryza sativa*), pea (*Pisum sativum*) cowpea (*Vigna unguiculata*) and barley (*Hordeum vulgare*) (Führs *et al.* 2010). The main symptoms of Mn toxicity in plants are Mn oxidation spots and shrivels on the leaves; the formation of Mn spots is mainly caused by the accumulation of Mn oxides or phenols in the epidermal outer cell walls of the leaves under conditions of Mn toxicity (Fecht-Christoffers *et al.* 2003). Under the stress of Mn toxicity, obvious Mn oxidation spots appeared in the primary leaves, old leaves and young leaves of soybean, indicating that a large amount of Mn oxides or phenols might accumulate in the leaves of soybean. In the present study it was found that when the concentration of exogenous Mn was 25 µmol/l, obvious Mn oxidation spots appeared on the old leaves and primary leaves, along with higher Mn accumulation in young leaves, old leaves and roots. Although the biomass was not obviously inhibited, the total length of root and total surface area of root were prominently reduced under 25 µmol/l Mn treatment, indicating that the critical treatment concentration of Mn toxicity in soybean might be 25 µmol/l. Chen *et al.* (2015) reported that instylo (*Stylosanthes guianensis*), cowpea (*Vigna sinensis*), alfalfa (*Medicago sativa*) and clover (*Trifolium pratense*), the critical concentrations of Mn toxicity were 400, 953, 1258 and 2384 µmol/l, respectively, indicating that soybean is more susceptible to Mn toxicity stress in acidic soil. In addition, it was also found that when the Mn concentration was 25 µmol/l, old leaves had obvious spots, while young leaves did not appear to have obvious Mn spots until the concentration of exogenous Mn was at or above 100 µmol/l. Meanwhile, the amount of Mn spots in young leaves was significantly lower than in old leaves and primary leaves in the meantime with exogenous Mn content, indicating that old and primary leaves were more sensitive to Mn toxicity than young leaves. On the other hand, under Mn toxicity stress, the concentration of Mn in old leaves of soybeans was significantly higher than that in young leaves and roots, indicating that the distribution of Mn in different parts of soybeans was different. The transportation or accumulation of excess Mn in old leaves of soybeans might be an important mechanism for soybeans to alleviate the toxic effect of Mn on the roots and young leaves. This finding differs from the report of Mora *et al*. (2009) who reported that the accumulation of Mn in the roots is one of the main mechanisms for the detoxification of Mn in ryegrass (*Lolium multiflorum*), but the specific physiological and molecular mechanisms need to be further studied.

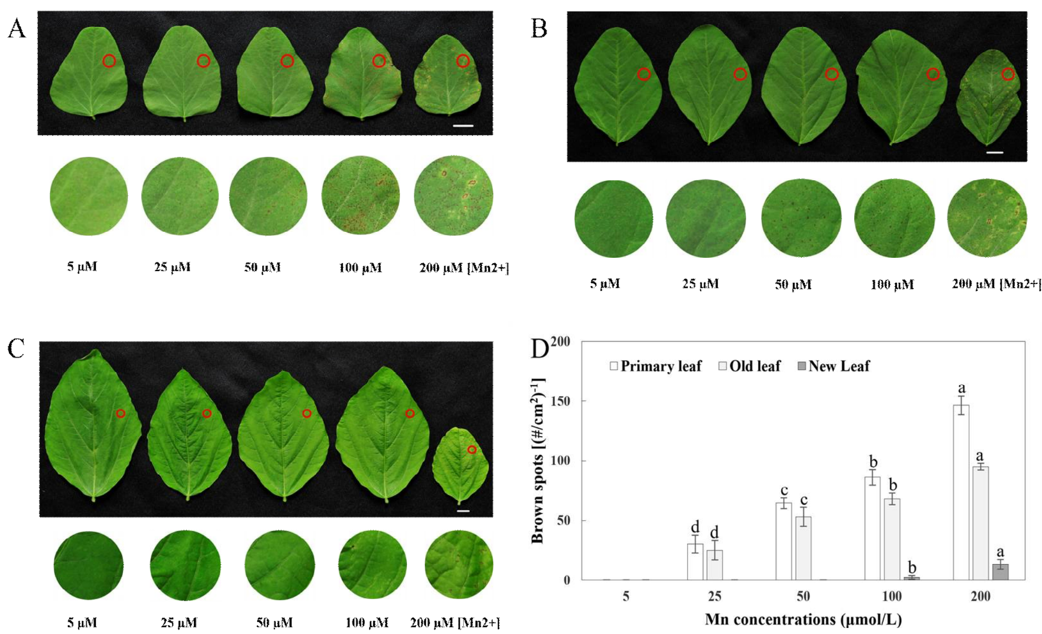

Fig. 1. Results of various concentrations of Mn on oxidation spot accumulation in various types of soybean leaves. (A) Primary leaf; (B) old leaf; (C) young leaf. The picture below displays enlarged image of the leaves with the red circle area into the above picture. (D) Brown spot numbers. The seedlings of soybean were cultivated with routine conditions for 5 days of culture, meanwhile, treated with 5 to 200 µM $MnSO_4$ for 16 days of culture (bars=2 cm). The bar indicates the average value of 4 independent experiment repeats and the standard error (SE). Data in the column charts accompanied with various letters are significant differences among different leaves treatments confirmed via Duncan's multiple range test at the level of p≤5%.

With the increase in the exogenous Mn concentration, the height of soybeans and the biomass of shoots and roots were significantly inhibited (Fig. 2A-E). When the concentration of exogenous Mn was 50 µmol/l, the fresh weights of the shoots and roots were significantly reduced by 7.98 and 10.19%, respectively, compared with the normal concentration of Mn (5 µmol/l). When the concentration of exogenous Mn increased to 100 µmol/l, the plant height and dry weight of the roots were significantly decreased compared with plants under normal Mn conditions, which decreased by 4.92 and 16.45%, respectively. When the concentration of exogenous Mn was 200 µmol/l, the height of plant, fresh and dry weights of the shoots, the fresh and dry weights of the roots were further reduced by 9.99, 49.42, 47.78, 39.44 and 39.14%, respectively. The results showed that soybean plant height, shoot and root biomass were sensitive to Mn toxicity.

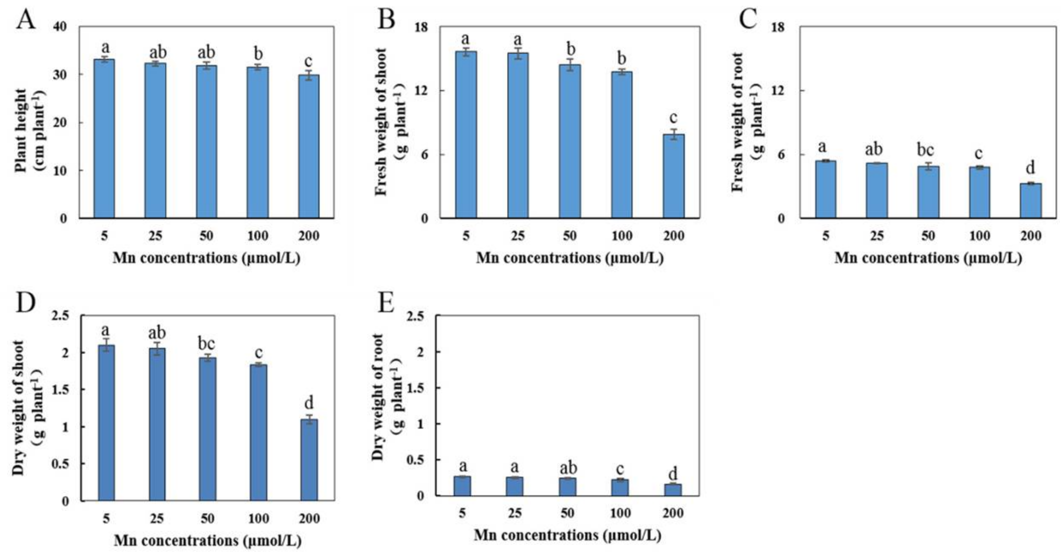

Fig. 2. Influences of various concentrations of Mn on the growth of soybean. (A) Plant height; (B) fresh weight of shoot; (C) fresh weight of root; (D) dry weight of shoot; (E) dry weight of root. The seedlings of soybean were cultivated with routine conditions for 5 days of culture, meanwhile, dealt with 5 to 200 µM $MnSO_4$ for 16 days of culture (bars = 2 cm). The bar indicates the average value of 4 independent experiment repeats and the standard error (SE). Data in the column charts accompanied with various letters are significant differences among different treatments confirmed via Duncan's multiple range test at the level of $p \leq 5\%$.

In addition, it was found that Mn toxicity stress significantly affected soybean root morphology. Compared with the normal Mn concentration (5 µmol/l), the total length of root, surface area of root and average diameter of root were significantly reduced by 6.02, 9.17 and 7.09% under 25 µmol/l exogenous Mn, respectively (Fig. 3A-F). When the concentration of exogenous Mn was 50 µmol/l, the taproot tip length decreased by 8.25% compared to the normal Mn concentration (Fig. 3C). Moreover, when the concentration of exogenous Mn was 100 µmol/l, the root length and root volume decreased by 6.98 and 12.56%, respectively. The total root length, taproot length, taproot tip length, surface area of root, mean diameter of root and volume of root decreased by 37.80, 12.16, 24.76, 40.47, 14.18 and 40.46%, respectively, when the exogenous Mn concentration was 200 µmol/l (Fig. 3A-F).

Excess Mn inhibits soybean root growth. Soybean root growth inhibition might be associated with alteration of root cell wall structure and lignification by Mn toxicity, which provides a relatively comprehensive understanding of the molecular responses of soybean roots to Mn stress (Chen *et al*. 2016).

Exogenous Mn levels significantly affected the Mn concentration in the young leaves, old leaves and roots of soybean, and under the same Mn concentration treatment, the content of Mn in old leaves was observably higher than in roots and young leaves (Fig. 4A). With the increase in the concentration of exogenous Mn, the Mn concentration in young leaves, roots and old leaves gradually increased. When the concentration of exogenous Mn was 200 µmol/l, the concentrations of Mn in the roots, old leaves and young leaves reached a maximum. At the same time, the content of Mn in the old leaves was 39.22 and 37.25% higher than that in the roots and young leaves, respectively, indicating that the Mn absorbed by the roots was mainly moved to the overground parts of the plant and mainly accumulated in the old leaves.

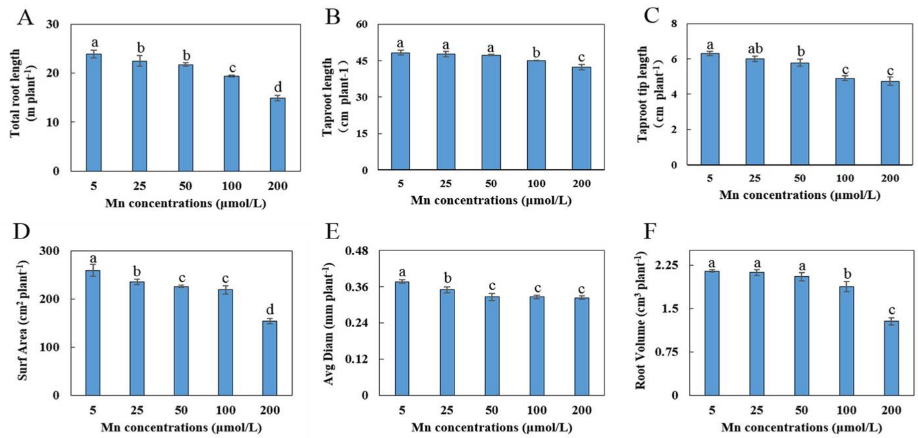

Fig. 3. Effects of the growth of root after dealing with various concentrations of Mn. (A) Total root length; (B) taproot length; (C) taproot tip length; (D) surface area of root; (E) average root diameter; (F) root volume. The seedlings of soybean were cultivated with routine conditions for 5 days of culture, meanwhile, dealt with 5 to 200 μM $MnSO_4$ for 16 days of culture (bars=2 cm). The bar indicates the average value of 4 independent experiment repeats and the standard error (SE). Data in the column charts accompanied with various letters are significant differences among different treatments confirmed via Duncan's multiple range test at the level of p≤5%.

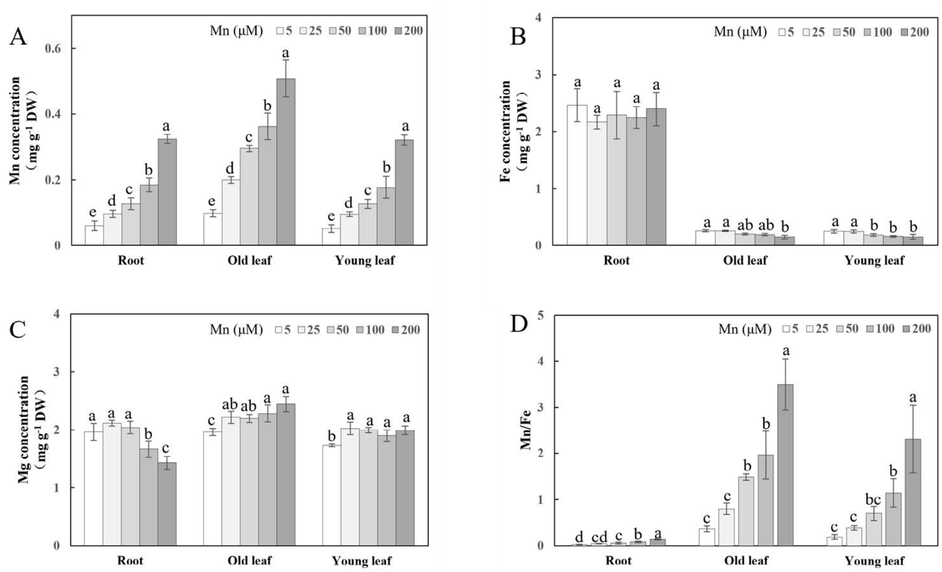

Fig. 4. Concentrations of Mn, iron, and magnesium and the ratio of Mn to iron in the roots, old leaves and young leaves at various levels of Mn. (A) concentrations of Mn; (B) concentrations of Fe; (C) concentrations of Mg; (D) the ratio of Mn to iron. The seedlings of soybean were cultivated with routine conditions for 5 days of culture, meanwhile, dealt with 5 to 200 μM $MnSO_4$ for 16 days of culture (bars=2 cm). The bar indicates the average value of 4 independent experiment repeats and the standard error (SE). Data in the column charts accompanied with various letters are significant differences among different treatments confirmed via Duncan's multiple range test at the level of p ≤ 5%.

With the increase in exogenous Mn concentration, the iron concentration in both old and young soybean leaves showed a gradually decreasing trend, but the iron concentration in the roots was observably higher than in the old and young leaves, and the change was not significant (Fig. 4B). When this concentration was increased to 200 μmol/l, the iron concentration in both old and young leaves was at a minimum, and the iron concentration in the root was 94.17 and 93.75% higher than that in the old and young leaves, respectively. This indicated that the root absorbed and stored most of the iron. Similar to the change in Mn concentration in young leaves, old leaves and roots, the increase in exogenous Mn concentration significantly increased the ratio of Mn and iron (Mn/Fe) (Fig. 4D). When the concentration of exogenous Mn increased to 200 μmol/l, the value of Mn/Fe in old leaves and young leaves increased by 96.43 and 93.90% compared with that in roots, respectively. The results showed that Mn treatment significantly reduced the iron concentration of old and young leaves.

In contrast with the changing trend in the content of Mn, the concentration of magnesium in soybean roots reduced with increasing exogenous Mn concentration (Fig. 4C). When the Mn concentration was 100 μmol/l, the magnesium concentration decreased by 15.31% compared with the normal Mn concentration (5 μmol/l). When the concentration of Mn was 200 μmol/l, the concentration of magnesium decreased by 27.04% compared with the normal concentration of Mn (5 μmol/l). With the increase of Mn concentration, the concentrations of magnesium in the old and young leaves increased gradually. When the concentration of Mn was 200 μmol/l, the concentrations of magnesium in the old and young leaves increased by 24.49 and 15.03%, respectively, compared with the normal concentrations of Mn.

Manganese can help to maintain the normal structure of the chloroplast membrane, participating in the photosynthetic electron transport system and the photodecomposition of water in the photosystem (Fecht-Christoffers *et al.* 2003). Iron (Fe) is directly or indirectly involved in photosynthetic and respiratory electron transfer and affects chloroplast formation, which is a prerequisite for chlorophyll formation (Wilmar *et al.* 1964). Magnesium (Mg) is an essential element for chlorophyll synthesis in plants and plays an important role in plant metabolism (Bot *et al.* 1990, Hauck *et al.* 2003). Therefore, the stability and balance of the relative contents of Mn, iron and magnesium are very important for the synthesis and photosynthesis of chlorophyll. In the present study, it was observed that when the concentration of exogenous Mn was increased from 25 to 50 μmol/l, the concentration of iron in the young leaves of plants was significantly reduced by 25%. This might be due to the antagonistic effect of Mn and iron, suggesting that excessive Mn may inhibit the absorption of iron and the transport process from underground to overground in the plant. Similarly, research results on broomcorn (*Sorghum bicolor*) showed that when the exogenous Mn concentration increased from 0.1 to 10 μmol/l, the absorption of iron in plants was inhibited, leading to a decrease in iron concentrations in plants (Kuo and Mikkelsen 1981). In contrast, promoting iron absorption and increasing iron concentrations will help plants better adapt to Mn toxicity. It has been reported that the iron concentration in Mn-tolerant cotton genotypes was higher than that in Mn-sensitive cotton genotypes (Foy *et al.* 1995). In the present study, the results indicated that under Mn toxicity stress, the iron concentration in soybean roots did not change significantly and remained at a high level, indicating that soybean roots could maintain a high iron concentration to alleviate Mn toxicity, which might be one of the physiological mechanisms of soybean adaptation to Mn toxicity.

Different from the varying trend of Mn concentrations in the plants, the magnesium concentration in soybean roots decreased with an increase in exogenous Mn concentration, indicating that the absorption of Mn^{2+} had certain antagonistic effects. Similar to previous research results, with the improvement in the effectiveness of exogenous Mn, the uptake of magnesium in stylo (*Stylosanthes guianensis*), tomato (*Solanum lycopersicum*), broomcorn (*Sorglum bicolor*) and other plants was inhibited, and the magnesium content in plants was significantly decreased (Bot *et al.* 1990). In the present study, although Mn toxicity stress significantly reduced the magnesium concentration in soybean roots, it increased the magnesium concentration in the aboveground parts (old and young leaves), suggesting that Mn toxicity stress mainly inhibited the uptake of magnesium by soybean roots but promoted the transport of magnesium from the roots to the aboveground parts of the plants. Increase of the concentration of magnesium in the leaves played an important role in relieving Mn toxicity and maintaining the normal function of the leaves, which might be one of the mechanisms of soybean adaptation to Mn toxicity.

Results revealed that soybean Mn tolerance might be related to the accumulation of more Mn in old leaves, the promotion of overground Mn transport, the existence of different Mn distribution patterns (higher in old leaves than in young leaves), and the using of antagonistic effect with iron or magnesium. The present study would lay foundation for the further study and analysis of the physiological and molecular mechanisms of soybean Mn toxicity tolerance.

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