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Physiological response of *Polygonum perfoliatum* L. following exposure to elevated manganese concentrations

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Abstract:

Polygonum perfoliatum L. is a Mn-tolerant plant having the potential to grow in mine wasteland with elevated manganese concentrations. The physiological changes of *P. perfoliatum* grown in different Mn concentrations (5, 500, 1000, 2000, 5000, 10000 μmol·L^{-1}) were investigated in glasshouse study to evaluate its tolerance and physiological response to accumulated manganese. A hydroponic study was carried out in order to study the changes in ultrastructure with increasing Mn concentrations (5, 1000, and 10000 μmol·L^{-1}). Absorption bands of *P. perfoliatum* differed greatly in lipids, proteins and carbohydrates. With elevated levels of Mn (5-2000 μmol·L^{-1}), absorbance changed little, which demonstrated that lower Mn concentrations had a negligible influence on transport functions. With Mn concentrations in excess of 2000 μmol·L^{-1}, absorbance increased slightly but then eventually decreased. Lower Mn concentrations (5 and 1000 μmol·L^{-1}) had no breakage function to the ultrastructure of *P. perfoliatum*. However, as Mn concentration increased to 10000 μmol·L^{-1}, visible damage became evident, the quantity of mitochondria in root cells increased and the grana lamellae of leaf cell chloroplasts revealed a disordered state. Compared with controls, black agglomerations were observed in cells of *P. perfoliatum* grown with 1000 and 10000 μmol·L^{-1} Mn for 30 days. As the Mn concentration reached 10000 μmol·L^{-1}, a novel acicular substance developed in leaf cells and intercellular spaces, possibly indicating a tolerance mechanism in *P. perfoliatum.* These results confirm that *P. perfoliatum* shows potential for the revegetation of abandoned Mn tailings.

Key words: *Polygonum perfoliatum* L.; Manganese tolerance; Chemical composition; Ultrastructure

Introduction

Large areas of metalliferous ore from mining and smelting contain highly toxic metal concentrations, e.g. lead, zinc and manganese, which are phytotoxic to many plant species, and therefore restrict vegetation establishment (Wu et al. 2016; Kong et al. 2017). Plants that have evolved to colonize heavy metal contaminated soils may be classified into two basic strategies, exclusion mechanisms and accumulation (Baker et al. 1989). Metal hyperaccumulating plants are less susceptible to the toxicity of heavy metals, and demonstrate tolerance which has become valuable for phytoremediation of contaminated soils (Hao et al. 2013; Wu et al.2017). It has been reported that more than 500 hyperaccumulators have been discovered, but less than 30 are applicable to manganese (Mn) tolerance (Fernando et al. 2013).

Generally, heavy metals with high concentration would induce damage of cellular ultrastructures in plants; such damage is mainly towards alterations of cellular organelles, e.g. chloroplast, mitochondria and vacuole (Weng et al. 2013; Liu et al. 2017; Chen et al. 2017). Additionally, the extent of damage was closely related to exposure time and concentration of the heavy metal (Keller et al. 2015). For example, the ultrastructure of *Sargassum pallidum* cells were irregular and abnormal following exposure to excessive concentrations of Cu, As, and Pb, whereas Cd particularly destroyed the ultrastructure of chloroplasts and inhibited Chl synthesis (Miao et al. 2014). Elevated Pb concentrations have been shown to adversely affect the cellular structure of *Caenotus canadensis* L. roots (Li et al. 2016) whilst Zn is sequestered in metallo-organic compounds located in leaf vacuoles of *Thlaspi caerulescens* to prevent Zn toxicity (Kupper et al. 1999). Physiological parameters of damage include decreased chlorophyll a production indicating less photosynthetic efficiency, an increase in lipid peroxidation and electrolyte conductivity indicating cell membrane injuries (Majumder et al. 2013). Zayneb (2015) discovered that superoxide dismutase, ascorbate peroxidase and catalase increased following exposure to excessive concentrations of Cd in *Trigonella foenum-graecum* (Zayneb et al. 2015).

Manganese is an essential trace element for plants. Nevertheless, plants exposed to increased Mn concentrations often suffer from Mn poisoning. Plants have developed various mechanisms, including compartmentalization, chelation, avoidance and exclusion, antioxidation, and ion interaction, to overcome Mn

toxicity (Fernando et al. 2015). The exudation of organic acid mainly contributes to Mn detoxification, both internally and externally. *Phytolacca acinosa* may enhance its tissues tolerance to Mn by the exudation and transportation of organic acid following lower Mn treatments (Xue et al. 2011). Absorption bands of *Phytolacca americana* differ greatly in carbohydrates and proteins, largely because of the exudation and transportation of organic substances (Ren et al. 2007). Mn^{2+} release from soils was critical to elucidate the formation of Mn oxides and to assess the biotoxicity of excess Mn^{2+} to plants in an acid soil. The ability of organic acids to promote Mn^{2+} followed the order: citric acid ＞pyritic acid ＞tartaric acid ＞malic acid ＞lactic acid (Yang et al. 2011). The conversion of Mn^{2+} to a metabolically inactive compound by the Mn-oxalate complex, was a key detoxification mechanism (Dou et al. 2009); Mn can be sequestered into a large, metabolically inert intracellular compartment, and is one of the main mechanisms of Mn tolerance and accumulation (Xu et al. 2015).

P. perfoliatum is a Mn tolerant plant found in manganese wasteland tailings in Southern China. It can tolerate Mn concentrations of approximately 41400 $mg \cdot Kg^{-1}$. In this paper *P. perfoliatum* was grown hydroponically in order to investigate if its chemical composition and ultrastructure were affected following exposure to varying Mn concentrations up to 10000 $\mu mol \cdot L^{-1}$. We also attempt to understand the plants response mechanisms for reducing elevated Mn concentrations in its tissues.

Materials and methods

Hydroponics culture

Seeds of *P. perfoliatum* collected from wasteland tailings in Southern China were spread on sand-filled pots. Following germination (~14 days), plants of the same size were selected and their roots thoroughly washed. Hoagland's nutrient solution (Xue et al. 2004) was used as the culture, which included 2.5mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.5 mM KCl, 0.5mM $(NH_4)H_2PO_4$, 2×10^{-4} mM $CuSO_4$, 1×10^{-3} mM $ZnSO_4$, 0.1mM EDTA Fe Na, 2×10^{-2} mM H_3BO_3, 5×10^{-6} mM $(NH_4)_6Mo_7O_{24}$, 1×10^{-3}mM $MnSO_4$. After a 7-day culture in 1/4 Hoagland's nutrient solution and an 8-day culture in 1/2 Hoagland's nutrient solution, plants were grown on in different Mn concentrations (5, 500, 1000, 2000, 5000, 10000 $\mu mol \cdot L^{-1}$), added as $MnCl_2$ (AR). Each treatment was replicated

three times. The process of collection and pretreatment of *P. perfoliatum* followed the standard procedure of Wang et al. (2016).

Plants, using pattern of solution culture (Full strength of Hoagland nutrient solution, , which included 2.5mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.5 mM KCl, 0.5mM $(NH_4)H_2PO_4$, 2×10^{-4} mM $CuSO_4$, 1×10^{-3} mM $ZnSO_4$, 0.1mM EDTA Fe Na, 2×10^{-2} mM H3BO3, 5×10^{-6} mM $(NH4)_6Mo_7O_{24}$, 1×10-3mM $MnSO_4$),

Mn content analysis

Electricity plate digestion was used with ICP-OES in the determination of manganese in subsamples of dried plant tissue (c. 0.1g). The experiment was repeated three times. The acid medium was 20 mL of aqua regia (HCl (AR, mass fraction=36-38%) : HNO_3 (AR, mass fraction=65%) =1:3) and 2 mL of $HClO_4$(AR, mass fraction=70-72%). Sample scouring time was 30s replicated three times. The wavelength of manganese was 2576 nm.

FTIR analysis

The spectral information of various tissues and organs were investigated using Fourier Transform Infrared (FTIR) spectroscopy in the mid-IR range with a Nicolet IS10 infrared spectrometer. The characteristic wavelength was 4000 to 400 cm^{-1} with a resolution of 1 cm^{-1}. Plant samples were finely blended with KBr (0.5/50mg) using an agate mortar.

Cellular ultrastructure analysis

Subsamples of fresh plant tissue were cut into approximately 1-2 mm pieces with a scalpel and subsequently subjected to fixation and embedding protocols. Pretreatment of samples followed the procedure of Xue *et al.* (2016b). Specimens were sliced into ultrathin sections (80 nm slices), and the specific ultrastructures were characterized under a transmission electron microscope (JEOL TEM-1230EX).

EDS analysis

Serial ultrathin sections (120 nm slices) of plant tissue were photographed for their electron cloud density distribution, followed by X - ray spectrum analysis with an EDAX-PHOENIX energy spectrum analyzer. The working condition of the energy spectrum analyzer was as follows: acceleration voltage 80kV, spot size 80 nm diameter, sample table dip 35°, CPS 1500, test time 100s.

Statistical analysis

All analyses were performed in quintuplicate. The data were statistically analyzed with Microsoft Excel 2016, SPSS version 22.0 and Origin 9.1.

Results and discussion

Effect of Mn concentration on biomass of *P. perfoliatum*

The total biomass of *P. perfoliatum* varied inversely with Mn concentration (Table 1). With elevated concentrations of Mn, biomass of *P. perfoliatum* significantly showed an overall reduction, but a slight increase was found at 2000 $\mu mol \cdot L^{-1}$ Mn. In comparison to controls, fresh leaf biomass from 10000 $\mu mol \cdot L^{-1}$ Mn decreased by 60%, and fresh root biomass decreased by 83.33%. Plant growth was not affected at low concentrations, but differences were revealed at high concentrations such as slow growth and a significant reduction in biomass; the plants life cycle was nevertheless still completed. Furthermore, the ratio of leaf to root fresh biomass was related to Mn treatment.

Mn uptake and accumulation characteristics

Manganese translocation was found to be in the order: leaves> roots>stems (Table 2). Manganese content in *P. perfoliatum* tissues increased with increasing Mn concentration. In leaves, Mn reached 13138 $mg \cdot kg^{-1}$ when grown in 500 $\mu mol \cdot L^{-1}$ Mn. At 10000 $\mu mol \cdot L^{-1}$, Mn content in stems and leaves reached its maximum, 16077 and 41400 $mg \cdot kg^{-1}$, respectively. Manganese was an essential trace element for plants in the range of 20-500 $mg \cdot kg^{-1}$,

but plants exposed to over 1500 mg·kg^{-1} Mn often suffer from Mn toxicity (Xue et al. 2010). *P. perfoliatum* showed stronger uptake and enrichment at low Mn concentrations as well as at high levels.

Translocation factor (hereafter referred to as TF) reflects the transportation and distribution of metals in plants from below to above ground. Manganese mainly accumulates in the leaves, which therefore increases its transportation. Plants can chelate Mn, which is then accumulated in the leaves and stems and is one of the important mechanisms by which its toxicity is reduced (Fernando et al. 2013). However, the TF between leaves and roots reached a maximum at 2000 μmol·L^{-1} Mn. A possible reason for this may be chelation and the results support *P. perfoliatum* as a Mn tolerant plant.

Effect of Mn treatments on the chemical composition of *P. perfoliatum*

There was no distinguishing peak displacement, and shoulder peak varied little. Changes of absorbance were not obvious at Mn concentrations below 2000 μmol·L^{-1}, which shows that the exudation and transportation were little influenced during lower Mn treatments. Above 2000 μmol·L^{-1} Mn, absorbance slightly increased but then decreased (Figure 1). This suggested that low concentrations of Mn stimulated the plants to produce organic acids and other exudates to overcome Mn toxicity, but high concentrations affected physiological process in cells.

The stretching vibration peak of 3420 cm^{-1} (free hydroxyl) is mainly reflected in root carbohydrate (cellulose, hemicelluloses, and polysaccharides) (Ren et al. 2008). The band height initially declined but then increased (Figure 1), probably because a large number of hydroxyls from root epidermal cell walls reacted with Mn thereby forming stable compounds. However, elevated exogenous Mn treatments appeared to damage this mechanism. Carboxylic acid O-H and methyl stretching vibration peaks overlapped near 2920 cm^{-1}, mainly as a result of vitamins, membrane and cell wall components. With elevated concentrations of Mn, the band height first decreased then increased. It may be that the production and transportation of organic compounds were associated with Mn treatments. Also, organic acids released from root cells chelated excessive Mn^{2+}. The peak in 1380 cm^{-1} is produced by the C=O stretching mode of carbonyl compounds in aliphatic ketones. Band height first decreased then increased (Figure 1). These results indicated that elevated exogenous Mn treatments increased soil cation

exchange capacity by demethylation of pectin in cell walls, which may increase the tolerance to Mn toxicity. The stretching vibration peak of 1060 cm^{-1} is mainly reflected in alcohol and ether-based ester or phenol group C-O bond. With elevated concentrations of Mn, the absorption peak first decreased then increased. The products of membrane lipid peroxidation accumulated in roots played the leading role in peak variation at Mn concentrations below 2000 $\mu mol \cdot L^{-1}$, but excess Mn^{2+} damaged the process.

There was no distinguishing peak displacement, and shoulder peak varied little in stems of *P. perfoliatum* (Figure 2). With elevated Mn (5-500 $\mu mol \cdot L^{-1}$), absorbance did not alter. Above 500 $\mu mol \cdot L^{-1}$ Mn, absorbance increased slightly then decreased, which appears to show that Mn^{2+} promoted carbohydrate production. The peak near 1735 cm^{-1} is a methyl absorption band (membrane and cell wall) found in oil containing compounds. With increasing Mn concentration, the absorption peak initially decreased then increased, and the peak reached a maximum at 1000 $\mu mol \cdot L^{-1}$. Early lipid peroxidation thereby reducing lipid content and production of aliphatic ketone compounds containing a carbonyl group which gradually increased may explain the increase in peak. Above 1000 $\mu mol \cdot L^{-1}$ Mn, absorbance decreased. Results showed that carbohydrate increased following low Mn exposure, and *P. perfoliatum* strengthened the tolerance by adjusting its osmotic potential, membrane lipid peroxidation was enhanced with lipid and carbohydrate production decreasing at high levels.

Absorption spectra (FTIR) in leaves revealed that the absorption peaks were forced to shift and shoulder peaks had shrunk (Figure 3). With elevated Mn (5-1000 $\mu mol \cdot L^{-1}$), absorbance increased dramatically, which indicated that lower Mn^{2+} had promoted the production and transportation of organic compounds. There was no significant change in absorbance from 2000 to 5000 $\mu mol \cdot L^{-1}$. Above 5000 $\mu mol \cdot L^{-1}$, absorbance decreased dramatically, indicating that excess Mn^{2+} clearly had an impact on the production and transportation of carbohydrates and other organic substances in leaves of *P. perfoliatum.*

Effect of Mn treatments on the ultrastructure of *P. perfoliatum*

P. perfoliatum was grown under glasshouse conditions in order to study its ultrastructure following supply of nutrient solutions supplemented with increasing Mn concentrations (5, 1000, and 10000 $\mu mol \cdot L^{-1}$). Lower Mn concentrations with 5 and 1000 $\mu mol \cdot L^{-1}$ had no breakage function to the ultrastructure of *P. perfoliatum* (Fig 4A,

Fig5A, Fig 6A, Fig 4B, Fig5B and Fig 6B). However, with an increase in Mn concentration of up to 10000 μmol·L^{-1}, visible damage was evident (Fig 4C, Fig 5C and Fig 6C), the quantity of mitochondria in root cells increased and the grana lamellae of leaf cell chloroplasts became disorganized (Fig 4C, 7C). While chloroplast structure and function had obvious damage under excess Mn^{2+}, *P. perfoliatum* still survived, suggesting that *P. perfoliatum* has a higher tolerance to excessive Mn concentrations.

Generally, excess Mn^{2+} has direct cytotoxicity such as to inhibit the uptake and activity of Ca^{2+}, Fe^{2+} and Mg^{2+} whilst inducing oxidative stress, leading to decreased chlorophyll and rubisco contents, damaged chloroplast ultrastructures, reduced photosynthetic rate, and even death. However, certain plant species have evolved in heavy metal contaminated soils which can tolerate excess Mn^{2+} especially in the plant shoot (Blamey et al. 2015). In the present study, lower Mn concentrations with 5 and 1000 μmol·L^{-1} had no breakage function to the ultrastructure of *P. perfoliatum*, and the effects on photosynthesis were minimal as observed by FTIR and TEM. In roots, the exudation of organic acids mainly contributes to Mn detoxification (both internally and externally), uptake and transport. The storage of Mn in the root cell walls may keep the ion sequestered from the root cytoplasm. In leaves, Mn preferentially accumulated in leaf epidermal cells which may be an avoidance mechanism to prevent damage to photosynthetic cells; epidermal cells lack chloroplasts. The conversion of Mn^{2+} to a metabolically inactive compound by organic acid or phenolic compounds, such as the Mn-oxalate complex, is an important detoxification mechanisms (Deng et al. 2010). Further understanding of the molecular mechanisms of Mn tolerance in plants requires further investigation.

P. perfoliatum had a high Mn tolerance, and it may be a result of its detoxification storage form in its cells. The metal transporters involved in removing Mn from the cytosol or moving it to the vacuolar membrane, where Mn can be sequestered into a large and relatively metabolically inert intracellular compartment, play important roles in Mn uptake, transportation and accumulation at the whole plant level (Zhang et al. 2010). Manganese accumulated in the supernatant part, accounting for 74%-82% of the total Mn in the leaves (Xu et al. 2009). Compared with controls, black agglomerations were found in cells of *P. perfoliatum* after treatment with 1000 and 10000 μmol·L^{-1} Mn after 30 days; these became obvious at higher Mn concentrations (Fig 5C and Fig 7C). Black agglomerations were found in cells of Mn tolerant plants, indicating that they were possibly manganese

oxides (Dou et al. 2009, Papadakis et al. 2007 and Xue et al. 2016b). This is consistent with our results in that black agglomerations appeared in the high Mn treatments but this still requires further research.

Acicular substances analysis in leaves of *P. Perfoliatum*

At 10000 μmol·L^{-1}, Mn content in leaves reached a maximum, 41404 mg·kg^{-1} indicating that *P. perfoliatum* strongly accumulates Mn after either long or short-term treatments. To avoid metal toxicity, plants have evolved mechanisms including efflux of metal ions from cells and sequestration into internal cellular compartments (Kim et al. 2004). At a Mn concentration of 10000 μmol·L^{-1}, a novel acicular substance developed in leaf cells and intercellular spaces, possibly indicating a tolerance mechanism in *P. perfoliatum*.

Through energy spectrum analysis Mn concentrations in acicular crystals were significantly greater than in other locations (Figure 8) and it might be a result of the compartmentation of Mn in the cells, possibly indicating a tolerance mechanism in *P. perfoliatum*.

Overexposure to Mn appears to be the basis of a more active extracellular covalent POD bound to the cell wall, being involved in the lignification process (Blamey et al. 2015). Manganese toxicity was also observed with reactions with other elements including phosphorus, calcium and ferrum. (Esteban et al. 2013). Manganese accumulation in epidermal cells suggests that the root endodermis hinders transportation of Mn, protecting the normal physiological processes of cells (Dučićet al. 2012). Phosphate contents in acicular substances by EDS were 7.92% and 11.46%. Phosphate may consume and precipitate Mn reducing its biological activity, but it should be stressed that although it is confirmed that phosphate may play a major role in heavy metal tolerance mechanisms and phytoremediation, the role of phosphate on manganese accumulation in *P. perfoliatum* still requires further research (Kochian et al. 2004; Hauck et al. 2003).

Conclusions

The growth of *P. perfoliatum* was not affected by low concentrations of Mn, whilst differences were revealed at high concentrations, such as slow growth and a significant reduction in biomass. Manganese

distribution was as follows: leaves> roots>stems, with a translocation factor >1. Effects of Mn on the plants chemical composition revealed that *P. perfoliatum* reduces Mn stress through a number of mechanisms including production and transportation of organic substances, organic acid complexation, and membrane lipid peroxidation. Lower Mn concentrations with 5 and 1000 $\mu mol \cdot L^{-1}$ had no breakage function to the ultrastructure of *P. perfoliatum*. However, as Mn concentration increased to 10000 $\mu mol \cdot L^{-1}$, visible damage began to appear in cells of *P. perfoliatum*, the quantity of mitochondria in root cells increased and grana lamellae of leaf cell chloroplasts became disorganized. An unknown acicular substance was also found in the intercellular space and cells, which might be through fixation and precipitation of Mn with phosphate.

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