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# Response of enzymatic and non-enzymatic antioxidant defense systems of *Polygonum hydropiper* to Mn stress

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**Abstract:** The response of enzyme and non-enzymatic antioxidants of Mn hyperaccumuator, *Polygonum hydropiper* (*P. hydropiper*), to Mn stress was studied using hydroponics culture experiments to explore the mechanism of Mn tolerance in this species. Results showed that both chlorophyll and carotenoid contents significantly ($p$<0.05) decreased with increasing Mn treatment levels (0, 0.5, 1, 2, 4, and 8 mg/L) in hydroponics. The concentrations of malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) in the root and shoot of *P. hydropiper* were accumulated under Mn stress. Meanwhile, the anti-oxidative functions of several important enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) in plants were stimulated by Mn spike in leaves and roots, especially at low Mn stress; while sulfhydryl group (—SH) and glutathion (GSH) were likely involved in Mn detoxification of *P. hydropiper* under high Mn stress.

**Key words:** *Polygonum hydropiper*; hyperaccumulation; enzymatic antioxidative defense; non-enzymatic antioxidative defense

## 1 Introduction

Phytoremediation is considered as a novel biological remediation technique for the heavy metals contaminated circumstances with potentially cost-effective, engineering economical and environmentally-friendly characteristics [1−6]. Up to date, researches on phytoremediation are mainly conducted on screening of proper hyperaccumulators and the mechanisms of hyperaccumulator's adsorption, translocation and accumulation as well as its tolerance and detoxification to the heavy metal, and Mn-hyperaccumulators are hardly clear for their mechanisms mentioned above [7]. Hence, it is necessary to explore the mechanisms of Mn accumulation, tolerance and detoxification in hyperaccumulators, considering that it may be highly useful for applying these plants in remediating Mn contaminated soil or water.

The toxic effects of heavy metals were linked to the production of reactive oxygen species (ROS), such as $O_2^-$, $\cdot OH^-$, and $H_2O_2$ [8]. ROS can react with lipids, proteins, pigments, and nucleic acid and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability [9]. On the other hand, the antioxidative defense system plays an important role in tolerance to heavy metal, as they could catch toxic free radicals to protect themselves from oxidative stress induced by heavy metals. The antioxidative defense system comprises enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) are major ROS-scavenging enzymes in plants [10−11]. Low relative molecular mass antioxidants containing thiol (—SH), such as glutathione (GSH) and phytochelatins (PCs) [12] are important non-enzymatic antioxidants.

*Polygonum hydropiper* (*P. hydropiper*) has recently been confirmed as a Mn-hyperaccumulator in field invstigation [13]. So far, little is known about the Mn accumulation, tolerance and antioxidative defenses of non-metalliferous population of hyperaccumulator *P. hydropiper*. The objective of this work is to study the effects of Mn on the enzymatic antioxidative and some non-enzymatic antioxidative defenses, which are very important for a plant in stressed environments: 1) the changes of chlorophyll and carotenoid contents, 2) the lipid peroxidation and other oxidative stress status, and

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3) some enzymatic antioxidants and non-enzymatic antioxidants in tissues of *P. hydropiper*.

## 2 Materials and methods

### 2.1 Plant material and growth conditions

Seedlings of *P. hydropiper* were collected at the Lijiang River in Guangxi Province, China, and cultured in 1/2 Hoglands. Then, new shoots without roots of these plants were cut and grew in 1/2 Hoglands for initiation of new roots. The 1/2 Hogland's solutions contained the following ingredients (mmol/L): $Ca(NO_3)_2{\cdot}4H_2O$ 2.50, $MgSO_4{\cdot}7H_2O$ 1.00, $KNO_3$ 2.50, $KH_2PO_4$ 0.50, $H_3BO_3$ 0.025, $ZnSO_4{\cdot}7H_2O$ $0.40\times10^{-3}$, $MnCl_2{\cdot}4H_2O$ $4.50\times10^{-3}$, $CuSO_4{\cdot}5H_2O$ $0.15\times10^{-3}$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ $0.01\times10^{-3}$ and Fe-EDTA 0.05. After 3 weeks, the fresh weights of seedlings of each treatment were recorded and then the plants were exposed to 0.003 (CK), 0.5, 1, 2, 4 and 8 mmol/L Mn (supplied as $MnSO_4{\cdot}H_2O$) for 21 d. There were 3 replicates for each treatment, and each replicate consisted of 3 plants.

During the period of culture and metal treatment, the solutions were adjusted daily with HCl and NaOH to pH 5.8, aerated every day and renewed once every three days. The plants were grown in a plant culture chamber with 14 h light period, 26/20 °C day/light temperature and 60%−70% relative humidity.

### 2.2 Plant harvest and measurements of plant biomass

The plants were harvested after they were exposed to Mn for 8 days. The harvested plants were rinsed with tap water, and the roots were immersed in 20 mmol/L $Na_2$-EDTA for 15 min to remove Mn adhered to the root surface. Plants were thoroughly washed 3 times with deionized water and blotted dry with a piece of tissue paper. The fresh mass, shoot and root length were recorded. Then, the plants were separated into roots, stems and leaves, some of them were preserved at −20 °C, and the others were dried at 105 °C for 30 min, then at 70 °C to a constant mass.

### 2.3 Determination of Mn concentration

Dried plant samples of *P. hydropiper* were ground, passed through a 60 mesh filter, and digested with a mixture of nitric acid ($HNO_3$) and perchloric acid ($HClO_4$) (3:1, volume ratio). Mn concentrations of all samples were determined by AAS (Hitachi 180-80).

### 2.4 Measurements of chlorophyll content, enzyme activity and lipid peroxidation

Chlorophyll content, enzyme activity and MDA contents were extracted and measured according to our previous report [6]. $H_2O_2$ contents were determined by the method of VELIKOVA et al [14].

### 2.5 Analysis of non-protein thiols

The concentrations of –SH, GSH and phytochelatins (PCs) were measured according to the methods by SNELLER et al [15].

### 2.6 Statistical analysis

Data analysis was processed with Microsoft Excel 2007 and SPSS13.0 software. All the values were expressed as means±standard deviation (S.D.) of three replicates. Data were statistically analyzed by the one-way ANOVA, taking $P<0.05$ as significance according to the LSD test.

### 2.7 Statistical analysis

The statistical analysis was carried out by one-way ANOVA using SPSS 13. The Duncan's method was used for multiple comparison at $p<0.05$ level between treatment means.

## 3 Results

### 3.1 Mn accumulation in plants

The leaves of *P. hydropiper* under hydroponic culture contained more than 10000 mg/kg Mn in all Mn treatments and the maximum Mn content in leaves reached 37490 mg/kg at 4 mmol/L Mn treatment (Table 1). Moreover, *P. hydropiper* also showed an efficient Mn translocation from root to shoot, as *L*/*R* (*L* represents leaf metal concentration, and *R* represents root metal concentration) and *S*/*R* (*S* represents stem metal concentration) were all higher than 1 (Table 1).

### 3.2 Effects of Mn addition on chlorophyll pigments

The results in the present work (Table 2) indicated that chlorophyll and carotenoid concentrations in leaves of *P. hydropiper* decreased greatly in various Mn treatments compared with the values of control. There were no significant ($p>0.05$) reductions in $Chl_a/Chl_b$ under all treatments, and no toxic symptoms were manifested during the whole experiment period.

### 3.3 Lipid peroxidation and $H_2O_2$ accumulation

Malondialdehyde (MDA) in both leaves and roots of *P. hydropiper* increased with elevated Mn levels, and its peak ranked under 8 mmol/L Mn treatment, increased by 92% and 262% against the control, respectively (Table 3). The generation rate of $H_2O_2$ in leaves and roots of *P. hydropiper* was similar to that of MDA, and also reached its maximum at 8 mmol/L Mn addition by an increase of 120% and 129% in comparison with the control, respectively.

### 3.4 Antioxidative enzymes

Changes of some antioxidative enzymes activities in leaves and roots of *P. hydropiper* are listed in Table 4.

**Table 1** Effects of Mn on biomass and Mn concentration in leaves, stems and roots of *P. hydropiper*

| $c$(Mn)/(mmol·L$^{-1}$) | Plant height/cm | Plant mass/g | Root length/cm | Mn concentration*/( mg·kg$^{-1}$) | | | Ratio** |
|---|---|---|---|---|---|---|---|
| | | | | Roots | Stems | Leaves | |
| CK | 17.5±0.3$^{a}$ | 3.8±0.2$^{a}$ | 37.3±0.9$^{a}$ | 52.1±10.8$^{e}$ | 41.5±14.4$^{e}$ | 72.7±17.7$^{d}$ | 2.2 |
| 0.5 | 16.5±0.4$^{b}$ | 3.3±0.2$^{b}$ | 27.7±1.0$^{b}$ | 924.8±31.1$^{d}$ | 5343.3±546.6$^{d}$ | 19542.1±1195.3$^{c}$ | 26.8 |
| 1 | 15.5±0.4$^{c}$ | 3.0±0.1$^{c}$ | 24.3±1.3$^{c}$ | 833.1±35.2$^{d}$ | 6073.9±259.3c$^{d}$ | 30462.8±2412.5$^{b}$ | 43.9 |
| 2 | 14.6±0.3$^{d}$ | 2.3±0.2$^{d}$ | 22.8±0.4$^{c}$ | 1522.5±41.4$^{c}$ | 7927.5±931.3$^{c}$ | 30244.7±1178.2$^{b}$ | 25.1 |
| 4 | 13.6±0.3$^{e}$ | 1.7±0.1$^{e}$ | 19.3±0.7$^{d}$ | 2699.6±153.8$^{b}$ | 12622.4±1939.3$^{b}$ | 37490.5±2495.2$^{a}$ | 18.6 |
| 8 | 11.8±0.3$^{f}$ | 1.0±0.1$^{f}$ | 16.4±0.9$^{e}$ | 4364.1±342.1$^{a}$ | 18273.2±1468.7$^{a}$ | 34794.4±1006.1$^{a}$ | 12.2 |

* means Mn concentration in roots, stems and leaves of *P. hydropiper* upon Mn exposure; Values represent mean±SE ($n$=3); Different letters indicate significant differences at $p$<0.05; ** means ratio of concentrations of Mn in leaves, stems and roots of *P. hydropiper* upon Mn exposure.

**Table 2** Photosynthetic pigments in leaves of *P. hydropiper* under Mn stress

| $c$(Mn)/(mmol·L$^{-1}$) | Photosynthetic pigment/(μg·g$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Chlorophyll *a* | Chlorophyll *b* | Total chlorophyll | Carotenoid | $Chl_a/Chl_b$ |
| CK | 2.47±0.09$^{a}$ | 0.70±0.08$^{a}$ | 3.18±0.06$^{a}$ | 0.43±0.03$^{a}$ | 3.55±0.55$^{a}$ |
| 0.5 | 1.68±0.26$^{c}$ | 0.53±0.21$^{ab}$ | 2.21±0.23$^{c}$ | 0.32±0.02$^{c}$ | 3.71±0.22$^{a}$ |
| 1 | 1.39±0.25$^{cd}$ | 0.37±0.06$^{b}$ | 1.75±0.25$^{d}$ | 0.30±0.01$^{cd}$ | 3.87±0.93$^{a}$ |
| 2 | 1.53±0.78$^{cd}$ | 0.35±0.06$^{b}$ | 1.87±0.15$^{d}$ | 0.28±0.01$^{d}$ | 4.52±0.41$^{a}$ |
| 4 | 2.00±0.10$^{b}$ | 0.55±0.08$^{ab}$ | 2.55±0.17$^{b}$ | 0.37±0.01$^{b}$ | 3.71±0.43$^{a}$ |
| 8 | 1.20±0.09$^{d}$ | 0.42±0.09$^{b}$ | 1.62±0.18$^{d}$ | 0.31±0.02$^{cd}$ | 2.96±0.47$^{a}$ |

Values are means ± S.D. ($n$=3); One-way ANOVA (1 factor: type of issue) is performed for each parameter and different letters in same column mean that there is significantly difference at $p$<0.05 based on LSD; The two letters of the same column mean no explicit difference between them.

**Table 3** Effects of different Mn levels on contents of MDA and $H_2O_2$ of *P. hydropiper*

| $c$(Mn)/(mmol·L$^{-1}$) | MDA/(μmol·kg$^{-1}$) | | $H_2O_2$/(μmol·kg$^{-1}$) | |
|---|---|---|---|---|
| | Leaves | Roots | Leaves | Roots |
| CK | 13.51±0.24$^{e}$ | 8.69±0.24$^{f}$ | 7.93±0.31$^{f}$ | 7.69±0.46$^{e}$ |
| 0.5 | 14.75±0.35$^{d}$ | 10.71±0.18$^{e}$ | 9.88±0.62$^{e}$ | 10.41±0.31$^{d}$ |
| 1 | 15.39±0.20$^{cd}$ | 11.58±0.31$^{d}$ | 11.28±0.19$^{d}$ | 11.16±0.48$^{d}$ |
| 2 | 16.13±0.16$^{c}$ | 12.39±0.47$^{c}$ | 23.12±0.16$^{c}$ | 12.26±0.34$^{c}$ |
| 4 | 18.70±0.33$^{b}$ | 17.07±0.25$^{b}$ | 27.07±0.86$^{b}$ | 13.55±0.51$^{b}$ |
| 8 | 25.97±0.90$^{a}$ | 19.16±0.14$^{a}$ | 28.72±0.35$^{a}$ | 17.61±0.50$^{a}$ |

**Table 4** Effects of different Mn levels on antioxidant enzymes activities of *P. hydropiper*

| $c$(Mn)/(mmol·L$^{-1}$) | SOD/(U·g$^{-1}$) | | POD/(U·g$^{-1}$·min$^{-1}$) | | CAT/(U·g$^{-1}$·min$^{-1}$) | | APX/(U·g$^{-1}$·min$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | Leaves | Roots | Leaves | Roots | Leaves | Roots | Leaves | Roots |
| CK | 209.1±0.7$^{a}$ | 256.8±3.7$^{bc}$ | 9541.5±129.3$^{c}$ | 167.7±4.8$^{e}$ | 279.1±11.7$^{c}$ | 183.4±3.8$^{d}$ | 231.1±11.0$^{d}$ | 382.7±10.0$^{c}$ |
| 0.5 | 197.9±0.8$^{b}$ | 267.4±5.1$^{a}$ | 11520.0±290.6$^{b}$ | 175.0±2.2$^{e}$ | 325.9±19.6$^{b}$ | 213.6±6.4$^{c}$ | 607.8±19.1$^{b}$ | 417.5±3.3$^{b}$ |
| 1 | 191.2±0.8$^{c}$ | 260.4±5.4$^{ab}$ | 12762.8±553.0$^{a}$ | 203.0±8.4$^{d}$ | 418.0±6.4$^{a}$ | 324.0±9.3$^{a}$ | 675.7±12.9$^{a}$ | 473.3±6.8$^{a}$ |
| 2 | 189.8±0.9$^{c}$ | 255.1±3.9$^{bc}$ | 12495.6±196.0$^{a}$ | 536.7±5.9$^{b}$ | 324.9±8.9$^{b}$ | 286.3±11.5$^{b}$ | 468.4±9.9$^{c}$ | 344.9±6.3$^{d}$ |
| 4 | 185.9±2.0$^{d}$ | 251.0±1.8$^{c}$ | 11008.7±811.7$^{b}$ | 669.4±5.6$^{a}$ | 272.1±8.2$^{c}$ | 208.6±8.0$^{c}$ | 226.3±6.2$^{d}$ | 271.0±11.0$^{e}$ |
| 8 | 180.6±1.3$^{e}$ | 240.3±3.0$^{d}$ | 10090.3±198.9$^{c}$ | 479.3±3.5$^{c}$ | 211.2±1.4$^{d}$ | 178.3±4.3$^{d}$ | 188.2±4.8$^{e}$ | 195.5±7.8$^{f}$ |

The activities of SOD in leaves were linearly decreased with Mn increase in culture solutions with a modeling equation of $Y$=5.14$X$+210.41 ($R^2$=0.9296), where $Y$ and $X$ is SOD and Mn content, respectively. Meanwhile, the changes of SOD activities in roots were rather complex, that is, it increased first, and then decreased with increasing the Cd supply in solutions and reached its peak value of 267.4 U/g at 0.5 mmol/L Mn level in

culture. The similar trends were found for CAT and APX in leaves and roots, and they reached the peak (418.0 and 324.0 U/(g·min) for CAT and 675.7 and 473.3 U/(g·min) for APX) when 1.0 mmol/L Mn was added into solutions. The activities of POD were significantly ($p$<0.05) increased by 5.75%−33.75% in leaves and by 4.35%−299.16% in roots, when being compared with the controls.

### 3.5 Sulfhydryl group (—SH), glutathion (GSH) and phytochelatins (PCs)

Effects of various Mn levels on the contents of —SH, GSH and PCs of *P. hydropiper* were listed in Table 5. Results showed that the contents of —SH and GSH in leaves and roots of *P. hydropiper* decreased progressively, and reached its nadir at 1 mmol/L Mn level, and then increased at 8 mmol/L Mn level. The influence on PCs activities in leaves and roots of *P. hydropiper* was negligible.

## 4 Discussion

It was reported that a manganese hyperaccumulator should have content in excess of 10000 mg/kg in its aboveground biomass [16]. In the present work, the leaves of *P. hydropiper* under hydroponic culture contained more than 10000 mg/kg Mn in all Mn treatments (Table 1), and this illustrated that *P. hydropiper* in solution remained its ability to hyperaccumulate Mn. These results showed that the plant had great tolerance to Mn pollution, and Mn exposure did not generate serious physiological injury. Some researches indicated that excess $Mn^{2+}$ can induce the generation of ROS [14, 17], which can initiate lipid peroxidation [15, 18]. MDA is an index of lipid peroxidation. In this work, MDA and $H_2O_2$ contents in leaves and roots were elevated under Mn treatments, so the plants were subjected to Mn-induced oxidative stress (Table 3).

As well known, SOD quenches $O_2^-$ to $H_2O_2$, and $H_2O_2$ could be further reduced to $H_2O$ by APX, CAT or POD [19]. In the present work, the SOD activity decreased gradually with increasing the level of Mn in leaves of *P. hydropiper*. However, both increase and decrease of SOD activity have been reported in other metal stress. TIRYAKIOGLU et al [20] observed that SOD activity increased in shoots of Hamidiye genotype of barely from 0 to 60 mmol/L Cd [20]. Reduction of SOD activity has been found in leaves of *Helianthus annus* [17] and *Pisum sativum* L [21]. In lower (<8 mmol/L) Mn treatment level in this work, all of POD, CAT and APX activities increased both in leaves and roots of *P. hydropiper*, which indicated that those enzymes all functioned in detoxifying cellular $H_2O_2$ (Table 4). POD activities in leaves and roots of plants in Mn treatments were all significantly ($p$<0.05) higher than those in control, indicating that POD was more sensitive to be activated under Mn stress.

Metals in plants are assumed to be more likely to form complexes with biomolecules, such as thiols, other than to exist as free ion within the cells [22]. Glutathione (*γ*-*L*-glutamyl-*L*-cysteine-*L*-glycine, GSH) is a low relative molecular mass non-protein compound with antioxidative properties, and also is the precursor of phytochelatins (PCs). Both of them play a very important role in controlling antioxidative defenses system and maintain the balances of active oxygen concentrations [22]. Accumulation of PCs is considered to be an important mechanism of heavy metal detoxification in terrestrial plants [23]. In this work, the contents of —SH and GSH in leaves and GSH in roots of *P. hydropiper* all were significantly ($p$<0.05) higher than those in controls under high (8 mmol/L) Mn pollution, while the differences between low Mn stress and controls were minor. In the view of antioxidative enzymes, —SH and GSH might play an important role in Mn detoxification at higher Mn treatment levels, and the antioxidative enzymes, especially POD, might play an important role in Mn detoxification at lower Mn treatments. This result was consistent with reports by MISHRA et al [22] and SETH et al [24]. Though PCs were supposed to be important for metal detoxification, the influences on the

**Table 5** Effects of different Mn levels on contents of —SH, GSH and PCs of *P. hydropiper*

| $c$(Mn)/(mmol·L$^{-1}$) | $w$(—SH)/(μmol·g$^{-1}$) | | $w$(GSH)/(μmol·g$^{-1}$) | | $w$(PCs)/(μmol·g$^{-1}$) | |
|---|---|---|---|---|---|---|
| | Leaves | Roots | Leaves | Roots | Leaves | Roots |
| CK | 0.63±0.02$^{b}$ | 0.46±0.05$^{ab}$ | 0.33±0.03$^{bc}$ | 0.12±0.02$^{bc}$ | 0.30±0.02$^{ab}$ | 0.34±0.04$^{a}$ |
| 0.5 | 0.63±0.08$^{b}$ | 0.46±0.05$^{ab}$ | 0.36±0.07$^{b}$ | 0.14±0.01$^{b}$ | 0.26±0.01$^{b}$ | 0.31±0.04$^{a}$ |
| 1 | 0.51±0.05$^{c}$ | 0.42±0.04$^{b}$ | 0.22±0.02$^{d}$ | 0.09±0.01$^{d}$ | 0.29±0.03$^{ab}$ | 0.33±0.04$^{a}$ |
| 2 | 0.55±0.04$^{bc}$ | 0.45±0.01$^{ab}$ | 0.25±0.01$^{cd}$ | 0.10±0.01$^{cd}$ | 0.30±0.03$^{ab}$ | 0.35±0.01$^{a}$ |
| 4 | 0.65±0.06$^{b}$ | 0.47±0.02$^{ab}$ | 0.35±0.08$^{b}$ | 0.13±0.01$^{b}$ | 0.30±0.01$^{ab}$ | 0.34±0.03$^{a}$ |
| 8 | 0.80±0.05$^{a}$ | 0.52±0.01$^{a}$ | 0.48±0.05$^{a}$ | 0.19±0.02$^{a}$ | 0.32±0.01$^{a}$ | 0.33±0.01$^{a}$ |

PCs activity in leaves and roots of *P. hydropiper* were found to be negligible under all Mn treatment levels in this work, indicating that PCs might not be activated or participate in Mn treatment for *P. hydropiper*. Studies on some other metal hyperaccumulators and hypertolerant species, such as *Sedum alfredii* [5], *Thlaspi caerulescens* [25], and *Pteris vittata* [23], also revealed that metal tolerance did not surely rely on PCs synthesis. Therefore, the relationship between PCs and Mn tolerance in *P. hydropiper* needs to be further studied.

## 5 Conclusions

Experimental study on the effects of Mn addition on enzymatic and non-enzymatic antioxidative defenses of *P. hydropiper* indicated that Mn stress induced significantly ($p$<0.05) negative impacts on plant growth. Additionally, elevation of chlorophyll and carotenoid, and high Mn accumulation and transformation occurred, which illuminated that *P. hydropiper* has a great tolerance and detoxification under Mn stress via its enzymatic defenses and non-enzymatic antioxidative defenses, i.e. POD, SOD, APX and CAT as the main role in decomposition of Mn hazards at lower Mn pollution levels. Meanwhile, non-enzymatic antioxidative defenses, i.e. –SH and GSH may play the main role at higher Mn pollution levels.

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