Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 21(2009) 1667–1672
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Soluble protein and acid phosphatase exuded by ectomycorrhizal fungi and seedlings in response to excessive Cu and Cd

ZHENG Weishuang, FEI Yingheng, HUANG Yi*

College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China. E-mail: zhengweishuang@pku.edu.cn

Received 26 January 2009; revised 30 March 2009; accepted 17 April 2009

Abstract

Fungi and their symbionts can alleviate heavy metal stress by exuding soluble proteins and enzymes. This study examined the role of soluble protein and acid phosphatase (APase) exuded by *Xerocomus chrysenteron*, an ectomycorrhizal fungus, and the seedlings of its symbiont, Chinese pine (*Pinus tabulaeformis*), under conditions of excessive Cu and Cd. The growth type showed that this poorly studied ectomycorrhizal fungus was capable of tolerating high concentrations of Cu, and may be useful in phytoremediation. *X. chrysenteron* grew well at 80 mg/L Cu, and the EC_{50} for Cd was 17.82 mg/L. *X. chrysenteron* also showed enhanced exudation of soluble protein in both isolated and inoculated cultivations under the influence of Cu and Cd. Soluble protein exudation, however, differed under Cu and Cd stress in isolates. In mediums containing Cu, soluble protein exudation increased with concentration, but in mediums containing Cd the content of soluble protein increased to a comparable level at all concentrations. This study demonstrated that soluble protein was related to heavy metal tolerance, although the different ions played different roles. While APase activity in exudates of fungi and seedlings decreased under Cu and Cd stress in comparison to the control, the APase activity in seedlings was maintained by inoculation. Thus, *X. chrysenteron* facilitated the ability of plant to maintain a normal nutrient uptake, and therefore to protect it from heavy metal toxicity.

Key words: soluble protein; acid phosphatase; exudate; Cu, Cd; *Xerocomus chrysenteron*

DOI: 10.1016/S1001-0742(08)62471-7

Introduction

Ectomycorrhizal fungi can alleviate heavy metal stress in their host plants (Bradley *et al*., 1981, 1982). Among the mechanisms of tolerance, protein is considered to be one of the most important chelators in the bioavailability reduction of heavy metals. Additionally, mycorrhizal fungi can detoxify high concentrations of heavy metals via exudation of metal-binding proteins (Morselt *et al*., 1986; Howe *et al*., 1997). When plant species grow in soils that have been artificially contaminated with heavy metals, mycorrhizal symbiosis markedly increases root and shoot protein content (Rabie, 2005).

In addition to chelation, proteins can act as enzymes to ameliorate metal toxicity. Acid phosphatase (APase), a group of enzymes that catalyze the hydrolysis of various phosphate esters in an acidic environment, are widely distributed in plants (Tabaldi *et al*., 2007) and can be enhanced by excessive heavy metals (Jeong and Macaskie, 1995; Macaskie *et al*., 1994; Tsekova and Galabova, 2003; Tsekova *et al*., 2002). Research on bacteria has found that APase is responsible for the precipitation of heavy metals (Jeong and Macaskie, 1995) and mediates metal uptake by the liberation of inorganic phosphate (Macaskie *et al*., 1994).

Very little research has been undertaken on ectomycorrhizal fungi in relation to the role of soluble proteins and enzymes in the resistance to heavy metals like Cu and Cd. The objective of this study was to determine whether the responses of soluble protein and APase in ectomycorrhizal fungi and inoculated seedlings are similar to those of other microbes and plants.

1 Materials and methods

1.1 Fungi and plant cultivation

The ectomycorrhizal fungi *Xerocomus chrysenteron* was separated from fruiting bodies collected from conifer forests in the Western Hills of Beijing, and identified at Beijing Forestry University, China.

X. chrysenteron was cultured in nutrient agar in Petri dishes (Kottke *et al*., 1987). To reproduce inoculums, fungal mycelia were transferred from Petri dishes into specifically designed tubes with Kottke solution (Kottke *et al*., 1987) and aerated with filtered air for one week (Huang and Tao, 2001). The mycelia and nutrient solutions were then mixed evenly for one minute, and the mycelia suspension was used for plant inoculation.

Chinese pine (*Pinus tabulaeformis*) seeds were provided

* Corresponding author. E-mail: yhuang@pku.edu.cn

for the study by the Seed Station of Beijing Forestry Bureau. The seeds were firstly steeped in 10% hydrogen peroxide for 30 min to sterilize the surface, and then steeped in hot water (60°C) for 12 h to soften the seed capsule. The treated seeds were transferred to Petri dishes with sterilized perlite as the medium for germination. Following germination, the seeds were then placed in plastic pots filled with sterilized perlite for pre-growth. Pots were kept in an incubator and experienced 14 h of daylight (25°C and 8000 lx) and 10 h of darkness (18°C). After four weeks, the young seedlings were divided into two groups. Half were inoculated with *X. chrysenteron* and the other half were treated with sterilized mycelia media as the control. The seedlings were then ready to undergo the metal treatments.

1.2 Resistance test

To assess the tolerance of *X. chrysenteron* to heavy metals, the fungi were cultured in nutrient agar (pH 5.0) containing heavy metals with 0, 10, 20, 40, or 80 mg/L of Cu, or 0, 2.5, 5, 10, or 20 mg/L of Cd. Each group had four replicates. Diameters of mycelia extension were measured by ruler and recorded every day. After 30 d, the mycelia (with agar) were firstly melted at 100°C, then filtered, and finally dried at 80°C for 24 h. After cooling in desiccators for 8 h, the final weights of mycelia were measured and recorded.

1.3 Extraction of fungal exudates

Mycelia were cultivated in Erlenmeyer flasks with nutrient solutions (pH 5.0) containing different concentrations of either Cu (15, 30 or 60 mg/L) or Cd (2.5, 5, 10 or 20 mg/L) for 25 d at 25°C. Mycelia were separated by a filter. It was assumed that the mycelia exudate dissolved in the nutrient solution. The nutrient solution was then stored at 4°C for protein and APase assay. Mycelium cultivated in media without added Cu or Cd served as the controls. Each treatment had three replicates.

1.4 Extraction of plant exudates

After ten weeks of cultivation in pots (four weeks for pre-growth and six weeks after inoculation), inoculated and non-inoculated seedlings were selected and transferred into Petri dishes containing one layer of sterilized glass beads and filled with 15 mL of 1:10 diluted Kottke solution without glucose as the medium (Ahonen-Jonnarth *et al*., 2000). Each dish contained one seedling. The treatments were 15 mL of 1:10 diluted Kottke solution without glucose and contained either Cu (15 or 30 mg/L) or Cd (2.5 or 5 mg/L). Dishes with 15 mL of solution alone were served as the control. After 15 days of incubation in Petri dishes, the solution was extracted by syringe and stored without roots for protein and enzyme analysis. All treatments had three replicates.

1.5 Soluble protein and APase assay

Soluble protein content was assayed according to the method described by Lowry *et al*. (1951).

APase activity was determined as described by Galabova *et al*. (1993), with *p*-nitrophenylphosphate (pNPP) as a substrate. The reaction mixture contained 100 μL of 3.8 mmol/L pNPP, 100 μL of 0.1 mol/L sodium acetate buffer (pH 5.5) and 100 μL exudate-containing solution. After incubation at 37°C for 15 min, the reaction was terminated with 1 mL of 0.2 mol/L NaOH and the absorbance at 405 nm was measured. One unit was defined as the amount of enzyme releasing 1 nmol *p*-nitrophenol (pNP) per minute at 30°C.

1.6 Statistics and calculations

Microsoft Office Excel 2003 and SPSS 7.0 were used for data calculation and statistical treatments. One-way ANOVA and Fishers least significant difference (LSD) test were conducted to define significant differences.

Origin 7.0 was used to draw growth curves and to fit the logistic formulas.

Growth data (dry weight of mycelia) under Cu and Cd conditions were curve-fitted to calculate the effective concentration inhibiting growth by 50% value (EC_{50}) for *X. chrysenteron* by the Trimmed Spearman Karber method.

2 Results

2.1 *X. chrysenteron* response to excessive Cu and Cd

With excessive concentrations of Cu in the cultivation environment, the growth mode of *X. chrysenteron* did not change; all measurements fit the logistic growth curve. At all concentrations of Cu, except the highest one, the growth curves of mycelia diameter were indistinguishable from the control. At the highest Cu concentration (80 mg/L), growth was partly inhibited (Fig. 1).

The mycelial biomass (dry weight) of *X. chrysenteron* did not change significantly with the exposure to different levels of Cu (Fig. 2). Based on the growth type and the final biomass, *X. chrysenteron* grew well at all concentrations and thus EC_{50} could not be estimated.

Although the growth mode of *X. chrysenteron* did not change under the stress of different concentrations of the non-essential trace element Cd, the mycelia showed a limited increase in diameter (Fig. 3). Under Cd stress, mycelial growth reached the asymptotic phase on day 17

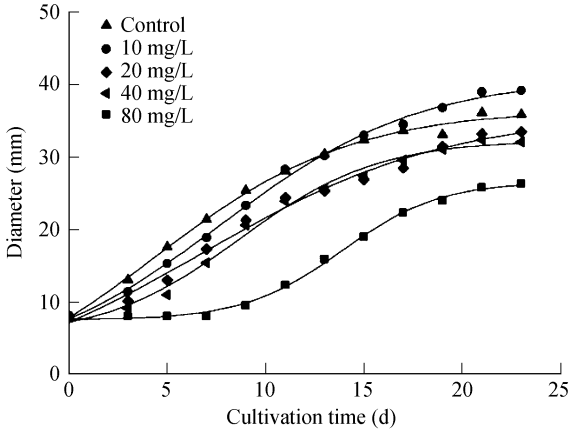

Fig. 1 Growth curves of *X. chrysenteron* under the stress of Cu.

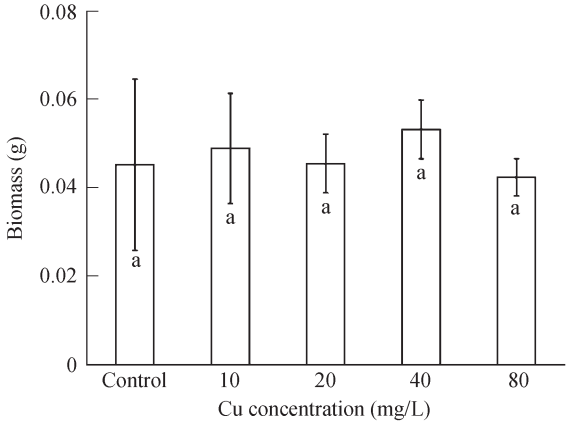

Fig. 2 Final biomass of mycelia under Cu stress. The bars with the same letter were not significantly different at $p = 0.05$.

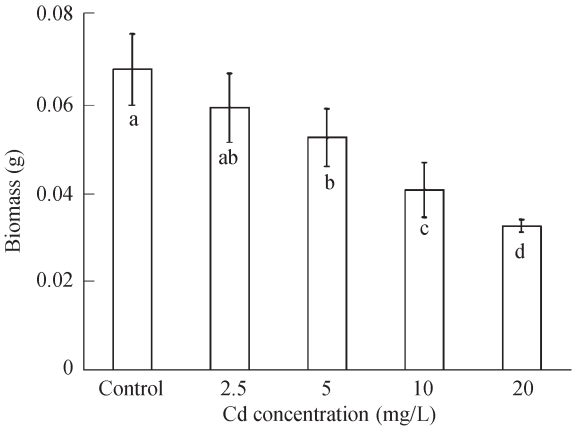

Fig. 4 Final biomass of mycelia under Cd stress. Bars with the same letter were not significantly different at $p = 0.05$.

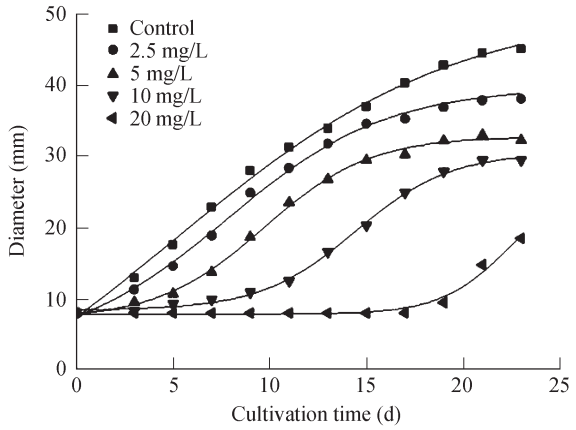

Fig. 3 Growth curves of *X. chrysenteron* under Cd stress.

at 5 mg/L, and growth was still in the logarithmic phase after day 25 at 20 mg/L, demonstrating a dose-dependent delay. The final diameter of the mycelia decreased with the increased concentration of Cd.

The biomass accumulation of *X. chrysenteron* also showed a marked decrease with excessive Cd (Fig. 4). When the concentration of Cd in the cultivation medium reached 5.0 mg/L, the final dry weight of mycelia decreased to about three-quarters, and at 20 mg/L to about one half that of the control group (Fig. 4). The EC_{50} value for *X. chrysenteron* was 17.82 mg/L.

Based on the tolerance capacity, Cu (30 and 60 mg/L) and Cd (5 and 10 mg/L) were selected to treat mycelia for assessment of the protein and enzyme responses to excessive levels of heavy metals.

2.2 Soluble protein content in exudates of fungi and roots

Soluble protein exudate per gram of biomass (mycelial dry weight) by fungi was enhanced by excessive Cu or Cd (Fig. 5). Soluble protein in the cultivation solution increased with Cu concentration. At 60 mg/L Cu, the amount increased by 72%. Under Cd stress, the soluble protein content in mycelial exudate was enhanced by 88%–112% at all concentrations.

The plant seedlings inoculated with *X. chrysenteron* exuded far more protein than those not inoculated, whether or not heavy metals were present in the environment. With excessive Cu or Cd, the exudation of soluble protein remained much higher in inoculated roots than in non-inoculated roots. Higher concentrations of Cu or Cd induced the exudation of more soluble protein (Table 1).

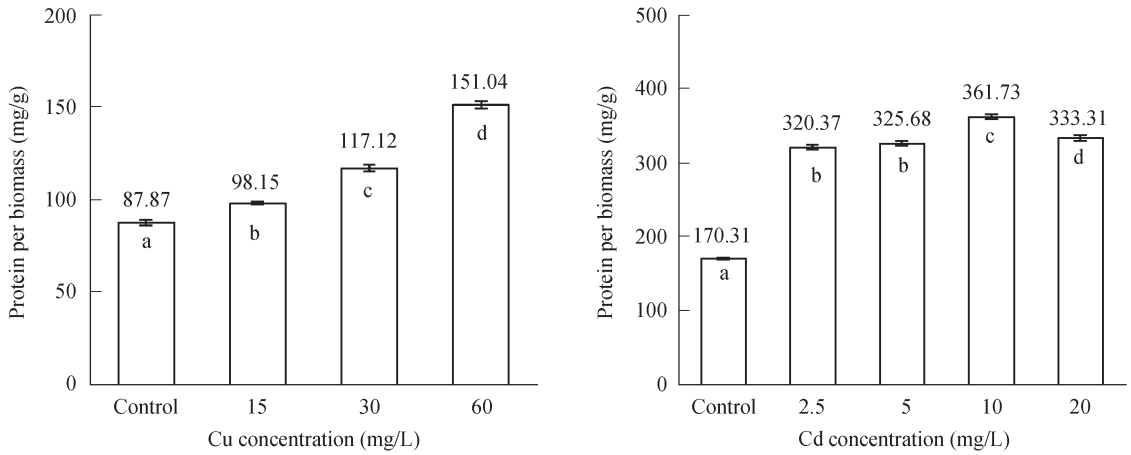

Fig. 5 Soluble protein content in mycelial exudate with Cu or Cd exposure. Means with the same letter in the bar were not significantly different at $p = 0.05$.

2.3 APase activity in fungi and root exudates

Under heavy metal stress, the activity of APase per gram of protein in fungal exudate decreased in both high Cu and high Cd treatments (Fig. 6). The activity of APase decreased by 44% in 30 mg/L Cu, 76% in 60 mg/L Cu, 65% in 5 mg/L Cd and 83% in 10 mg/L Cd, compared with the control.

Inoculation with *X. chrysenteron* also enhanced the activity of APase in plants without Cu or Cd treatment. Under the stress conditions of excessive Cu or Cd, the APase activity of inoculated seedlings increased 1.3–1.5 times (Table 2). The degree of increase was similar at all concentrations.

Table 1 Soluble protein exudate per gram of dry root (mg/g)

Treatment	Non-inoculated seedlings	Inoculated seedlings	Ratio*
Control	1.64 ± 0.12 a	1.88 ± 0.21 a	1.15
15 mg Cu/L	1.89 ± 0.08 b	2.19 ± 0.08 ab	1.16
30 mg Cu/L	1.38 ± 0.03 c	2.40 ± 0.05 b	1.75
2.5 mg Cd/L	2.05 ± 0.07 d	2.41 ± 0.35 b	1.18
5 mg Cd/L	1.47 ± 0.09 c	2.26 ± 0.05 b	1.54

* Fold increase of soluble protein content after inoculation. All values are mean ± SD of three replicates. The data were analyzed by one-way ANOVA and compared by LSD. The values with the same letter within a column were not significantly different at $p = 0.05$.

Table 2 Root APase activity per gram protein (U/g)

Treatment	Non-inoculated seedlings	Inoculated seedlings	Ratio*
Control	49.63 ± 4.89 a	66.38 ± 2.21 a	1.34
15 mg/L Cu	38.12 ± 2.85 b	54.41 ± 3.29 b	1.43
30 mg/L Cu	24.84 ± 4.07 c	35.65 ± 3.04 c	1.44
2.5 mg/L Cd	30.95 ± 3.23 c	42.41 ± 1.79 c	1.37
5 mg/L Cd	26.73 ± 3.03 c	40.39 ± 6.75 c	1.51

* Fold increase of APase activity after inoculation. All values are means ± SD of three replicates. The data were analyzed by one-way ANOVA and compared by LSD. The values with the same letter within a column were not significantly different at $p = 0.05$.

3 Discussion

3.1 High tolerance of *X. chrysenteron* under excessive Cu and Cd conditions

The most important and original finding from these experiments was the discovery that the ectomycorrhizal fungus *X. chrysenteron* was extremely resistant to high concentrations of the essential element Cu. The growth mode of *X. chrysenteron* did not change under excessive Cu stress. Following the normal logistic curve (Fig. 1), the final biomass under various Cu concentrations did not show significant changes, with the fungus even growing well when exposed to 80 mg/L Cu. Although many ectomycorrhizal isolates, such as *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus bovinus*, *S. luteus* and *S. variegates*, grow well and even show increased growth in 10 mg/L Cu, few have higher tolerance (Blaudez *et al*., 2000b). The most tolerant isolates found by Blaudez *et al*. (2000b) were *Paxillus involutus*, *Pisolithus tinctorius* and *S. luteus* with EC_{50} values > 60 mg/L Cu, markedly less than that of *X. chrysenteron*.

Cd also did not change the logistic growth mode of *X. chrysenteron*. Although the radial growth and final dry weight showed remarkable inhibition by Cd stress, the EC_{50} was 17.82 mg/L, which is much higher than the average level in contaminated soils (Herawati *et al*., 2000). Compared with the results of Blaudez *et al*. (2000b), in which the highest EC_{50} for the tested species of ectomycorrhizal fungi was around 1 mg/L of Cd, *X. chrysenteron* had a much higher capacity to resist excessive Cd stress. This suggests that *X. chrysenteron* has great potential for the remediation of metal-contaminated soils.

3.2 Soluble protein in exudate was enhanced by *X. chrysenteron*

The results obtained in this study demonstrated that Cu and Cd enhanced soluble protein exudation. Similar results have been found in previous research. Heavy metal ions, such as Zn, induce a general change in the array of exuded proteins in an ericoid mycorrhizal fungus (Martino

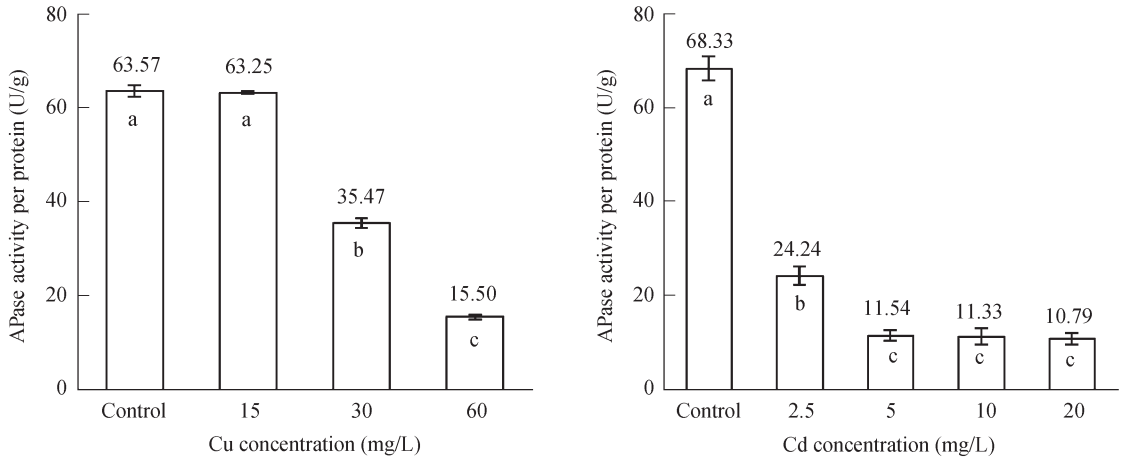

Fig. 6 APase activity under Cu and Cd stress. Means with the same letter were not significantly different at $p = 0.05$.

et al., 2002). Protein production is stimulated in activated sludge bacteria cultured in a growth medium containing Cu, leading to a 14% increase in the quantity of extracted crude proteins (Antsuki *et al*., 2003). Soluble protein exudation is assumed to be one of the mechanisms of heavy metal tolerance and varies depending on species, the specific heavy metal, and its concentration. *X. chrysenteron* showed tolerance to Cu and Cd but behaved differently in response to each. With excessive Cu, *X. chrysenteron* showed a dose-response relationship, and grew well at all concentrations. Conversely, at all concentrations of Cd soluble protein content was enhanced to the same degree, and was greater than that induced by Cu. This shows that *X. chrysenteron* is more actively tolerant to Cu than Cd. In relation to heavy metal tolerance in *X. chrysenteron*, soluble protein exudation played more of a role in response to Cu rather than Cd.

Additionally, protein exudation triggered by heavy metal stress is assumed to be a mechanism of heavy metal tolerance in symbionts. In intolerant species, the total protein content decreases with heavy metal concentration, and thus negatively affects seedling growth (Zengin and Munzuroglu, 2006). Mycorrhizal symbiosis can, however, help plant species growing in environments contaminated with heavy metals (Zhang *et al*., 2006) and increase root and shoot protein content (Rabie, 2005). Research has demonstrated that exposure to increasing concentrations of Cd results in an accumulation of proteins in *Lepidium sativum* L., a hyper-accumulative species (Gianazza *et al*., 2007), and an enhancement of soluble protein in cucumber seedlings (Talanova *et al*., 2000). Such findings suggest that the enhanced soluble protein content in the exudate is related to an enhanced tolerance to excessive Cd. In our study, inoculation with *X. chrysenteron* helped seedlings maintain the protein content in the exudate (Table 1). Without Cu or Cd treatment, the inoculated seedlings exuded more soluble protein than non-inoculated ones. After exposure to excessive Cu and Cd, the soluble protein concentration of the exudate in non-inoculated seedlings decreased with increased concentration of both Cu and Cd. Conversely, in inoculated roots the soluble protein concentration in the exudate increased.

Soluble protein exudation may be involved in metal-binding and antioxidant mechanisms. Reports suggest that metal-binding proteins are affected by Cd exposure (Kammann *et al*., 1996) and excessive Cu concentrations can affect the accumulation of Cu in protein fractions (Lin and Wu, 1994). In addition, Zn induces a protein shift towards the production of more basic, low molecular weight polypeptides including antioxidant enzymes, which play a role in heavy metal responses of plants and microorganisms (Martino *et al*., 2002). Mycorrhizal treatment decreases oxidative stress by triggering antioxidative systems, including soluble protein (Zhang *et al*., 2006). In *X. chrysenteron*, Cu may play a triggering role. Further studies are required to identify and characterize these soluble proteins and to discover their possible roles in Cu and Cd tolerance.

3.3 Activity of APase changes with excessive Cu and Cd

In sensitive species, the uptake of nutrients like nitrogen and phosphate are often inhibited by metal pollution (Blaudez *et al*., 2000a; Adriaensen *et al*., 2003). A lack in phosphate and other nutrients is suggested to be one of the crucial factors of heavy metal toxicity in plants (Wong, 2003). Improving nutrient conditions, therefore, may help plants resist heavy metal stress. For example, adding phosphorus to contaminated soil alleviates Zn toxicity (Shetty *et al*., 1995) and As toxicity (Knudson *et al*., 2003). We found that the activity of APase was much higher in inoculated roots than in non-inoculated ones (Table 2). These results are similar to those obtained by Tarafdar and Marschner (1994), who showed that plant dry weight increased after inoculation and APase activity was strongly correlated with hyphal length. As the main function of APase is to catalyze the hydrolysis of various phosphate esters in an acidic environment (Tabaldi *et al*., 2007), it promotes the nutrient cycle. The presence of mycorrhizae notably improves the phosphorus nutrient status of plants (Zhang *et al*., 2006; Tarafdar and Marschner, 1994) and may provide the plants with greater tolerance to heavy metal toxicity.

Other studies have reported that excessive Cu concentration causes a reduction in phosphorus uptake (Lin and Wu, 1994). When seedlings are cultivated without nutrient elements in the medium, the lack of a phosphorus source stimulates the exudation of large amounts of APase (Lee, 2004). Thus, some investigators have suggested that APase activity increases under the stress of high levels of heavy metals, and is one possible process of detoxification and resistance (Tsekova and Galabova, 2003; Tsekova *et al*., 2002). The function of APase in heavy metal resistance, however, is not yet clear and we did not find any information to support this presumption. Heavy metal ions are also considered to be inhibitors for enzyme activity (Huang and Shindo, 2000). Under the stress of excessive Cu and Cd, APase activity in the fungal exudate was greatly inhibited; whereas the APase activity decreased in both inoculated and non-inoculated seedlings, but inoculation alleviated Cu and Cd stress (Table 2). After inoculation, the APase activity ratio increased with concentration, which indicated that some unknown adjustment process related to the synthesis of APase occurs in plants resisting Cu and Cd stress.

The decrease of enzyme activity, however, cannot support the presumption that APase is directly involved in Cu and Cd detoxification, although it might help the plant to resist the nutrient inadequacy that often accompanies heavy metal stress. Further research is required to clarify how the synthesis of APase is inhibited by excessive heavy metals, and whether the resistance of mycorrhizal roots influences any factors during this process.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 20777004). We are grateful to Prof. Iain C. Bruce for his

advice on preparing the manuscript.

References

Adriaensen K, van der Lelie D, Van Laere A, Vangronsveld J, Colpaert J V, 2003. A zinc-adapted fungus protects pines from zinc stress. *New Phytologist*, 161(2): 549–555.

Ahonen-Jonnarth U, Van Hees P A W, Lundstrom U S, Finlay R D, 2000. Organic acids produced by mycorrhizal *Pinus sylvestris* exposed to elevated aluminium and heavy metal concentrations. *New Phytologist*, 146(3): 557–567.

Antsuki T, Sano D, Omura T, 2003. Functional metal-binding proteins by metal-stimulated bacteria for the development of an innovative metal removal technology. *Water Science and Technology*, 47(10): 109–115.

Blaudez D, Botton B, Chalot M, 2000a. Effects of heavy metals on nitrogen uptake by *Paxillus involutus* and mycorrhizal birch seedlings. *FEMS Microbiology Ecology*, 33(1): 61–67.

Blaudez D, Jocob C, Turnau K, Colpaert J V, Ahonen-Jonnarth U, Finlay R *et al*., 2000b. Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. *Mycological Research*, 104(11): 1366–1371.

Bradley R, Burt A J, Read D J, 1981. Mycorrhizal infection and resistance to heavy metal toxicity in *Calluna vulgaris*. *Nature*, 292(5821): 335–337.

Bradley R, Burt A J, Read D J, 1982. The biology of mycorrhiza in the Ricacacea VIII, the role of mycorrhizal infection in heavy metal resistance. *New Phytologist*, 91(2): 197–209.

Galabova D, Tuleva B, Balasheva M, 1993. Phosphatase activity during growth of *Yarrowia lipolytica*. *FEMS Microbiology Letter*, 109(1): 45–48.

Gianazza E, Wait R, Sozzi A, Regondi S, Saco D, Labrac M *et al.*, 2007. Growth and protein profile changes in *Lepidium sativum* L. plantlets exposed to cadmium. *Environmental and Experimental Botany*, 59(2): 179–187.

Herawati N, Suzuki S, Hayashi K, Rivai I F, Koyama H, 2000. Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia and China by soil type. *Bulletin of Environmental Contamination And Toxicology*, 64(1): 33–39.

Howe R, Evans R L, Ketteridge S W, 1997. Copper-binding proteins in ectomycorrhizal fungi. *New Phytologist*, 135(1): 123–131.

Huang Q, Shindo H, 2000. Effects of copper on the activity and kinetics of free and immobilized acid phosphatase. *Soil Biology and Biochemistry*, 32(13): 1885–1892.

Huang Y, Tao S, 2001. Excessive Cu and Zn affecting on distribution of the metals and activities of glycolytic and nitrogen incorporating key enzymes in mycelia of ectomycorrhizal fungi *Suillus bovinus*. *Journal of Environmental Sciences*, 13(3): 337–341.

Jeong B C, Macaskie L E, 1995. PhoN-type acid phosphatase of a heavy metal accumulating *Citrobacter* sp.: resistance to heavy metal and affinity towards phosphomonoester substrates. *FEMS Microbiology Letter*, 130(2-3): 211–214.

Kammann U, Friedrich M, Steinhart H, 1996. Isolation of a metal-binding protein from ovaries of dab (*Limanda limanda* L.) distinct from metallothionein: Effect of cadmium exposure. *Ecotoxicology and Environmental Safety*, 33(3): 281–286.

Knudson J A, Meikle T, DeLuca T H, 2003. Role of mycorrhizal fungi and phosphorus in the arsenic tolerance of basin wildrye. *Journal of Environmental Quality*, 32(6): 2001–2006.

Kottke I, Guttenberger M, Hampp R, Oberwinkler F, 1987. An *in vitro* method for establishing mycorrhizae on coniferous tree seedlings. *Trees*, 1(3): 191–194.

Lee T M, 2004. Induction of acid phosphatase activity by phosphorus deficiency in *Gracilaria coronopifolia* (Gigartinales, Rhodophyta). *Phycologia*, 43(5): 493–500.

Lin S L, Wu L, 1994. Effects of copper concentration on mineral nutrient-uptake and copper accumulation in protein of copper-tolerant and copper-nontolerant *Lotus purshianus* L. *Ecotoxicology and Environmental Safety*, 29(2): 214–228.

Lowry O H, Rosebrough N J, Farr A L, Randall R J, 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemisty*, 193(1): 265–275.

Macaskie L E, Bonthrone K M, Rouch D A, 1994. Phosphatase-mediated heavy metal accumulation by a *Citrobacter* sp. and related enterobacteria. *FEMS Microbiology Letter*, 121(2): 141–146.

Martino E, Franco B, Piccoli G, Stocchi V, Perotto S, 2002. Influence of zinc ions on protein secretion in a heavy metal tolerant strain of the ericoid mycorrhizal fungus *Oidiodendron maius*. *Molecular and Cellular Biochemistry*, 231(1-2): 179–185.

Morselt A F W, Smits W T M, Limonard T, 1986. Histochemical demonstration of heavy metal tolerance in ectomycorrhizal fungi. *Plant and Soil*, 96(3): 417–420.

Rabie G H, 2005. Contribution of arbuscular mycorrhizal fungus to red kidney and wheat plants tolerance grown in heavy metal-polluted soil. *African Journal of Biotechnology*, 4(4): 332–345.

Shetty K G, Hetrick B A D, Schwab A P, 1995. Effects of mycorrhizae and fertilizer amendments on zinc tolerance of plants. *Environmental Pollution*, 88(3): 307–314.

Tabaldi L A, Ruppenthal R, Cargnelutti D, Morsch V M, Pereira L B, Schetinger M R C, 2007. Effects of metal elements on acid phosphatase activity in cucumber (*Cucumis sativus* L.) seedlings. *Environmental and Experimental Botany*, 59(1): 43–48.

Talanova V V, Titov A F, Boeva N P, 2000. Effect of increasing concentrations of lead and cadmium on cucumber seedlings. *Biologia Plantarum*, 43(3): 441–444.

Tarafdar J C, Marschner H, 1994. Phosphatase activity in the rhizosphere and hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. *Soil Biology and Biochemistry*, 26(3): 387–395.

Tsekova K, Galabova D, 2003. Phosphatase production and activity in copper(II) accumulating *Rhizopus delemar*. *Enzyme and Microbial Technology*, 33(7): 926–931.

Tsekova K, Galabova D, Todorova K, Ilieva S, 2002. Phosphatase activity and copper uptake during growth of *Aspergillus niger*. *Process Biochemistry*, 37(7): 753–758.

Wong M H, 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. *Chemosphere*, 50(6): 775–780.

Zengin F K, Munzuroglu O, 2006. Effects of heavy metals (Pb^{++}, Cu^{++}, Cd^{++}, Hg^{++}) on total protein and abscisic acid content of bean (*Phaseolus vulgaris* L. cv. Strike) seedlings. *Fresenius Environmental Bulletin*, 15(4): 277–282.

Zhang X H, Lin A J, Chen B D, Wang Y S, Smith S E, Smith F A, 2006. Effects of *Glomus mosseae* on the toxicity of heavy metals to *Vicia faba*. *Journal of Environmental Sciences*, 18(4): 721–726.