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Effects of insoluble Zn, Cd, and EDTA on the growth, activities of antioxidant enzymes and uptake of Zn and Cd in *Vetiveria zizanioides*

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Abstract

A root-bag experiment was conducted to study the effects of insoluble Zn, Cd, and ethylenediaminetetraacetic acid (EDTA) on the plant growth, activities of antioxidant enzymes, proline, glutathione (GSH), water-soluble proteins and malondialdehyde (MDA) of *Vetiveria zizanioides*. The *V. zizanioides* uptake capacity of Zn and Cd also determined. The results showed that plant growth of *V. zizanioides* was inhibited by Zn and Cd. The shoot dry weight (SDW) and root dry weight (RDW) decrease by 14.2%, 14.1%, 17.0% and 17.3%, 32.5%, 35.7%, respectively, compared to the control without EDTA addition. After adding EDTA, shoot and root dry weight decreased over 10% and 15%, respectively. The toxicity from insoluble Zn and Cd in soil on SDW and RDW of *V. zizanioides* was in order: Zn+Cd > Cd > Zn. The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), and contents of MDA and proline increased significantly, while the contents of GSH and water-soluble proteins decreased markedly with increasing Zn and Cd toxicity. With EDTA, shoot and root Zn concentrations increased in the Zn treatment by 7.3% and 37.4% , and Cd concentrations in the combined Zn and Cd treatment increased by 18.6% and 391.9% compared to the treatment without EDTA. However, Zn and Cd concentrations in shoot and roots decreased in the Cd treatment compared to the plants grown in absence of EDTA, with exception of root Cd concentration in the presence of EDTA.

Key words: *Vetiveria zizanioides*; antioxidant enzymes; glutathione; malondialdehyde; praline; concentration of Zn and Cd
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Introduction

Cadmium (Cd) and zinc (Zn) are common heavy metal pollutants in mine lands and they often occur together in the environment because of their similar chemical properties (Hutton, 1983). Biologically, however, these two elements have different properties. Cadmium is nonessential and toxic, and can disturb the ionoregulatory system of aquatic organisms (McGeer *et al*., 2000). It has also been found to cause oxidative stress (Hendry *et al*., 1992). Cadmium-induced enhancement of lipid peroxidation has been reported for sunflower, bean, and barley (Shaw, 1995). However, the studies on the response of plants to Cd-induced oxidative stress provided contrasting results (Chaoui *et al*., 1997). In contrast, Zn is essential as a cofactor for many enzymes (e.g., Cu/Zn-SOD, a superoxide dismutase) and also serves as an important function in transcription of many genes (Chung *et al*., 2005). At high level, Zn can cause osmoregulatory disturbances in aquatic organisms (McGeer *et al*., 2000) and may also cause cytotoxic effects in the presence of hydrogen peroxide (Chung *et al*., 2005).

Studies undertaken to understand the interaction between Zn and Cd have reported inconsistent results. White and Chaney (1980) reported that Cd uptake was reduced in both soybean roots and shoots through application of Zn. Wu *et al*. (2002) reported that the physiological damage caused by Cd toxicity could be alleviated by application of Zn. Eriksson (1990) found that the Zn-Cd interaction varied with the crop species.

The restoration of the wastelands resulting from mining has become an important issue because they not only occupy the vast tillable lands but also cause environmental hazards (Whiting *et al*., 2004). It is well known that the insoluble components of heavy metal contaminants constitute a major proportion of their total content. Therefore, removing the insoluble fractions of heavy metals from soils is important in long-term the restoration and phytoremediation of mining lands.

Vetiveria zizanioides has a high tolerance for heavy metal(loid)s such as As, Cu, Cd, Pb, Hg, Ni, Se, and Zn, and can accumulates a substantial amount of metal(loid)s in roots (Tian *et al*., 2004). It is an important plant for the phytoremediation of heavy metal contaminated soil and restoration of mined wasteland in tropical or subtropical

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regions.

Mobilizing or chelating agents, such as ethylenediaminetetraacetic acid (EDTA), have been added to contaminated soils in an attempt to increase plant metal uptake and shoot accumulation, especially when the metal bioavailability is low (Pastor *et al*., 2007). Several reports emphasized the feasibility of using chelate-induced phytoextraction to remediate soils polluted with a mixture of heavy metals, such as Zn and Cd (Meers *et al*., 2005). Most of the studies on uptake and accumulation of heavy metals by plants have been conducted on using soluble sources of Zn and Cd. A little is known about the role of EDTA in the uptake of insoluble Zn and Cd. The main objectives of the present studies were to investigate effects of insoluble Zn and Cd, and EDTA on biomass, activities of antioxidant enzymes, concentrations of proline, glulathione (GSH), water-soluble proteins and malondialdehyde (MDA) of *V. zizanioides* in calcareous soil. The uptake of Zn and Cd by *V. zizanioides* in a calcareous soil was also investigated.

1 Materials and methods

1.1 Plant, soil and heavy metal treatments

Vetiveria zizanioides was grown in a calcareous soil collected from Tongnan, Chongqing, China. The soil was characterized as having a pH of 8.32 (soil:water, 1:2.5, *m/V*), an organic matter content of 11.8 g/kg and a cation exchange capacity (CEC) of 185 $mmol_c$/kg. Available N, P, K and Zn were 22.7, 20.1, 161.0, and 2.0 mg/kg, respectively. Total N, Zn, and Cd were 0.41 g/kg, 96.1 mg/kg, and 0.98 mg/kg, respectively. The available Cd was below the detection level (< 0.01 mg/kg).

The experiment was conducted in a glasshouse from April to July 2005. Zn and Cd were added at 0 (control), 80 mg/kg Zn, 40 mg/kg Cd, and 80 mg/kg Zn + 40 mg/kg Cd as $ZnCO_3$ and $CdCO_3$ in combination with 0.8 mmol/kg EDTA as Na_2-EDTA. Soil sample (2.50 kg) was mixed with the designated amounts of Zn, Cd, EDTA and fertilizers containing 100 mg/kg N in the form of urea, 75 mg/kg P as KH_2PO_4, and 100 mg/kg K as KCl. A root-bag technique was used to separate the rhizosphere from bulk soil. The root-bags (12 cm in depth, and 5 cm in diameter) made of 500-mesh nylon screen, were filled with 200 g rhizosphere, and then buried in the centre of a plastic pot (14 cm in depth, 19 cm in diameter) containing 2.30 kg bulk soil (Xu *et al*., 2007). Three uniform seedlings (3 cm in root length, 15 cm in seedling height) were transplanted into the root-bag of each pot. The pots were arranged in a completely randomized design on a bench, and each treatment was replicated three times. Distilled water was added daily to maintain soil moisture. After 90 d of growth, plants were harvested and washed thoroughly with distilled water, then separated into roots and shoots. The activity of antioxidant enzymes, the concentrations of proline, GSH, water-soluble proteins and MDA were analyzed. The plant samples then oven-dried at 65°C for 72 h and dry weights of roots and shoots were recorded, respectively.

1.2 Protein and enzyme analyses

Tissue samples were homogenized in ice-cold deoxygenated 20 mmol/L Tris-HCl buffer (pH 7.4, 1:9, *W/V*) and centrifuged at 10000 ×*g* for 10 min. Aliquots of 100 μL were used for enzyme activity measurements. The activity of catalase (CAT) was measured using a microtiter plate assay as previously described (Hansen *et al*., 2006). The activity of peroxidase (POD) was determined according to Nakano and Asada (1981). The activity of superoxide dismutase (SOD) was determined by the method of Minami and Yoshikawa (1979) with 50 mmol/L Tris-Ca-codylic sodium salt buffer at pH 8.2, containing 0.1 mmol/L EDTA. Total water-soluble proteins in samples were measured by the Bradford assay (Bradford, 1976).

1.3 Analysis of proline and glutathione

Proline was extracted and determined by the method of Bates *et al*. (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 ×*g* for 10 min. After acetic acid and acid ninhydrin were added, the supernatant was boiled for 1 h and then absorbance of the supernatant was determined at 520 nm. Total glutathione was estimated by the modified procedures according to Griffith (1980). Leaf sample (0.5 g) was homogenized in 1.5 mL of 5% (*W/V*) sulpho-salicylic acid. The homogenate was centrifuged at 10000 ×*g* for 10 min at 25°C. The supernatant (1.0 mL) was neutralized with 1.5 mL of 0.5 mol/L potassium phosphate buffer (pH 7.5) containing EDTA 15 mmol/L, 0.2 mL of 6 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 mL of 2 mmol/L NADPH and 0.1 U of Baker's yeast glutathione reductase (GR) (Sigma Aldrich Co., USA). The change in the absorbance at 412 nm was measured at (25 ± 2)°C. Proteins of tissue homogenate were precipitated with 40% (*W/V*) trichloracetic acid (TCA). The MDA assay was based on the condensation of one molecule of malondialdehyde with two molecules of thiobarbituric acid (TBA) in the presence of reduced reagent volumes to increase sensitivity, generating a chromogen with UV absorbance. The TBA + MDA complex was analyzed by HPLC essentially as described by Bird *et al*. (1983). The HPLC system consisted of a Hewlett-Packard 1050 gradient pump (Avondale, USA) equipped with an automatic injector, a 1050 diode-array absorption detector and the computer program was Chemical Station Software (Hewlett-Packard, Japan). Aliquots of the TBA + MDA samples were injected into a 5-mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5-μm filter (Millipore, Bedford, USA). The flow rate was 2 mL/min. MDA + TBA standards were prepared using tetraethoxypropane. The absorption spectra of standards and samples were identical with a characteristic peak at 540 nm.

1.4 Analyses of Zn and Cd

Dried plant samples were ground (< 1 mm). Approximately 0.1000 g were transferred into 120-mL Teflon

pressure digestion vessels, and digested in concentrated HNO_3-$HClO_4$ (2:1, *V/V*). Zn and Cd concentrations in the extracts were analyzed using a flame atomic absorption spectrometer (FAAS) (Perkin Elmer SIMMA 6000, USA). Soil Zn and Cd concentrations were determined by digesting approximately 1.000 g in triacid mixture (nitric acid:sulphuric acid:perchloric acid was 10:1:0.5, *V/V/V*).

1.5 Statistical analysis

Experimental data were analysed using a two-way analysis of variance (ANOVA), followed by the least significant difference test ($P = 0.05$). The statistical software SPSS 12.0 was performed.

2 Results

2.1 Growth response

The shoot dry weight (SDW) and root dry weight (RDW) of *V. zizanioides* decrease by 14.2%, 14.1%, 17.0% and 17.3%, 32.5%, 35.7% compared to the control without EDTA addition for Zn, Cd, Zn + Cd treatments, respectively (Table 1). The decrease was greater in roots than in shoots. The toxicity of soil insoluble Zn and Cd on SDW and RDW of *V. zizanioides* was in order: Zn + Cd > Cd > Zn. Addition of EDTA to the soil enhanced the toxic effects of Zn and Cd on *V. zizanioides* growth (Table 1). The toxicity order for metals in the presence of EDTA was similar to the treatments without EDTA addition.

2.2 Activities of antienzymes and contents of MDA

Activities of SOD, CAT and POD, and MDA contents of shoots and roots were increased for all Zn and Cd treatment in both the absence and presence of EDTA (Table 1), with the order of Zn + Cd > Cd > Zn. The higher values were shown in the presence of EDTA.

2.3 Contents of proline, glutathione and proteins in shoots

The proline content of *V. zizanioides* shoots was increased for all the treatments (Table 2). Shoot proline contents were in the order of Zn + Cd > Cd > Zn for both EDTA absence and presence, and the contents were higher in the presence of EDTA.

The contents of shoot total soluble protein and GSH were decreased by exposure to Zn and Cd for both with and without EDTA treatments (Table 2). The contents of shoot total soluble protein were decreased in the order of Zn > Cd > Zn + Cd for both the absence and presence of EDTA. Shoot GSH contents in the presence of EDTA were higher than in the absence of EDTA, in contrast the shoot total soluble protein contents in the presence of EDTA were lower.

2.4 Plant Zn and Cd concentrations

Shoot and root Zn concentrations and uptake of *V. zizanioides* are shown in Figs. 1 and 2. Without EDTA addition, shoot and root Zn concentrations and accumulations increased when exposed to Zn alone, and combined Zn and Cd. After EDTA addition, shoot and root Zn concentrations increased in the Zn alone treatment by 7.3% and 37.4%, whereas decreased in combined Zn and Cd and Zn alone treatment, especially root Zn concentration. Zn concentrations were in the order of Zn > Zn + Cd > Cd for both EDTA absence and presence, with exception of root

Table 2 Effect of soil insoluble Zn and Cd and EDTA on concentrations of proline, glutathione (GSH), and water-soluble proteins in shoot of *V. zizanioides*

	Treatment	Proline (μg/g)	GSH (μg/kg)	Proteins (μg/g)
–EDTA	Control	2.36	0.62	77.4
	Zn	3.23	0.52	73.8
	Cd	4.58	0.46	62.8
	Zn + Cd	4.79	0.42	53.4
+EDTA	Control	3.37	0.82	74.7
	Zn	8.21	0.73	49.8
	Cd	9.34	0.67	43.8
	Zn + Cd	10.12	0.65	37.8
LSD0.05 among metals		0.52	0.09	0.29
LSD0.05 between EDTA treatments		0.74	0.13	0.41
LSD0.05 interaction between metals and EDTA treatments		0.43	0.08	0.24

+ EDTA and – EDTA are the same as in Table 1.

Table 1 Effect of soil insoluble Zn and Cd and ethylenediaminetetraacetic acid (EDTA) on biomass, activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) concentration of malondialdehyde (MDA) in shoots of *Vetiveria zizanioides*

Sample	Treatment	Biomass (g/pot dw)		POD (U/g·min)		SOD (U/g)		CAT (U/g·min)		MDA (μmol/g)	
		+EDTA	–EDTA	+EDTA	–EDTA	+EDTA	–EDTA	+ EDTA	–EDTA	+EDTA	–EDTA
Shoot	Control	14.82	14.14	101.5	126.0	28.21	35.84	220.9	190.5	5.23	6.10
	Zn	14.78	12.13	139.8	176.9	46.69	51.32	273.3	311.4	5.89	6.79
	Cd	13.64	12.15	189.7	199.6	59.88	67.78	344.1	365.7	6.64	7.05
	Zn + Cd	13.43	11.74	244.9	286.3	81.72	95.30	393.6	411.1	6.89	7.85
Root	Control	5.57	4.98	109.8	120.9	32.23	37.29	190.7	193.6	3.03	3.27
	Zn	4.99	4.12	165.2	167.9	43.56	55.34	265.2	269.8	3.30	3.80
	Cd	4.73	3.36	167.1	225.5	49.01	58.58	287.2	291.6	3.51	4.15
	Zn + Cd	4.22	3.20	176.7	254.8	70.33	90.61	351.7	377.1	4.01	5.01
Shoot	Metals	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
	EDTA	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
	Metals vs. EDTA	0.001		< 0.001		0.005		< 0.001		0.317	
Root	Metals	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
	EDTA	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
	Metals vs. EDTA	0.157		< 0.001		< 0.001		< 0.001		0.052	

+EDTA: treated with EDTA addition; –EDTA: treated without EDTA addition.

Zn concentrations in the absence of EDTA. The ratios of shoot Zn concentration and root Zn concentration ranged from 1.3 to 1.9 for all treatments. Shoot Zn accumulations were greater than root Zn contents in both the absence and presence of EDTA, however, shoot Zn concentration in all the treatments was lower than root Zn concentration.

Shoot and root Cd concentrations and accumulations were increased for Cd alone and the combination of Zn and Cd in the absence of EDTA treatment (Figs. 3 and 4). Shoot and root Cd concentrations were in the order of Cd > Zn + Cd > Zn for EDTA absence. With the EDTA addition, shoot and root Cd concentration in the combined Zn and

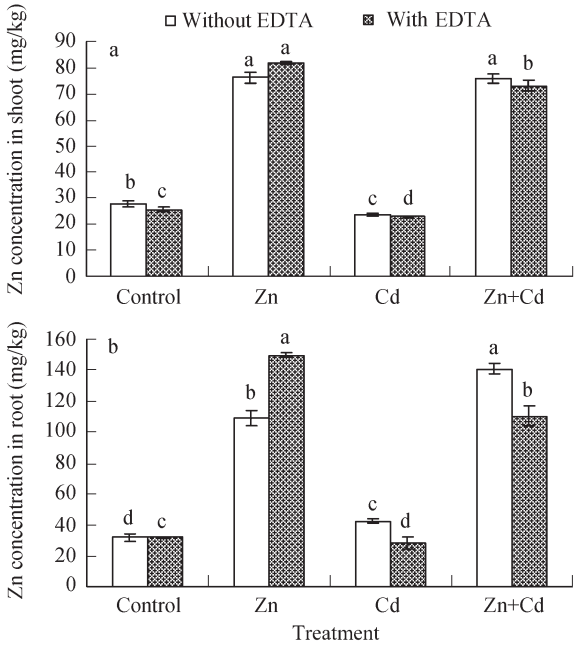

Fig. 1 Zn concentration in shoot (a) and root (b) of *Vetiveria zizanioides*.

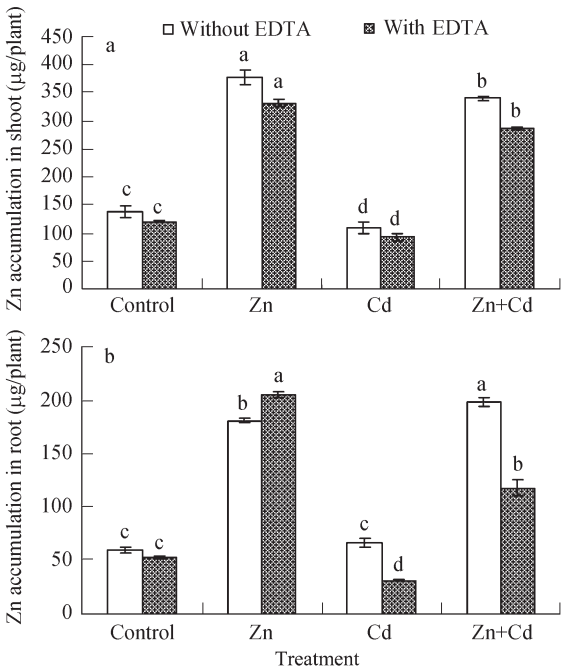

Fig. 2 Accumulation of Zn in shoot (a) and root (b) of *Vetiveria zizanioides*.

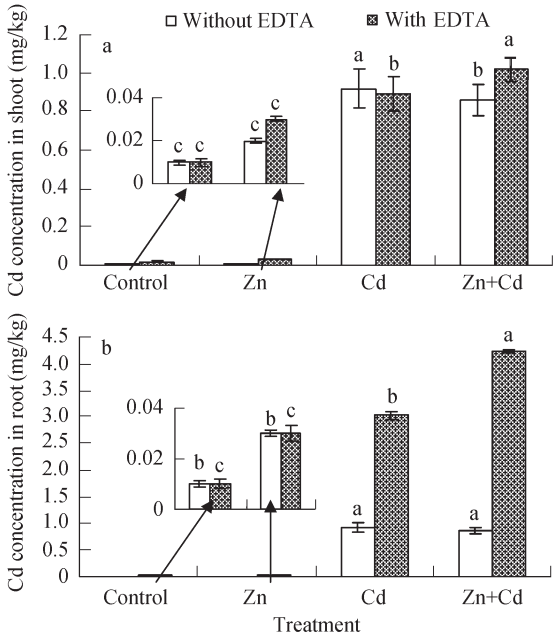

Fig. 3 Cd concentrations in shoot (a) and root (b) of *Vetiveria zizanioides*.

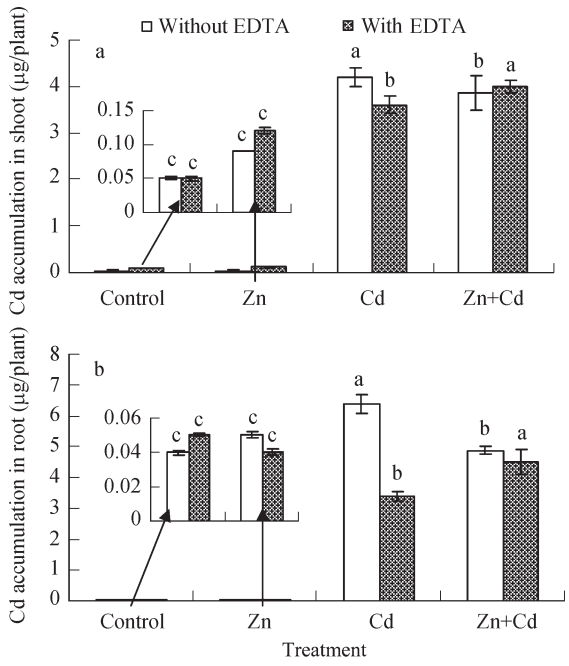

Fig. 4 Accumulation of Cd in shoot (a) and root (b) of *Vetiveria zizanioides*.

Cd treatment increased by 18.6% and 391.9%; root Cd concentration in Cd alone treatment increased by 229.3%, whereas shoot Cd concentration decreased by 3.3%. Shoot and root Cd concentrations were in the order of Zn + Cd > Cd > Zn in the presence of EDTA. The ratios of shoot Cd concentration and root Cd concentration ranged from 1.0 to 4.4 for all treatments. Root Cd concentrations and accumulations were higher than shoot Cd contents both in the absence and presence of EDTA when exposed to Cd alone and combined Zn and Cd (except for root Cd accumulation in the presence of EDTA).

3 Discussion

In this study, the toxicity from combined application of Zn and Cd on SDW and RDW of *V. zizanioides* was more severe than that of their alone. Similar results have been shown by plant dry weight of ryegrass (*Lolium perenne* L.) (Xu *et al*., 2006). Chelating agents such as EDTA are frequently used to increase the bioavailability of heavy metals to enhance their uptake by plants, although this may also decrease their biomass due to the toxicity of the extra uptake (Madrid *et al*., 2003). The results of this study show that the biomass of *V. zizanioides* was markedly decreased by EDTA supply. This may be due to the increased solubility of insoluble Zn and Cd by EDTA which led to increasing bioavailability of Zn and Cd in the soil, and thus enhanced the toxicity of Zn and Cd to plants.

High metal concentration can also enhance oxidative stress (Chung *et al*., 2005). It has been proposed that the protective mechanism involves metal-induced transcription of genes encoding proteins with key roles in antioxidant defense, including POD, SOD and CAT (Kling and Olsson, 2000). The POD, SOD and CAT activities of shoots and roots significantly increased in current study. Increase of POD, SOD and CAT activities has been considered as an adaptive response of plants to toxic environmental factors (Camp, 1996). Antioxidant enzymes have been shown to work in a cooperative or synergistic way to protect against oxidative stress (Bagnyukova *et al*., 2006). After adding EDTA, greater increases of POD, SOD and CAT activities result in high EDTA concentration which is toxic to plants (Luo *et al*., 2006). The higher activities of POD, SOD and CAT of shoots and roots all found in combined Zn and Cd treatment in both the absence and presence of EDTA may be due to a synergistic interaction of Zn and Cd on antioxidant enzymes. Similar results have been reported by Hassan *et al*. (2005).

Oxidative stress due to the existence of the toxic metals can be demonstrated by MDA content, which is considered to be a general indicator of lipid peroxidation (Chaoui *et al*., 1997). In this study, significant increases in MDA content were also observed in root and shoot after the plants were exposed to Zn, Cd and EDTA due to lipid peroxidation. Shoot and root MDA contents in combined Zn and Cd treatment were greater than that for Zn and Cd alone treatments in both the absence and presence of EDTA. The results show that the interaction effect of Cd-Zn combined stress on membrane lipid peroxidation is mainly displayed as mutualism, or antagonism under the combined stress of Zn and Cd.

Proline has been closely related to the antioxidation of plants and could eliminate active oxygen in plants (Prasad and Saradhi, 1995). In this study, shoot proline contents were increased markedly by insoluble Zn and Cd due to oxidation stress. Similar results of proline increases in plants exposed to Zn and Cd were reported by Schat *et al*. (1997). Proline content induced by Cd alone was greater than Zn alone possibly because Zn is an essential nutrient element to plants. Proline contents in combined Zn and Cd treatments were higher than that for Zn and Cd alone possibly due to their synergistic effect on proline content in plants.

In this study, the contents of GSH in shoots showed a dramatic decrease when exposed to Zn and Cd. The GSH contents in combined Zn and Cd treatments were higher than that for Zn and Cd alone in both the absence and presence of EDTA. Reduced GSH is also an important antioxidant, which is readily oxidized by reactive oxygen species (ROS) to oxidized glutathione (GSSG), but can also bind metals that might induce oxidative stress. The reduction of GSSG to yield GSH is catalyzed by glutathione reductase (GR) (Stohs and Bagchi, 1995). In contract, increased GSH in plants in response to applied Zn and Cd was reported by Schat *et al*. (1997) and increased GSH in response to applied Cd was reported by Pietrini *et al*. (2003).

There is evidence to suggest that high levels of heavy metals individually affect protein metabolism (Demirevska-Kepova *et al*., 2004). In this study, a significant reduction was found in the total soluble protein content of shoots in *V. zizanioides* seedlings after plants were exposed to Zn and Cd. This may be due to a significant decrease in the activity of nitrate reductase resulting in decreased NO_3^- reduction and subsequent inhibition of protein metabolism. The total soluble protein content of combined Zn and Cd treatments are higher for Zn and Cd alone both in the absence and presence of EDTA. Similar results of single and combined heavy metals induced a reduction in soluble protein contents were also reported by Tian *et al*. (2007).

Several studies have indicated that the interaction of Zn and Cd on the uptake of Zn and Cd by plants is different due to different ratios of Zn and Cd in soils (Huebert and Shay, 1992; Xu *et al*., 2006). In the present study, application of Cd treatment both in the presence and absence of EDTA generally decreased Zn concentration in shoots and roots, with the exception of an increased root Zn concentration with Cd application in the absence of EDTA. The results indicated that the presence of Cd in soil inhibited Zn uptake by plants. The Cd concentration in shoots and roots decreasing with the combined Zn and Cd treatment in the absence of EDTA compared to Cd treatment alone shows that Zn addition inhibited Cd uptake by plants in the absence of EDTA. While Cd concentrations in shoots and roots increased with the combined Zn and Cd treatment in the presence of EDTA due to the application of synthetic chelators to contaminated soils which can enhance the uptake and accumulation of heavy metals in plant shoots. This phenomena was also been reported by Penalosa *et al*. (2007). With EDTA addition, Zn and Cd concentrations of shoots and roots increased obviously in Zn alone and Cd alone treatments, and Cd concentrations of shoots and roots increased in combined Zn and Cd treatment, which may be due to metal-chelator interactions (Huebert and Shay, 1992). The findings suggest that the interaction of Zn and Cd on the uptake of Zn and Cd by plants is different due to the presence of chelators such as EDTA (Huebert and Shay, 1992). However, in shoot and root, Zn and Cd accumulations of EDTA addition decreased

due to declines of biomass. The similar results were also observed by Duo *et al*. (2005) where metals accumulation decreased in turfgrass after EDTA supply. A suitable level of the application of mobilizing/chelating agents, such as EDTA, should result in efficient accumulation of metals without reduction in the biomass of plants to enhance phytoextraction.

4 Conclusions

In this study, the plants showed a greater sensitivity to combined Zn and Cd application, and plant toxicity was greater with Cd than Zn. After adding EDTA, *V. zizanioides* growth was decreased further, possibly due to increased the bioavailability and toxicity of Zn and Cd. The activities of antioxidant enzymes (SOD, POD, and CAT), contents of MDA and proline significantly increased, while contents of GSH and water-soluble proteins markedly decreased when plants were exposed to Zn, Cd, and EDTA. Interactions of synergy on antioxidant enzymes, proline, GHS, protein and MDA were found between Zn and Cd.

High Zn accumulation was detected in shoots of *V. zizanioides* seedlings both in the absence and presence of EDTA, while Cd mainly accumulated in roots due to differences in the metal distribution in plants and in transferring metals from roots to shoots. The application of the synthetic chelator EDTA to contaminated soils greatly enhanced the uptake of Zn and Cd by plants, but in this study, Zn and Cd accumulations in plants decreased after adding EDTA due to decreased plant biomass. Therefore, determination of an optimum level for the application of EDTA is important in the phytoextraction of soil polluted with Zn and Cd.

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