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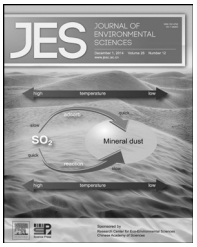

Enhanced efficiency of cadmium removal by *Boehmeria nivea* (L.) Gaud. in the presence of exogenous citric and oxalic acids

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ABSTRACT

Boehmeria nivea (L.) Gaud. is a potential candidate for the remediation of Cd contaminated sites. The present investigation aims to explore Cd tolerance threshold and to quickly identify the role of exogenous organic acids in Cd uptake and abiotic metal stress damage. Elevated Cd levels (0–10 mg/L) resulted in an obvious rise in Cd accumulation, ranging from 268.0 to 374.4 in root and 25.2 to 41.2 mg/kg dry weight in shoot, respectively. Citric acid at 1.5 mmol/L significantly facilitated Cd uptake by 26.7% in root and by 1-fold in shoot, respectively. Cd translocation efficiency from root to shoot was improved by a maximum of 66.4% under 3 mmol/L of oxalic acid. Citric acid exhibited more prominent mitigating effect than oxalic acid due to its stronger ligand affinity for chelating with metal and avoiding the toxicity injury of free Cd ions more efficiently. The present work provides a potential strategy for efficient Cd remediation with *B. nivea*.

Introduction

Industrial mining, smelting and agricultural activities such as long-term wastewater irrigation have caused prevalent soil Cd contamination and hazardous human diseases through edible plants. The persistence of Cd in soils and transfer into human body *via* food chain have aroused a major concern due to its great toxicity (Liu et al., 2008), mutagenicity, carcinogenicity and high risk to food safety. Cd contamination is extremely severe in Hunan Province (in central-south of China) because of frequent mining activities, which has led to farmland pollution, reduction of grain production and economic loss. For example, 36% of rice grown in Hunan Province was detected to have Cd levels above China's food standard regulation according to a research report (Lei et al., 2010). In order to alleviate the problem, efficient phytoremediation seems to be an appropriate route. *Boehmeria nivea* (L.) Gaud. (ramie), a perennial Urticaceae species and a primary fiber crop for textile as well as a dominant plant of mining sites in Hunan Province, has been identified as a new Cd tolerance plant species with deep root and high biomass (Wang et al., 2008). Compared to other herbaceous and woody plants, ramie has great superiority in phytoremediation of Cd contaminated sites especially the farmland for its particular capability of Cd accumulation, fast growth, ease of cultivation and propagation. All the above makes ramie not only an advantageous plant species to achieve economic and eco-environmental benefits, but also a valuable organism for studying Cd-induced physiological mechanisms to strengthen plant abiotic stress tolerance.

Due to its strong phytotoxicity, Cd can pose adverse symptoms on plants such as leaf chlorosis, browning of root tips and growth retardation, even resulting in generation of reactive oxygen species (ROS), inhibition of photosynthesis (López-Millán et al., 2009), membrane lipid peroxidation (Li et al., 2012), protein degradation, ultra-structural damage and ultimately cell death

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(Daud et al., 2009). To survive Cd-induced stress, plants have evolved protective mechanisms to mitigate and repair the oxidative impairment (Edreva, 2005), including morphological changes, physiological adaptations and antioxidative defense system. Generally, the antioxidative defense mechanisms include antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antioxidants like glutathione (GSH), ascorbic acid (AsA) and carotenoids (Fernández et al., 2013). SOD is the key enzyme involved in catalyzing the dismutation of highly reactive superoxide radicals $O_2^{\bullet -}$ to O_2 and H_2O_2, which is further decomposed to non-toxic forms like O_2 and H_2O by APX of the ascorbate–glutathione cycle or by GPX and CAT in the cytoplasm and other cellular compartments (Jin et al., 2008).

Phytoextraction is defined as the removal of contaminants from soil by plant roots and their subsequent translocation to harvestable shoot tissues (Clabeaux et al., 2011). As an eco-friendly and cost-effective remediation technology, using plants to extract toxic metals from soil can be less destructive to soil structure and plant rhizosphere, more esthetic and more suited to impacted communities than conventional methods (Meighan et al., 2011). The extraction efficiency was closely related with contaminant availability for root uptake and translocation to the aerial parts (Bhargava et al., 2012). However, low bioavailability and limited translocation of some metals become the predominant restrictions in phytoextraction (Najeeb et al., 2011; Yang et al., 2013). Then high biomass yielding plant species with chemically assisted phytoextraction such as adding chelating agents have been used to enhance metal extraction efficiency. Chelators like low-molecular-weight organic acids (LMWOA) are capable of forming chemical complexes with metal ions and modifying the bioavailability of heavy metals in soil. LMWOA (such as citric, oxalic and malic acid), natural compounds derived from root exudates, are characterized as lower toxicity and higher biodegradability, which makes them more suitable for assisted phytoremediation than synthetic chelators.

Extensive studies have indicated that organic acids are involved in tolerance, transport and storage of heavy metal and played a key role in maintaining cellular homeostasis (Irtelli and Navari-Izzo, 2006). In particular, 25 μmol/L of citric acid could enhance Cd uptake and translocation by 61% and 2.2-fold at 4 μmol/L Cd level in *Halimione portulacoides*, respectively (Duarte et al., 2007). It could also counter Cd toxicity by improving plant growth, restoring shape and structure of root cells, and eliminating plasmolysis in *Juncus effusus* L. (Najeeb et al., 2011). Najeeb et al. (2009) found that citric acid was capable of enhancing Mn phytoextraction by 20% and alleviating metal toxicity from the reduced number of plastoglobuli in plant chloroplast. Similarly, Jean et al. (2008) reported that citric acid strengthened Cr and Ni uptake and translocation in *Datura innoxia*. Citric acid triggered more positive impact on Cu bioavailability and a visibly higher concentration of Cu uptake in tobacco shoot compared to oxalic and tartaric acid (Evangelou et al., 2006). Comparatively, citric acid (10 mmol/L) demonstrated a higher Cu mobilization (from 1 in the control to 42 mg/kg) than tartaric acid (Pérez-Esteban et al., 2013). These studies put forward an insight into the positive effect of exogenous organic acids on plant biomass, metal uptake and translocation, and metal tolerance for improving phytoremediation efficiency. In this work, a new Cd tolerant plant was studied to extend the range of plant species for better understanding of its potential application in phytoremediation.

Previous studies mainly focused on the accumulation, transport and tolerance mechanisms of ramie and subcellular distribution of heavy metals in plant tissues (Liu et al., 2007; Xia et al., 2009; Wang et al., 2008, 2011). However, to our best knowledge, little information is available on the effect of LMWOA on Cd phytoextraction and tolerance. Therefore it seems significant to demonstrate the natural chelator–Cd–plant interaction in this species. The objectives of this study were to: (1) explore the potential of ramie in phytoextraction of Cd; (2) compare the effect of different concentrations of exogenous citric and oxalic acids on plant growth, Cd uptake and translocation; and (3) determine the role of citric and oxalic acids in Cd tolerance of ramie by regulating the antioxidant defense system involved in stress endurance.

1. Materials and methods

1.1. Plant collection and hydroponic culture

A batch of ramie seedlings was collected from Ramie Research Institute (Changsha, Hunan Province, China). Plants were acclimatized to hydroponic condition in 1/8 Hoagland nutrient solution (pH 6.0 ± 0.5) for 2 weeks. The solution was aerated continuously and renewed every 2 days. The experiments were conducted in a growth chamber with 25/20°C, day/night temperature, 60%–70% relative humidity and 14 hr photoperiod at light intensity of 300 μmol/(m^2·sec).

1.2. Cadmium treatment and the addition of organic acids

After preculturing for 2 weeks, the plants were treated in triplicates in a completely randomized design as follows: 0 (Cd_0), 2 (Cd_2), 5 (Cd_5), 10 (Cd_{10}) mg/L Cd concentrations as $Cd(NO_3)_2$ and Cd_{10} + CA_1, Cd_{10} + CA_2, Cd_{10} + OA_1, Cd_{10} + OA_2 (CA_1 and CA_2 refer to 1.5, 4 mmol/L citric acid; OA_1 and OA_2 refer to 3, 9 mmol/L oxalic acid, respectively). Organic acids were applied at highest Cd level (10 mg/L) after Cd exposure for 1 week. Plant tissues were harvested after one month. Fresh samples were frozen immediately in liquid nitrogen and stored at −80°C for subsequent analysis.

1.3. Analysis of cadmium uptake

Plant roots and shoots were dried at 80°C till constant weight. About 0.5 g dry samples were digested with 10 mL HNO_3 and 3 mL $HClO_4$ at 160°C by heating to be transparent. Digested liquid was washed several times into a 100 mL volumetric flask and diluted with de-ionized water to the calibration line for measurement. The Cd content was determined by atomic absorption spectrometer (Analyst 700, PerkinElmer, Massachusetts, USA) and calculated as mg/kg of dry weight (dw). Translocation factor (TF) is determined as the ratio of heavy metal concentration in plant shoot to that in root.

1.4. Plant growth and root activity analysis

Fresh weight (fw) and dry weight of plant were measured by electronic balance as an indicator of plant growth. The water content (C_w, %) was determined on the basis of Eq. (1):

$$C_w = \frac{W_f - W_d}{W_f} * 100\% \quad (1)$$

where, W_f (g) and W_d (g) refer to fresh weight and dry weight of plant, respectively.

Root activity (RA) was determined by the triphenyl tetrazolium chloride (TTC) method (Clemensson-Lindell, 1994). Fresh roots (about 0.5 g) were immersed in beakers with the addition of 10 mL of 0.4% TTC and phosphate buffer (1/15 mol/L, pH 7.0). After incubation at 37°C for 2 hr in the dark, 2 mL of 1 mol/L H_2SO_4 was added to terminate the reaction. Then the samples

were ground homogeneously in 4 mL of 95% (V/V) ethyl acetate to extract the reduced triphenyl tetrazolium formazan (TTF). The extracts were transferred into a graduated test tube and the residues were washed three times with the extract buffer. The absorbance of the color was recorded at 485 nm and RA was calculated as mg TTF / (g fw · hr).

1.5. Physiological assay

Extraction of chlorophyll and carotenoid was carried out according to the method of Lichtenthaler (1987). About 0.5 g frozen leaves were ground homogeneously with 80% chilled acetone in the dark and centrifuged at 2000 r/min for 10 min. Then chlorophyll and carotenoid content were read by ultraviolet spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) on the supernatant at 470, 646 and 663 nm. Chlorophyll and carotenoid content were expressed as mg/g fw.

Following the method of Chaoui et al. (1997), the malondialdehyde (MDA) content was determined by the 2-thiobarbituric acid (TBA) reactive metabolites. Leaf and root samples (about 1 g fw, respectively) were ground homogeneously in 10 mL of 10% (W/V) trichloroacetic acid (TCA). The extract was centrifuged at 10,000 r/min, 4°C for 10 min. Absorbing 2 mL of the supernatant, 3 mL of 0.5% TBA (made in 10% TCA) was added. After incubation at 95°C for 30 min, the mixture was ice-cooled quickly and then centrifuged at 10,000 r/min, 4°C for 15 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity at 600 nm. The MDA content was calculated as nmol/g fw.

Soluble protein was assayed by the binding of Coomassie Brilliant Blue G-250 method (Bradford, 1976). Leaf and root samples (about 0.5 g fw, respectively) were treated with 3 mL of solution containing 50 mmol/L Tris–HCl (pH 7.8), 0.5 mmol/L $MgCl_2$, 1 mmol/L EDTA and 1 mmol/L dithiothreitol in ice precooled mortar. The mixture was homogenized under ice-bath condition and centrifuged at 10,000 r/min, 4°C for 20 min. Then the supernatant (0.1 mL) was mixed with 0.9 mL of Tris buffer solution and 5 mL of Coomassie Brilliant Blue G-250 staining solution (Philadelphia, USA) completely. After being kept for 2 min, the absorbance of the extract was measured at 595 nm corresponding with the bovine serum albumin calibration curve. The soluble protein content was measured as mg/g fw.

Frozen leaf samples (0.5 g fw) were homogenized under chilled condition in the buffer: 50 mmol/L, pH 7.8 phosphate buffer containing 0.1% (V/V) Triton X-100 and 1% (W/V) polyvinylpyrrolidone for extracting SOD and POD; 0.9% (W/V) NaCl was used as an extraction buffer for CAT extraction. Then the homogenate was centrifuged at 15,000 r/min, 4°C for 20 min and the supernatant was used for determining enzyme activities, which were processed at 25°C. SOD activity was assayed by photochemical nitroblue tetrazolium (NBT) method. Photoreduction of NBT (formation of purple formazan) at 50% inhibition was recorded at 560 nm using 3 mL of mixture containing 50 mmol/L phosphate buffer (pH 7.8), 750 μmol/L NBT, 130 mmol/L methionine, 100 μmol/L EDTA-Na_2 and 20 μmol/L riboflavin. POD activity was determined using Guaiacol method by monitoring the increase in absorption at 470 nm as a consequence of guaiacol oxidation in a reaction solution (3 mL final volume) consisting of 50 mmol/L phosphate buffer (pH 7.0), 0.3% H_2O_2, 0.2% guaiacol and 0.5 mL of enzyme extract. For CAT activity determination, the H_2O_2 reduction method was used to measure the decrease in absorbance at 240 nm due to H_2O_2 consumption (Aeby, 1984). The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0), 0.1 mol/L H_2O_2 and 0.5 mL of leaf enzyme extract solution in a 3 mL volume. These enzyme activities were expressed as U/mg protein, OD_{470} / (g fw · min) and OD_{240} / (g fw · min), respectively, where 1 U meant enzyme content when the relative inhibition ratio of NBT photoreduction reached 50%.

1.6. Data analysis

The results from a representative experiment were presented as mean values ± S.E. of three replications. Graphical work was carried out using Origin v.8.0 (USA). Statistical significance was conducted by t-test at a probability level of $p < 0.05$.

2. Results

2.1. Cadmium accumulation and translocation

In the hydroponic condition, Cd accumulation in ramie roots and shoots exhibited a linear increase in response to elevated Cd in the medium as shown in Table 1. Cd accumulated in plant root was approximately 9–10 times that in the aerial part. It appeared to change significantly from Cd_2 to Cd_5 group ($p < 0.05$), with Cd uptake from 268.0 to 370.8 mg/kg dw in root and 25.2 to 35.6 mg/kg dw in shoot. The presence of citric and oxalic acids promoted Cd uptake in plant aerial and underground parts. Especially, the low dose of citric acid dramatically increased Cd uptake by 26.7% in root and 1-fold in shoot ($p < 0.01$). A slight increase in Cd transport from roots to shoots was observed, ranging from 9.4% to 11.0%. Compared to Cd_{10} treatment, the low dose of citric and oxalic acids significantly increased TF from 11.0% in the control to 17.8% and 18.3% ($p < 0.05$), respectively.

2.2. Plant growth attributes and RA

Plants showed slight symptoms of Cd phytotoxicity, as seen from trivial chlorosis in young leaves and browned symptom in root tips after exposure to Cd for 1 week. Plant growth retardation was observed at all Cd levels with a reduction in both fresh biomass and dry biomass, as well as shoot/root ratio (Table 2). Particularly fresh biomass and dry biomass at 2 mg/L of Cd level were declined by 30.5% and 46.7%, respectively. Compared to Cd_{10} treatment, the citric and oxalic acid addition alleviated the inhibition to plant growth, as seen from the increase in fresh biomass and dry biomass of plant tissues. The low level of citric acid enhanced plant fresh biomass and dry biomass by 44.6% and 74.4%, respectively, which were more distinct than the high one and two levels of oxalic acid. The water content increased from 74.4% in the control to 84.4% at 5 mg/L of Cd level, and decreased to 84.0% at highest Cd level. Citric acid decreased it to 80.8%, whereas oxalic acid slightly elevated it to 86.2%. The changes are not significant.

RA is a reliable indicator of root respiration and mineral substance absorption capability. As shown in Fig. 1, RA reached

Table 1 – Effects of Cd, citric and oxalic acids on Cd uptake and translocation in ramie (mean ± SE, n = 3).

Treatment	Cd concentration (mg/kg dw)		Root/shoot ratio of Cd concentration	TF value (%)
	Shoot	Root		
Cd_0	ND	ND	–	–
Cd_2	25.2 ± 2.6	268.0 ± 24.3	10.6	9.4
Cd_5	35.6 ± 4.2*	370.8 ± 36.5*	10.4	9.6
Cd_{10}	41.2 ± 4.0	374.4 ± 35.3	9.1	11.0
Cd_{10} + CA_1	84.2 ± 8.7**	474.2 ± 45.8**	5.6	17.8*
Cd_{10} + CA_2	56.6 ± 5.8	409.0 ± 37.5*	7.2	13.8
Cd_{10} + OA_1	84.0 ± 8.0**	460.2 ± 43.8**	5.5	18.3*
Cd_{10} + OA_2	53.0 ± 4.7	389.4 ± 34.7	7.4	13.6

Cd_0: without addition of Cd in nutrient solution; Cd_2, Cd_5, Cd_{10}: 2, 5, 10 mg/L Cd concentration; Cd_{10} + CA_1: 10 mg/L Cd concentration + 1.5 mmol/L citric acid; Cd_{10} + CA_2: 10 mg/L Cd concentration + 4 mmol/L citric acid; Cd_{10} + OA_1: 10 mg/L Cd concentration + 3 mmol/L oxalic acid; Cd_{10} + OA_2: 10 mg/L Cd concentration + 9 mmol/L oxalic acid.
ND: not detectable.
TF: translocation factor, the ratio of heavy metal concentration in plant shoot to that in root.
* and ** indicate significant differences in parameters between the control group and the experimental group at $p < 0.05$ and $p < 0.01$, respectively.

the highest value in the control (4.1 mg TTF / (g fw · hr)) and decreased progressively to 62.2%, 42.2% and 12.5% of the control with Cd level increasing. It was improved significantly to 15.2% of the control in the presence of low citric acid level ($p < 0.05$). But no visible changes occurred in oxalic acid treatment.

2.3. Chlorophyll and carotenoid content

Chlorophyll is regarded as a key indicator of plant photosynthetic capacity. Carotenoid, a cell endogenous antioxidant, plays an important role in scavenging ROS and preventing membrane lipid peroxidation. Chlorophyll content reached maximum (7.8 mg/g fw, 2.3 times of the control) at 5 mg/L of Cd level and declined sharply (5.3 mg/g fw) at highest Cd level. However, carotenoid content increased with Cd levels, reaching maximum (1.3 mg/g fw, 4.5 times of the control) at 10 mg/L of Cd level (Fig. 2). The low dose of citric acid (1.5 mmol/L) significantly increased chlorophyll and carotenoid content by 17.9% and 35.7% ($p < 0.05$), respectively, which exhibited a better mitigating effect on photosynthetic pigments than the high dose and two doses of oxalic acid.

2.4. Lipid peroxidation

The extent of lipid peroxidation in plant leaves and roots was estimated by determining MDA content. The much higher MDA content of ramie leaves revealed severer lipid peroxidation emerging in leaves. There was a dose-dependent increase in MDA content with a maximum of 57.1% increase in leaf and 72.1% in root at 10 mg/L of Cd level compared to the control (Fig. 3). Citric and oxalic acids ameliorated lipid peroxidation with MDA content decrease comparably in contrast to Cd_{10} treatment alone, especially the high citric acid level with 26.2% and 29.4% reduction in leaf and root, respectively. Citric acid exhibited a better alleviation on lipid peroxidation than oxalic acid, and the high dose of acid was better than the low one.

2.5. Soluble protein

Soluble protein content increased significantly by 31.8% in leaf and 76.3% in root at 5 mg/L of Cd level compared to the control ($p < 0.05$). Then it declined slightly by 7.3% in leaf and sharply by 46.2% (the minimum value 30.8 mg/g fw) in root at highest Cd level probably due to protein degradation (Fig. 4). The presence of citric acid resulted in enhancement of soluble protein content with an average of 2.9, 6.4 mg/g increment in leaf and root, respectively. While the high dose of oxalic acid declined the content by 3.1 mg/g in leaf.

2.6. Antioxidative enzymes

The SOD activity increased dramatically at low Cd level in response to Cd stress and this rise trend became gentle under higher Cd concentrations (Fig. 5). It reached 1.8-fold of non-Cd treatment at highest Cd level. The presence of citric and oxalic acids further enhanced the SOD activity. Especially the low citric

Table 2 – Effects of Cd, citric and oxalic acids on fresh biomass, dry biomass and water content of ramie (mean ± SE, n = 3).

Treatment	Fresh biomass (g/plant)			Dry biomass (g/plant)			Water content (%)
	Shoot	Root	Shoot/root ratio	Shoot	Root	Shoot/root ratio	
Cd_0	17.62 ± 1.61	9.25 ± 0.95	1.90	4.83 ± 0.52	2.06 ± 0.23	2.34	74.4
Cd_2	11.06 ± 1.27	7.61 ± 0.69	1.45	2.41 ± 0.21	1.26 ± 1.39	1.91	80.3
Cd_5	8.28 ± 0.76	7.93 ± 0.72	1.04	1.42 ± 0.15	1.11 ± 0.09	1.28	84.4
Cd_{10}	7.72 ± 0.73	5.50 ± 0.58	1.40	1.29 ± 0.14	0.82 ± 0.07	1.57	84.0
Cd_{10} + CA_1	10.72 ± 1.06	8.40 ± 0.83	1.28	2.36 ± 0.25	1.32 ± 0.14	1.79	80.8
Cd_{10} + CA_2	9.10 ± 0.98	8.38 ± 0.81	1.09	1.76 ± 0.16	1.27 ± 0.12	1.39	82.7
Cd_{10} + OA_1	9.86 ± 0.92	6.48 ± 0.65	1.52	1.56 ± 0.13	0.95 ± 0.08	1.64	84.6
Cd_{10} + OA_2	8.72 ± 0.85	6.78 ± 0.66	1.29	1.30 ± 0.12	0.84 ± 0.06	1.55	86.2

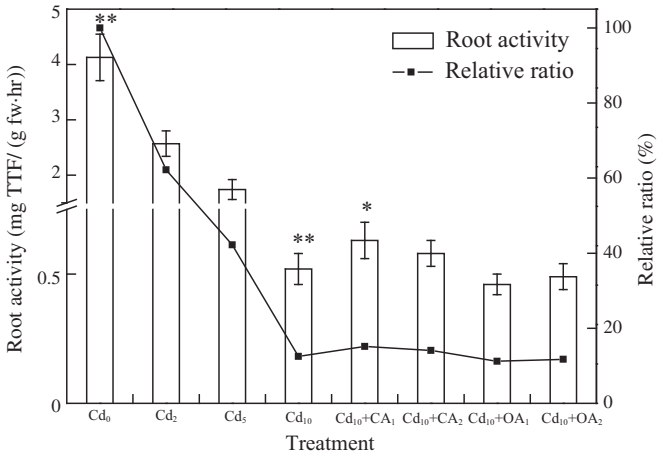

Fig. 1 – Root activity of ramie exposed to different levels of Cd, citric and oxalic acid application at the highest Cd level. Vertical bars means the standard error of mean values (n = 3). * and ** indicate significant differences in parameters between the control group and the experimental group at $p < 0.05$ and $p < 0.01$, respectively.

acid level improved SOD activity by 7.8%, more significantly than the high level as well as two levels of oxalic acid. Activities of POD and CAT decreased gradually with elevated Cd concentration and reached a minimum at highest Cd level (81.6% and 64.5% of its control, respectively). Changes in CAT activity followed the similar pattern as POD activity. A considerable enhancement of the two enzyme activities was observed in the presence of organic acids, which facilitated the efficiency of scavenging ROS and alleviated oxidative stress from Cd. Particularly, low citric acid level exhibited a better alleviation (7.5% increase in POD activity and 7.4% in CAT activity) than the high level as well as two levels of oxalic acid.

3. Discussion

3.1. *Prompting effects on Cd uptake and translocation by citric and oxalic acids*

It is meaningful to study the accumulation and transport mechanism of metals in plant for the high efficiency of phytoremediation. Cd content in plant tissues increased in a dose-dependent manner, which are in accordance with the results reported by Fidalgo et al. (2011). Compared to the aerial tissues, ramie showed a tendency to accumulate a higher amount of Cd in roots that were primary sites for metal access, as a natural protective response to lessen Cd toxicity in plant aboveground tissues especially leaves. Therefore, the translocation factor of Cd from roots to shoots remained at a relatively low value. Citric acid at 1.5 mmol/L and oxalic acid at 3 mmol/L significantly increased Cd uptake to both 2-fold in the shoots and 1.3 and 1.2-folds in the roots of the controls, respectively. They also improved the TF by 61.8% and 66.4%, respectively. Citric and oxalic acids promoted Cd uptake and transport, in consistent with the findings of Duarte et al. (2007), who reported enhanced Cd uptake and translocation by *Halimione portulacoides* in the presence of citric acid.

The above fact was probably due to the following reasons. Firstly, Cd solubility and availability were increased by the increased pH and the release of strong organic ligands from organic acids into the plant–solution interface, which facilitated Cd uptake by ramie roots and translocation to the shoots. Secondly, they activated ATPases in the plasma membrane resulting in alteration of ions transport in charge of metal

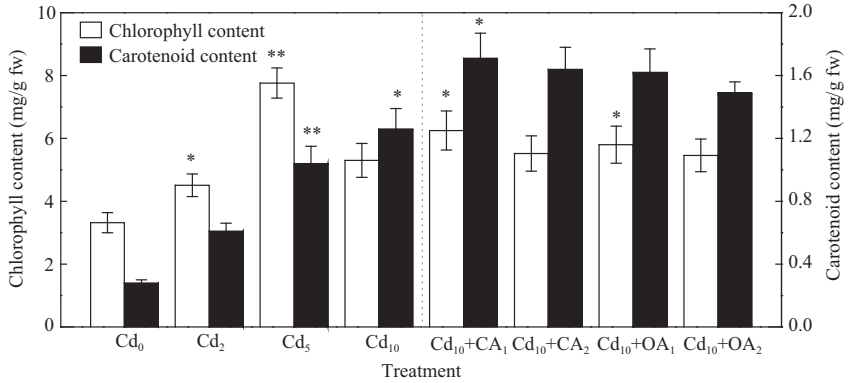

Fig. 2 – Total chlorophyll and carotenoid content in leaves of ramie exposed to different levels of Cd, citric and oxalic acid application at the highest Cd level. Vertical bars refer to the standard error of mean values (n = 3). * and ** indicate significant differences in parameters between the control group and the experimental group at $p < 0.05$ and $p < 0.01$, respectively.

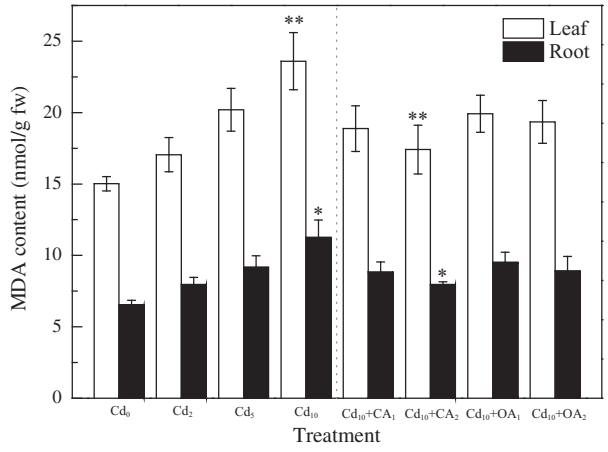

Fig. 3 – Malondialdehyde (MDA) content in leaves and roots of ramie exposed to different levels of Cd, citric and oxalic acid application at the highest Cd level. Vertical bars refer to the standard error of mean values (n = 3). * and ** indicate significant differences in parameters between the control group and the experimental group at $p < 0.05$ and $p < 0.01$, respectively.

translocation. Furthermore, organic acid chelating with heavy metal should be viewed as an essential process of efficiently participating in metal long-distance translocation in the xylem and avoiding the toxicity of free metal ions in plants (Evangelou et al., 2006; Montargès-Pelletier et al., 2008; Wei et al., 2009; Ghnaya et al., 2013). The chelant–metal complex might enter into plant tissues through breaking in the root endodermis and Casparian strips, and subsequently translocating upward to the shoots with the assistance of transpiration.

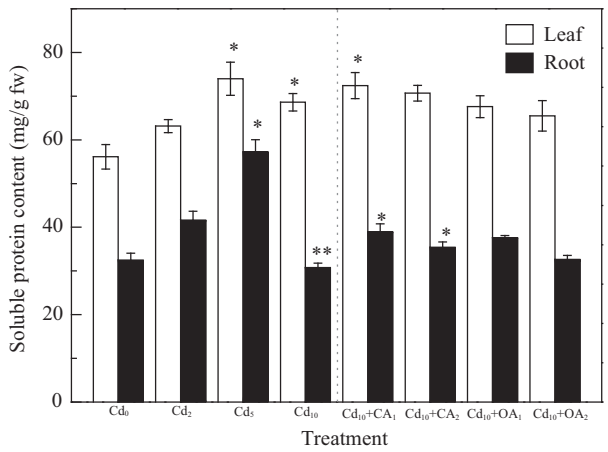

Fig. 4 – Soluble protein content in leaves and roots of ramie exposed to different levels of Cd, citric and oxalic acid application at the highest Cd level. Vertical bars refer to the standard error of mean values (n = 3). * and ** indicate significant differences in parameters between the control group and the experimental group at $p < 0.05$ and $p < 0.01$, respectively.

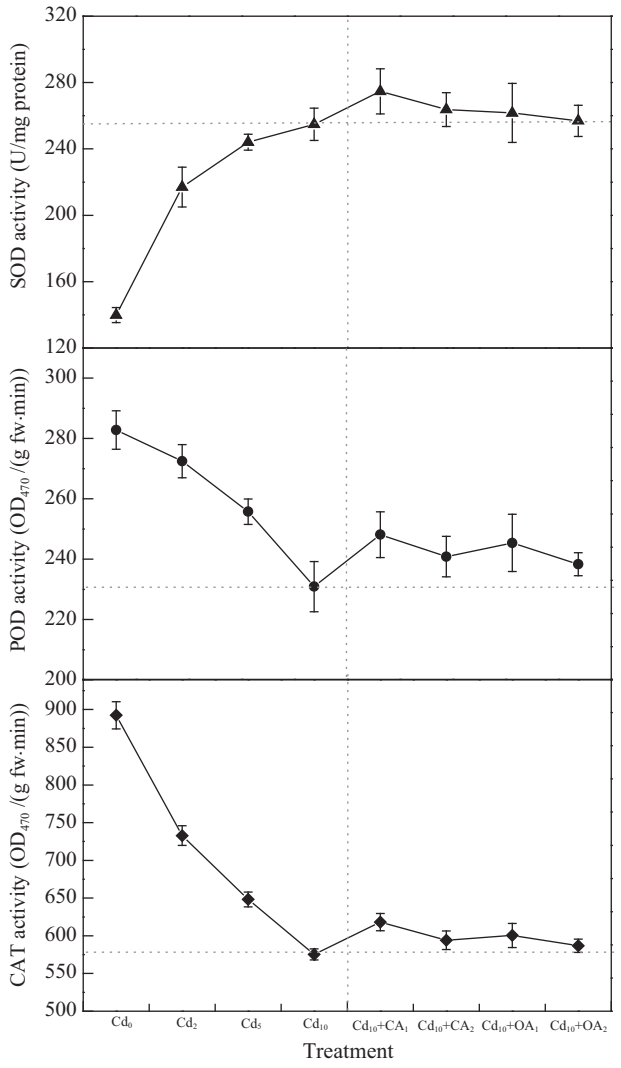

Fig. 5 – Superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) activities in leaves of ramie exposed to different levels of Cd, citric and oxalic acid application at the highest Cd level. Vertical bars means the standard error of mean values (n = 3).

3.2. *Effects of citric and oxalic acids on plant physiological properties*

The present study was carried out in hydroponics for the reason that the medium provided a homogeneous condition in favor of quick identification of Cd-induced plant physiological characters. Results showed that ramie was able to survive moderate Cd oxidative injury with some repairment of self-defense system and 10 mg/L was regarded as its tolerance threshold to Cd. Citric acid at 1.5 mmol/L could play a remarkable role in regulating Cd-induced physiological alterations in ramie.

A better comprehension of heavy metal toxicity on physiological metabolism contributes to investigating the approaches for Cd tolerance enhancement. An increasing metal sequestration, tissue tolerance and antioxidative

response determine the ability of plant to defy metal destructive damage (Jin et al., 2008). Heavy metal might interfere with numerous biochemical and physiological processes including respiration, nutrient uptake, photosynthetic system, protein metabolism and activities of antioxidative enzymes (Shamsi et al., 2008; Daud et al., 2009; Rai et al., 2013). Reduced plant biomass could reflect an irreversible plant growth inhibition (Srivastava et al., 2004), which appeared to be correlated with the decrease in RA under water stress induced by Cd stress. Reduced RA in turn resulted in weaker root respiration, less ATP production and nutrient elements absorption, as well as the reduction in POD activity involving in plant growth and development processes. The gradually brownish tissue and the sharp decrease of RA exhibited its sensibility to Cd toxicity. Cd stress resulted in a distinct decrease of chlorophyll and soluble protein synthesis. Particularly, the higher Cd concentration not only caused a dramatic breakdown of chlorophyll by inhibiting protochlorophyllide reductase and the photosynthetic electron transport (Aibibu et al., 2010), but also resulted in protein metabolism degradation in excess of Cd toxicity beyond plant's tolerance extreme. The changes of chlorophyll content and soluble protein content in this study coincided with the findings of Liu et al. (2007).

Antioxidative enzymes and certain metabolites play a crucial role in adaptation and ultimate survival of plant to Cd oxidative damage. With the Cd stress rising, there was an increase in the activity of SOD and a decline in the activities of POD and CAT, in line with the results of Najeeb et al. (2011). Enhanced relative water content (generally as the osmotic influence of abiotic stress), photosynthetic pigments, protein synthesis and SOD activity could be viewed as an adaptive regulation in response to Cd stress. Especially the SOD activity in the antioxidant system increased progressively with increasing Cd level, and acted as the first defense line of scavenging ROS to avoid excess oxidative impairment in response to the stimulation of lipid peroxidation. However, POD and CAT activities showed similar descending tendency probably due to the inhibition of enzyme synthesis or an alteration in the assemblage of enzyme subunits. Furthermore, inhibition of POD and CAT synthesis limited the efficiency of eliminating H_2O_2 produced in chloroplasts, which would lead to membrane lipid peroxidation demonstrated by the continuous elevated MDA content (an indicator of oxidative injury), implying that oxidative stress from Cd (probably the deleterious effect of H_2O_2) did exist in plant. Generally, the lipid peroxidation exhibited a concentration-dependent increasing trend. Particularly, it was relatively severer in ramie leaves than roots (Fig. 3). This could be explained by the following scenario: the generation of $NADP^+$ (a key electron acceptor in photosynthesis) decreased due to damaged CO_2 assimilation resulting from stomatal closure under Cd stress in leaves, further the lacking of $NADP^+$ with continuous light reaction form $O_2^{\bullet -}$ and subsequent H_2O_2 by facilitating the electron transfer from PS-I to O_2 (Demiral and Türkan, 2005; Liu et al., 2007).

In the presence of organic acids, all antioxidative enzyme activities were enhanced and lipid peroxidation was mitigated compared with the controls, which suggested that organic acids might have interfered in combating Cd-induced oxidative stress in plant as a consequence of the less toxicity of organic acid-Cd chelates. Citric acid posed a more effective influence due to its stronger binding affinity for Cd than oxalic acid. The contents of chlorophyll, carotenoid and soluble protein were also elevated owing to the alleviation of Cd oxidative damage. This could be explained by the prevention of free metal ion transmission in the cytosol. Cd damaged plant cells through cytoplasmic shrinkage and metal deposition and citric acid restored structure and shape of cells and eliminated plasmolysis (Najeeb et al., 2011). Thus the insight was proposed that ramie was able to survive more easily under high Cd stress, and exerted stronger defense in the presence of organic acids especially the citric acid, without showing visible phytotoxicity symptoms, which had prospect to be a useful strategy for the phytoremediation of Cd contaminated area, particularly the Cd polluted farmland concerning safety grain production.

Cd has previously been reported to induce phytochelatin (PC) synthesis using GSH as its precursor in several plant species (Srivastava et al., 2004; Vurro et al., 2011) particularly in root as a detoxify mechanism. In the subsequent work, we would like to investigate the interactions between PC induction and plant detoxification under abiotic Cd stress and the role of organic acids in this process to examine plant physiological mechanism more deeply (Mishra et al., 2006; Seth et al., 2008).

4. Conclusions

The results obtained from the present study demonstrated that exogenous citric and oxalic acids had the capability of facilitating Cd uptake and transport, and alleviating the physiological toxicity from free metal ions. As a Cd tolerance plant, ramie accumulated more Cd in root tissue than that in shoot tissue in order to help plant survive Cd toxicity. High Cd level reduced plant biomass, root activity, chlorophyll content, synthesis of protein, antioxidative enzyme activities (POD and CAT) and enlarged lipid peroxidation. Furthermore, citric and oxalic acids acted as a ligand to chelate with Cd and entered in the form of organic acid-Cd chelation, which efficiently alleviated the physiological toxicity from free metal ions. Taken together, as a non-edible crop and a pioneering plant species of abandoned mining area, ramie in combination with citric acid has promise to be applied in the phytoremediation of moderate Cd contaminated site due to the increased plant biomass, Cd uptake and endurance.

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