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Subcellular cadmium distribution and antioxidant enzymatic activities in the leaves of two castor (*Ricinus communis* L.) cultivars exhibit differences in Cd accumulation

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ABSTRACT

The aims of this study were: (1) the study of cadmium (Cd) accumulation and toxicity in different castor cultivars (*Ricinus communis* L.); (2) to investigate changes in antioxidant enzymatic activities and the subcellular distribution of Cd in young and old leaves from two different castor cultivars, after exposure to two different Cd concentrations, and explore the underlying mechanism of Cd detoxification focusing on antioxidant enzymes and subcellular compartmentalization. The Cd concentration, toxicity, and subcellular distribution, as well as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities were measured in Zibo-3 and Zibo-9 cultivars after exposure to two different concentrations of Cd (2 mg/L and 5 mg/L) for 10 days. This research revealed Cd accumulation characteristics in castor are root > stem > young leaf > old leaf. Castor tolerance was Cd dose exposure and the cultivars themselves dependent. Investigation of subcellular Cd partitioning showed that Cd accumulated mainly in the heat stable protein (HSP) and cellular debris fractions, followed by the Cd rich granule (MRG), heat denatured protein (HDP), and organelle fractions. With increasing Cd concentration in nutrient solution, the decreased detoxified fractions (BDM) and the increased Cd-sensitive fractions (MSF) in young leaves may indicate the increased Cd toxicity in castor cultivars. The BDM-Cd fractions or MSF-Cd in old leaves may be linked with Cd tolerance of different cultivars of castor. The antioxidant enzymes that govern Cd detoxification were not found to be active in leaves. Taken together, these results indicate Cd tolerance and toxicity in castor can be explained by subcellular partitioning.

1. Introduction

Cadmium (Cd) is an environmental toxicant (Thévenod, 2009) and one of the main pollutants in soil (Huang et al., 2011). Cadmium is not degraded in the environment and is easily transferred from the soil to the human body via the food chain, thus endangering human health (Jarup, 2003). Hence, it is essential to remediate Cd contaminated soil, preventing Cd from entering the human body via consumption of contaminated crops.

Castor (*Ricinus communis* L.), a highly valuable and renewable resource, can be cultivated for phytostabilization and for bioenergy production. Its use can simultaneously addresses two critical global problems: the need to meet increasing energy demands and the remediation of Cd polluted soils (Olivares et al., 2013). Thus, this plant has extensive potential, and researchers are giving it increasing attention worldwide.

Subcellular partitioning of metals in plants represents the internal mechanism of the plant to prevent toxic effects from metals (Wang and Rainbow, 2006; Miao and Wang, 2007). On the basis of differential centrifugation of tissue, five defined subcellular fractions containing metals were obtained and identified as; metal-rich granule (MRG), cellular debris, organelle, heat denatured protein (HDP), and heat stable protein (HSP) (Wallace et al., 2003). Subcellular partitioning under metal stress has been studied in marine diatoms (Miao and Wang, 2006), bivalves (Wallace et al., 2003), and wheat (Li et al., 2011b). Miao and Wang (2006) demonstrated by partitioning Cd to the soluble fraction (HDP and HSP) helped to prevent Cd toxicity in marine diatoms under different nutrient conditions. Wallace et al. (2003) reported that

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partitioning Cd to the biologically detoxified metal (MRG and HSP) fractions was shown to be the primary mechanism of detoxification in the diatom under moderate metal stress.

The antioxidant enzymatic system represents a defense strategy that plants use against Cd stress. Cd stress in plants induces oxidative stress in cells (Sandalio et al., 2001), leading to the enhancement of reactive oxygen species (ROS) and damage to cellular constituents (Li et al., 2011a). In order to combat oxidative stress caused by metal toxicity, the antioxidant defense system, which is comprised of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), plays an important role in scavenging ROS (Vanassche and Clijsters, 1990; Chaoui et al., 1997). Moreover, the activity levels of these antioxidant enzymes could reflect the biological process and indicate the metal toxicity (Li et al., 2011b).

To date, the subcellular partitioning of Cd and the antioxidant defense system in castor cultivars, with differing degrees of metal accumulation capacities, has never been studied. The main aims of this research are: (1) to determine the pattern of Cd accumulation in castors; (2) to investigate changes in antioxidant enzymatic activities and subcellular Cd distribution in young leaves and old leaves in two castor cultivars after exposure to different Cd concentrations, and explore the underlying mechanism of Cd detoxification, focusing on antioxidant enzymes and subcellular compartmentalization.

2. Materials and methods

2.1. Castor cultivars and experimental design

Seeds of castor cultivars, Zibo-3 and Zibo-9, were acquired from the Zibo Academy of Agricultural Sciences, Zibo, Shandong province, China. These two cultivars displayed different Cd accumulation capabilities in our previous research (Table S1). Based on these results, Zibo-9 and Zibo-3 were classified as a high Cd accumulator and a low Cd accumulator, respectively.

Castor seeds were initially grown on artificial, non-polluted soil for 2–3 weeks until seedlings developed two healthy tender leaves. These uniformed seedlings were used as research subjects for hydroponic experiments in a greenhouse located at the Center for Environmental Remediation, Institute of Geographical Sciences and Natural Resources, Chinese Academy of Sciences, Beijing, China.

The uniformed seedlings were transplanted in to 1 L pots containing 400 mL of Hoagland's solution (half strength) of the following composition: 2.5 mmol/L $Ca(NO_3)_2$, 2.5 mmol/L KNO_3, 0.5 mmol/L KH_2PO_4, 0.5 mmol/L $MgSO_4$, 25 μmol/L H_3BO_3, 2.25 μmol/L $MnCl_2$, 1.9 μmol/L $ZnSO_4$, 0.15 μmol/L $CuSO_4$, 0.05 mmol/L $(NH_4)_6Mo_7O_{24}$, and 5 μmol/L Fe-EDTA. A cadmium salt, $CdCl_2 \cdot 2.5H_2O$ (guaranteed reagent) was used as the pollutant source. The Cd salt was added to the hydroponic culture. The pH of the nutrient solution was maintained at 6.0 ± 0.1 by the addition of 0.1 mol/L NaOH. Eight replicates of each cultivar were treated with 0, 2, and 5 mg/L Cd: three replicates were used to determine biomass and Cd concentrations; the remaining replicates were used for analysis of physiological indexes. Plants were kept in a greenhouse at temperatures of 25/15 °C (day/night) and a 16 h photoperiod of approximately 300 mE/m^2/s intensity, and at 65% average relative humidity. The seedlings grew for 10 days in nutrient culture, and the nutrient culture was replaced every 3 days.

2.2. Plant sample preparation and chemical analysis

Each harvested plant was divided into root, stem, old leaf, and young leaf sections. The individual plant parts were first washed with tap water and then with 10 mmol/L ethylenediaminetetraacetic acid (EDTA) for 5 min to remove Cd from the roots' surface, and finally rinsed three times with deionized water. The plant parts used for determination of biomass and Cd concentration were oven dried at 70 °C to a constant weight. The dry weights were measured (to 0.1 mg accuracy) with an electronic balance. The fresh leaves used for analysis of antioxidant enzymes, soluble protein and subcellular distribution were stored in liquid nitrogen.

Roots, stems, old leaves, and young leaves were separately ground in a mill, and then digested in flasks at 60 °C, on an electric heating plate, with concentrated HNO_3. The temperature was then increased to 110 °C, and the mixture was maintained at that temperature until the solution became clear. The sample volume was adjusted to 25 mL with ultrapure water. The Cd concentration in each sample was measured by inductively coupled plasma optical emission spectrometry (Optima 5300 DV; Perkin-Elmer, Norwalk, CT, USA). Standard reference material for bush twigs and leaves (GBW07603: GSV-2) was used to monitor the Cd recovery from the plant samples (recovery: $90 \pm 10\%$). The chemical analysis was carried out at the Physical and Chemical Analysis Center, Institute of Geographical Sciences and Natural Resources, Chinese Academy of Sciences, Beijing, China.

2.3. Subcellular distribution of Cd in castor cultivar leaves

According to the differential centrifugation method (Lavoie et al., 2009; Li et al., 2011a, 2011b), intracellular Cd was separated into 5 subcellular fractions: Cd-rich granule (MRG), cellular debris, organelle, heat denatured protein (HDP), and heat stable protein (HSP) fractions. The method is as follows: a 0.2 g leaf sample, from each treatment, was ground to powder using liquid nitrogen and a mortar and pestle, and then homogenized in 5.0 ml of buffer solution containing 0.25 M sucrose, 1.0 mM dithioerythritol, and 50 mM Tris–HCl (pH 7.5). The homogenate was centrifuged at 15,000*g*, for 15 min, at 4 °C. The resulting pellet contained granules and cellular debris. The separation of the granules fraction from the pellet was conducted after resuspension in 2.0 ml ultrapure water and heating for 2 min at 100 °C, followed by the addition of 2.0 ml of 1.0 M NaOH and heating for 1 h at 70 °C, and then samples were centrifuged at 10,000*g*, at 4 °C, for 15 min. The resulting pellet was designated as the cellular debris fraction. The supernatant of the first centrifugation step, containing the cytosol and the organelle fractions, was then centrifuged at 100,000*g*, at 4 °C, for 60 min to sediment organelle components (i.e., chloroplasts, mitochondria). The resulting pellet was designated as the organelle fraction. The 100,000*g* supernatant containing the cytosol fraction was heat at 80 °C for 10 min, then cooled on ice for an hour, and finally centrifuged at 50,000*g*, for 15 min, at 4 °C, separating the heat stable proteins (HSP) in the supernatant and the heat denatured proteins (HDP) into the pellet. All five fractions were digested with 5.0 ml pure concentrated HNO_3.

The Cd concentration of the five fractions was measured by flame atomic absorption spectroscopy (Contra AA 700, Germany). The presumed metal-sensitive fractions (MSF) are defined as the organelle and HDP fractions, and the biologically detoxified metal (BDM) fractions are defined as the MRG and HSP fractions (Wang and Rainbow, 2006). The data presented are the mean values of triplicates.

2.4. Determination of antioxidative enzyme activities and soluble proteins

Fresh leaves (0.2 g) were homogenized with an extraction buffer solution (0.05 mmol/L $NaH_2PO_4 + Na_2HPO_4$ in 1 percent polyvinylpyrrolidone, pH 7.4), ground with a chilled mortar and pestle, and then centrifuged at 10,000*g*, for 10 min, at 4 °C (Li et al., 2011a). The supernatant was used to analyze soluble proteins and

antioxidant enzymes. The Bradford method was used to determine soluble protein concentrations and these concentrations are expressed as mg protein/g FW.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) (Giannopolitis and Ries, 1977). SOD activity was defined as the amount of protein inhibiting 50% of the initial reduction of NBT under light. One unit of SOD activity was expressed as U/mg protein. Catalase (CAT) activity was determined by measuring the H_2O_2 conversion rate at 240 nm (Aebi, 1984). Reduction of 0.1 units at A240 per min was considered as one unit of enzyme activity (U), and CAT activity was expressed as U/mg protein. Guaiacol peroxidase (POD) was determined by H_2O_2 induced guaiacol oxidation at 470 nm (Chance and Maehly, 1955). One unit of POD activity was expressed as U/mg protein.

2.5. Tolerance index, bioconcentration factor and translocation factor

The tolerance index (TI) was defined as the ratio of plant biomass after Cd treatments to that of the control group's biomass (Zacchini et al., 2009). The Cadmium bioconcentration factor (BCF) was defined as the ratio of Cd in the shoot or root of a plant to that in the nutrient solution or soil. The cadmium translocation factor (TF) was described as the ratio of Cd in the shoot to that in the root. The indexes are defined as follows:

$$\mathrm{TI} = \frac{W_{\mathrm{Cd}}}{W_{\mathrm{control}}}$$

where, W_{Cd} (g) and W_{control} (g) represent the biomass after Cd treatment and the biomass of the control group, respectively.

$$\mathrm{BCF} = \frac{C_{\mathrm{tissue}}}{C_{\mathrm{medium}}}$$

where, C_{tissue} (mg/L) and C_{medium} (mg/L) represent the Cd concentration in the aerial parts or root and the Cd concentration in the nutrient solution, respectively. Cd concentration in the aerial parts was the average concentration of Cd concentration in stem, old leaves, and fresh leaves.

$$\mathrm{TF} = \frac{C_{\mathrm{aerial}}}{C_{\mathrm{root}}}$$

where, C_{aerial} (mg/kg) and C_{root} (mg/kg) represent the Cd concentration in the aerial parts and the Cd concentration in the root, respectively. C_{aerial} was calculated on the basis of total Cd in the aerial parts divided by total dry weight of aerial parts (stem+old leaves+fresh leaves).

2.6. Statistical analysis

Data were analyzed using ANOVA testing and SPSS 16.0 software (SPSS, Chicago, IL, USA). Data are presented as mean values ± SE, and were compared by Duncan's test. A p value less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. The biomass analysis of two castor cultivars

The biomass of cultivars decreased with increasing Cd exposure concentrations from 0 to 5 mg/L (Table 1). Compared with 0 mg/L samples, biomass decreased in the Zibo-3 cultivars 22.5% and 28.0% after exposure to 2 and 5 mg/L Cd, respectively; biomass decreased in the Zibo-9 cultivars 14.0% and 30.6%, respectively. According to the two-way analysis of variance test analysis, the biomasses of the two cultivars were significantly influenced by Cd doses ($p < 0.01$) and between the two cultivars ($p < 0.01$). With increasing Cd concentrations the TI decreased from 77.5 to 72.0 for Zibo-3 and from 86.0 to 69.4 for Zibo-9.

Table 1
Effects of Cd stress on biomass and TI in two castor cultivars.

Cultivars	Cd treatments	Biomass (g/kg)	TI(%)
Zibo-3	0 mg/L	0.489 ± 0.01ab	
	2 mg/L	0.379 ± 0.02c	77.5
	5 mg/L	0.352 ± 0.03c	72.0
Zibo-9	0 mg/L	0.585 ± 0.05a	
	2 mg/L	0.503 ± 0.03ab	86.0
	5 mg/L	0.406 ± 0.03bc	69.4
Analysis of variance			
Cd		12.6**	
Cultivar		12.4**	
Cd × cultivar		0.621	

TI: tolerance index
Mean values ($n=3$) with different letters in the same column are significantly different between the treatments according to the Duncan's test ($p < 0.05$).
The effects of Cd dose and differences in cultivar were analyzed by the two-way analysis of variance test;
** $p < 0.01$.

3.2. Cd accumulation and subcellular distribution

The Cd accumulation in various tissues of the two castor cultivars after exposure to Cd (2 mg/L and 5 mg/L) is shown in Table 2. The Cd accumulation in the tissues of the Zibo-9 was higher than in the same tissues from Zibo-3, at comparable Cd exposure levels. As Cd concentration increased in the nutrition solution, the Cd accumulation in roots and stems increased while Cd concentrations in old and young leaves decreased. In both cultivars, Cd accumulation in the roots greatly exceeded the accumulation in other tissues. The order of Cd accumulation in different tissues was root > stem > young leaf > old leaf. According to the two-way analysis of variance test analysis, Cd concentrations in the tissues examined (roots, stems, old leaves, and young leaves) were significantly influenced by the dose of Cd ($p < 0.01$) and between the two cultivars used ($p < 0.01$). As Cd concentration increased in the nutrition solution, the BCF in the aerial part decreased from 56.9 to 19.2 in Zibo-3 and from 69.3 to 32.3 in Zibo-9, and from 501 to 416 in Zibo-3 and from 598 to 474 in Zibo-9 in the root. Both castor cultivars had a low Cd TF (4.62%–11.6%). The TF decreased from 11.4% to 4.62% in Zibo-3 and from 11.6% to 6.81% in Zibo-9, as Cd dosing increased in the nutrition solution.

The subcellular Cd concentrations and their relative distribution in the five fractions are shown in Fig. 1. With the increased Cd exposure, subcellular Cd concentrations in the HSP fraction of young leaves decreased from 2460 to 720 ng/g FW in Zibo-3 and from 3169 to 2181 ng/g FW in Zibo-9, with significant differences between the two Cd doses. However, subcellular Cd concentrations in the HDP fraction of young leaves in Zibo-3 significantly decreased from 405 to 218 ng/g FW while those in Zibo-9 significantly increased from 464 to 858 ng/g FW (Fig. 1a). The amount of Cd bound to HSP in Zibo-9 was higher than in Zibo-3, in the same tissue at the same Cd level (Fig. 1a). With increasing Cd exposure, from 2 to 5 mg/L, the relative Cd distribution in the HSP fraction in the young leaves of Zibo-3 significantly decreased from 48.3% to 31.6%, but a significant difference was not detected in the Zibo-9 young leaves (Fig. 1b). However, in old leaves, the percentage of Cd associated with the HSP fraction in Zibo-3 significantly

Table 2
Cd concentrations in different tissues of the two castor cultivars.

Cd treatments	Cultivars	Cd concentrations in different tissues (mg/kg)				BCF		TF
		Root	Stem	Old leaf	Young leaf	Aerial part	Root	
2 mg/L	Zibo-3	1002 ± 28.0b	259 ± 11.3b	19.7 ± 1.9b	48.8 ± 5.0b	56.9	501	0.114
	Zibo-9	1197 ± 70.3b	289 ± 18.3b	29.4 ± 3.6a	73.4 ± 5.8a	69.3	598	0.116
5 mg/L	Zibo-3	2082 ± 217a	264 ± 36.8b	13.2 ± 2.0b	30.2 ± 4.1c	19.2	416	0.0462
	Zibo-9	2369 ± 174a	366 ± 12.0a	18.6 ± 2.7b	51.4 ± 5.4b	32.3	474	0.0681
Analysis of variance								
Cd	61.1**	3.42	10.8*	15.8**				
Cultivar	2.80	8.86*	8.29*	20.1**				
Cd × cultivar	0.105	2.64	0.658	0.108				

BCF: bioconcentration factor; TF: translocation factor
Mean values ($n=3$) with different letters in the same column are significantly different between the treatments according to the Duncan's test ($p<0.05$).
The effects of Cd dose and differences between the cultivars used on Cd concentrations in different tissues (root, stem, old leaf, and young leaf) were analyzed by the two-way analysis of variance test;
* $p<0.01$.
** $p<0.01$.

increased from 40.9% to 46.7%, while those from Zibo-9 significantly decreased from 55.7% to 51.0% as the Cd dose increased (Fig. 1b), In addition, the percentage of Cd in the HDP fractions in old leaves from Zibo-3 decreased from 5.03% to 1.04%, but increased in the corresponding Zibo-9 samples from 3.15% to 6.90%, with significant differences between the two Cd doses (Fig. 1b).

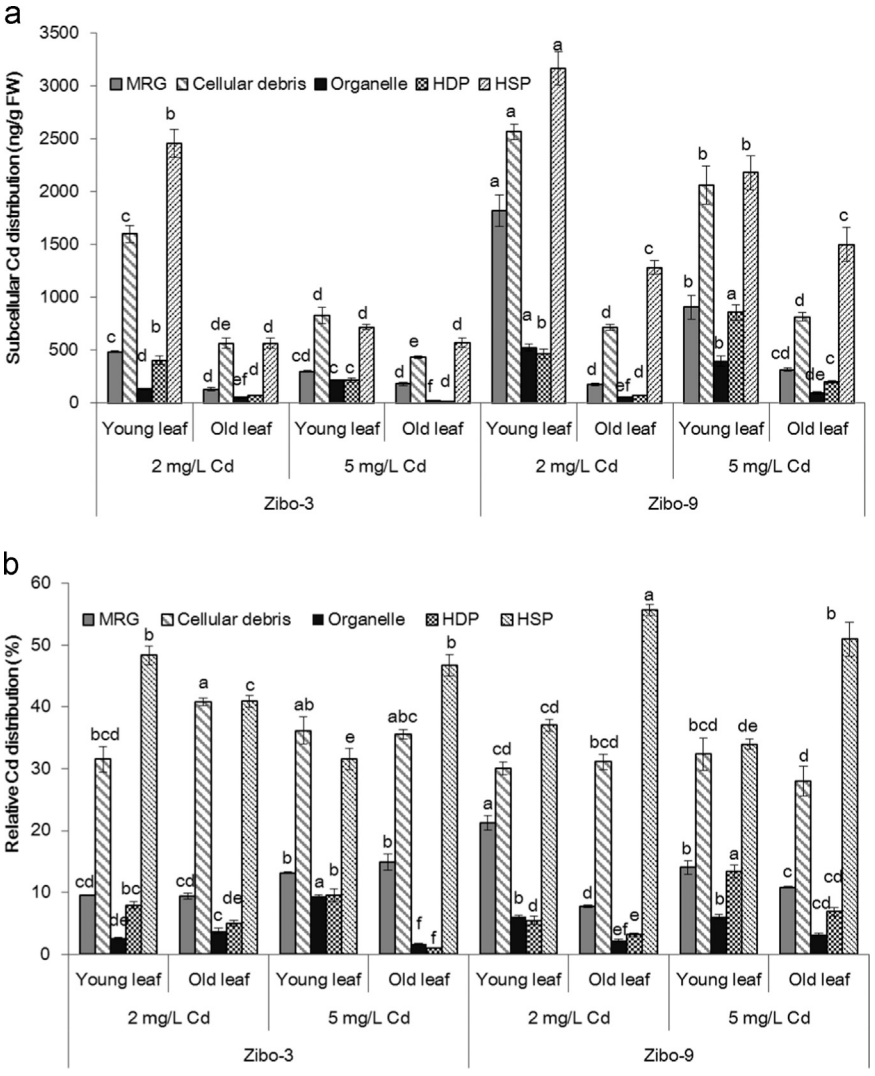

Fig. 1. Cd concentrations (a) and relative Cd distribution (b) in the five subcellular fractions from leaves. Bars show means ± SE ($n=3$). Different letters on the bars for the same tissue between the treatments show significant difference. MRG: Cd-rich granule; HDP: heat denatured protein; HSP: heat stable protein.

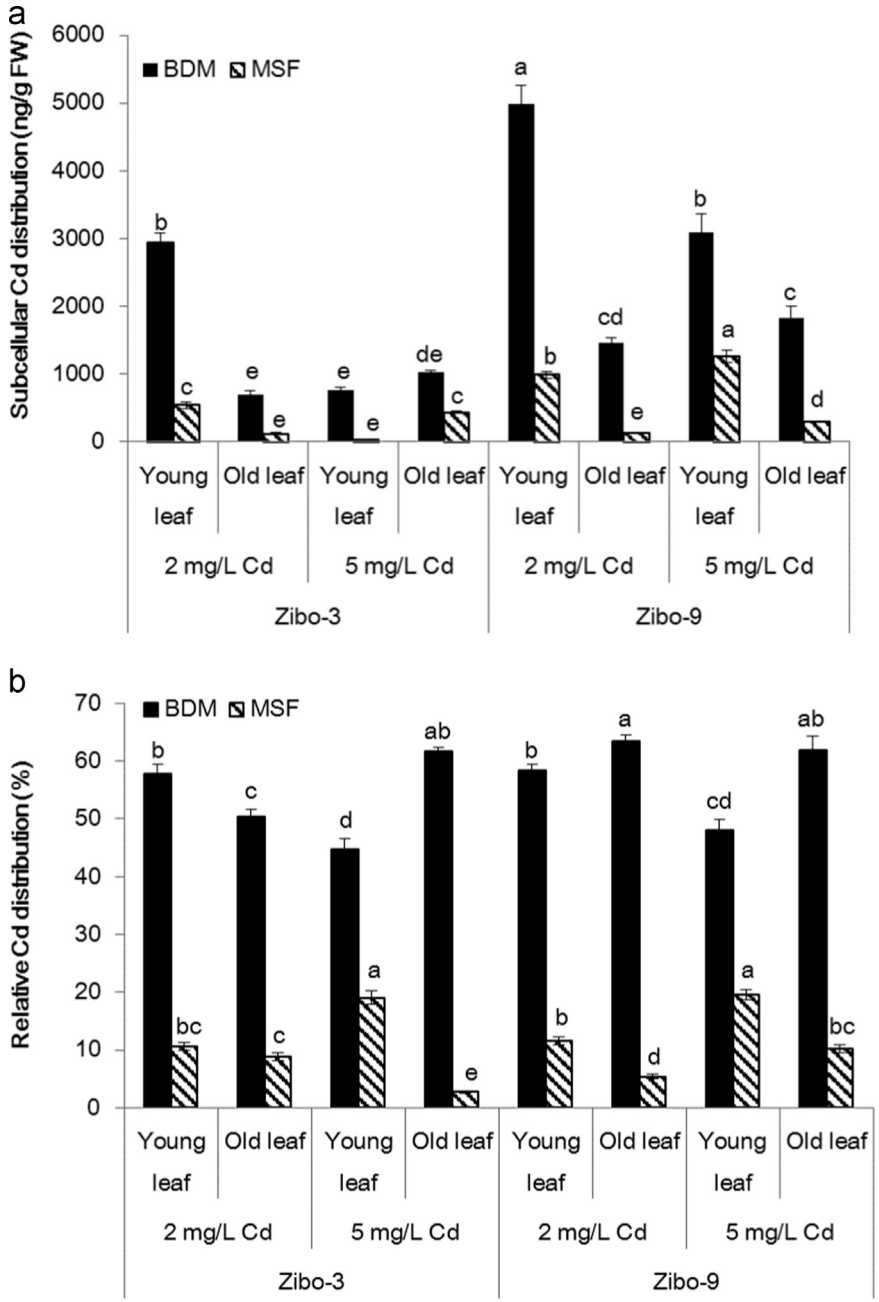

Fig. 2. Cd concentration (a) and relative Cd distribution (b) in the metal-sensitive fractions (MSF) and the biologically detoxified metal (BDM) fractions from leaves. Bars show means ± SE ($n=3$). Different letters on the bars for the same tissue between the treatments show significant difference. MSF: organelle and HDP; BDM: MRG and HSP.

The Cd concentrations and its relative distribution in the BDM and MSF are shown in Fig. 2. As Cd concentrations increased, subcellular Cd concentrations in BDM and MSF from the young leaves of Zibo-3 decreased from 2947 to 758 ng/g FW and from 538 to 33.3 ng/g FW respective. The subcellular Cd concentrations in MSF from the old leaves of Zibo-3 increased from 122 to 434 ng/g FW (Fig. 2a). However, subcellular Cd concentrations in young leaf BDM fractions from Zibo-9 decreased from 4989 to 3088 ng/g FW, but the MSF increased from 991 to 1257 ng/g FW, while subcellular Cd concentrations in old leaf MSF from Zibo-9 increased from 124 to 297 ng/g FW (Fig. 2a). Along the Cd exposure gradient, the BDM-Cd percentage in the young leaves significantly decreased from 57.9% to 44.8% in Zibo-3 cultivars and from 58.3% to 48.0% in Zibo-9 cultivars. The MSF-Cd from young leaves significantly increased from 10.6% to 19.0% in Zibo-3 cultivars and from 11.6% to 19.6% in Zibo-9 cultivars (Fig. 2b). As Cd exposure increased, the Cd-BDM fractions in old leaves significantly increased from 50.3% to 61.7% in Zibo-3 samples but decreased from 63.5% to 61.8%, with no significant difference, in Zibo-9 samples. The MSF-Cd percentage in old leaves significantly decreased from 8.84% to 2.71% in Zibo-3 samples and significantly increased from 5.40% to 10.2% in Zibo-9 samples (Fig. 2b).

3.3. Antioxidative enzyme activities

The changes in activity in antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), in old leaves and young leaves, induced by Cd exposure (2 mg/L to 5 mg/L) are presented in Fig. 3. Compared to the control group, SOD activity significantly decreased from 2.46 to 1.96 U/mg protein in young leaves from Zibo-3, at a 2 mg/L Cd dose (Fig. 3a). At a Cd level of 5 mg/L the CAT activity in the young leaves from Zibo-3

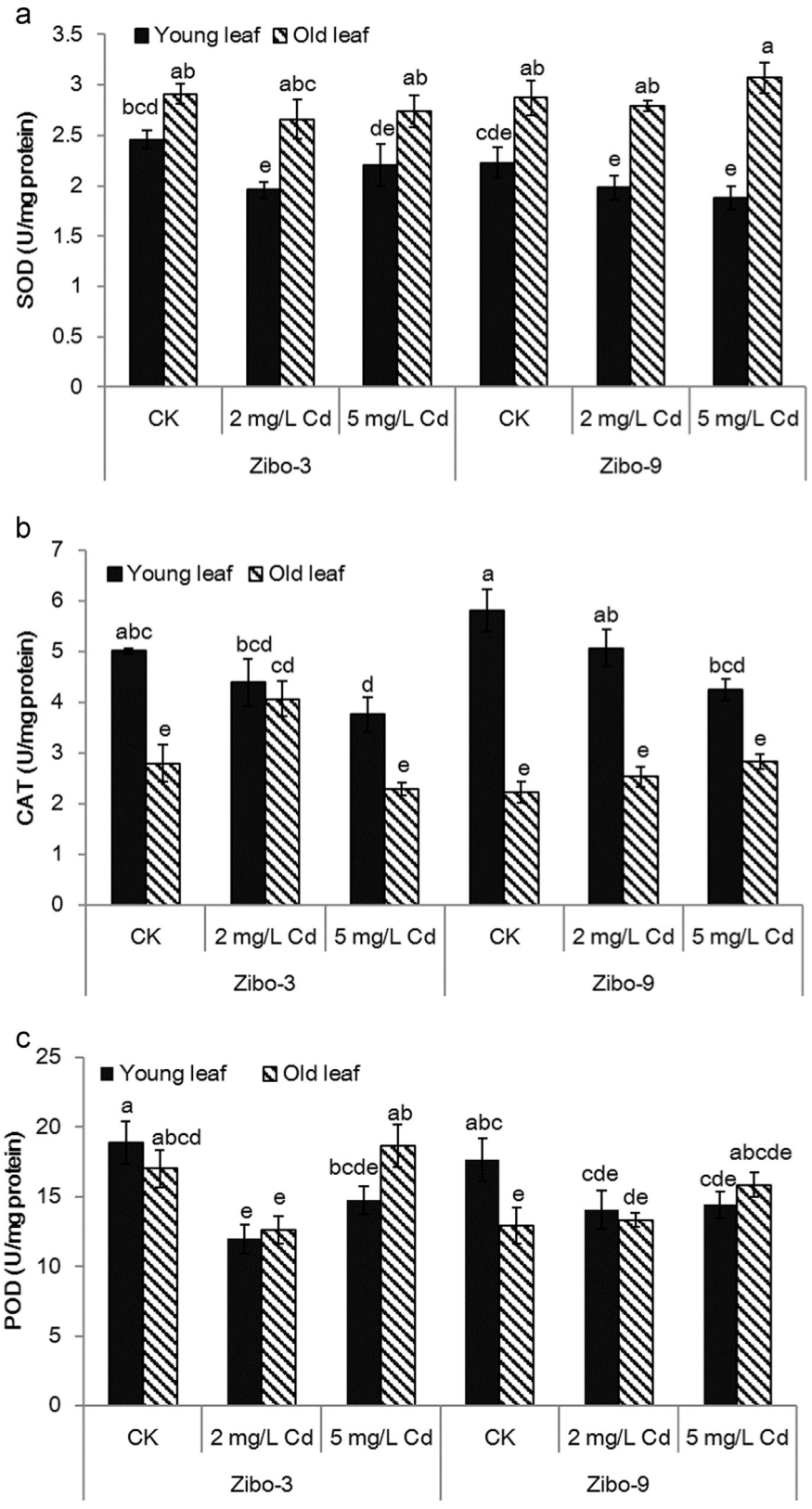

Fig. 3. SOD (a), CAT (b), and POD (c) activity levels from leaves. Bars show means ± SE ($n=3$). Different letters on the bars for the same tissue between the treatments show significant difference.

and Zibo-9 was decreased from 5.01 to 3.36 U/mg protein and from 5.81 to 4.43 U/mg protein, respectively, with significant differences, compared to the control group (Fig. 3b). The POD activity in young leaves from Zibo-3 significantly declined from 18.9 to 12.0 U/mg protein and from 18.9 to 14.8 U/mg protein, at Cd exposure levels of 2 mg/L and 5 mg/L, respectively, as compared to the control group (Fig. 3c).

4. Discussion

4.1. Cd accumulation and toxicity

Previous reports have characterized castor to have a high Cd tolerance (Shi and Cai, 2009) and higher remediation efficiency than Indian mustard (*Brassica juncea* L.). Thus it is considered to be a potential phytoremediator (Bauddh and Singh, 2012). However, great differences in Cd accumulation have been observed among various castor cultivars (Huang et al., 2011). The application of castor cultivars capable of high Cd accumulation may be an effective way to perform phytoremediation in Cd-polluted soil. Currently, research screening castor cultivars for high Cd accumulation capabilities is relatively rare. In previous research, through the evaluation of 30 castor bean cultivars for their cadmium phytoextraction ability, the cultivars were divided into low-accumulators and high-accumulators. The cultivars Zibo-9 and Zibo-3 were designated high as a Cd accumulator and a low Cd accumulator, respectively (Table S1). Hydroponic experiments revealed that Zibo-9 has higher accumulation capability than Zibo-3 (Table 2). Here we observed young leaves accumulate more Cd than old leaves (Table 1) suggesting size specific metabolic rates (Ringwood, 1989; Wallace and Luoma, 2003). Dunbar et al. (2003) also showed that Cd concentrations in young leaves were higher than those in old leaves in two potato cultivars (*Solanum tuberosum* L.) characterized with different Cd-accumulated capabilities. Here, Cd concentrations in roots were higher than in aerial parts (stem, leaf) of the plants, and the TFs of both castor cultivars were lower than 1 (Table 2). Zhang et al. (2014) reported that the Cd TF of castor ranged from 4.90% to 13.8% at a 1 or 2 mg/L Cd dose. According to its TFs (<1), castor is not a Cd phytoextraction plant. However, Olivares et al. (2013) proposed that castor could be a Cd phytostabilization plant participating in Cd stabilization, and it can be useful for oil production making effective use of Cd-polluted areas and reducing Cd mobilization and hazards to the ecosystem, securing human health.

Biomass has previously been used to assess Cd toxicity in castor (Shi and Cai, 2009). Earlier studies have suggested that Cd concentration in plant tissue reached 3–10 mg/kg dry weight which plants can suffer (Ghosh and Singh, 2005; Shi and Cai, 2009). Here, when Cd concentration in roots reached approximately 2000 mg/kg dry weight, the TI values were approximately 0.7 (Table 1), and both castor cultivars grew normally. Zhang et al. (2014) also reported normal growth of castor (cultivar Zibo no. 5) (TI=0.65), at a root Cd concentration of 2538 mg/kg dry weight. The current study suggested that castor is highly tolerant to Cd stress.

4.2. Cd subcellular distribution

Cd subcellular partitioning within organisms reflects the internal processes occurring during Cd accumulation, and provides some mechanistic information about tolerance and toxicity (Li et al., 2011b; Wallace et al., 2003). The methodology of metal subcellular fractionation involving differential centrifugation was originally based on animal models (Wallace et al., 2003), and later optimized for algal (Lavoie et al., 2009) and wheat (Li and Zhou, 2012). Nevertheless, these results should be interpreted with caution, considering various potential artefacts could distort these findings. For instance the following events could all impact these findings, the disruption of organelles during harsh homogenization, the leakage of Cd from organelles in to the cytosol, and/or an overestimation of Cd in MRG and cellular debris fractions (Wang and Wang, 2011; Lavoie et al., 2009). Despite these potential problems, this method has been widely used in metal partitioning studies and can provide useful information (Wang and Wang, 2011).

After entering cells, metals bind to different subcellular compartments and exhibit different ecotoxicological significances (Wang and Wang, 2011). Cd bound to the HSP fraction has been shown to have an important role in metal detoxification in diatoms (Lavoie et al., 2009; Wang and Wang, 2008). The increase of detoxified fractions (HSP and MRG) in the old leaves with increased Cd dose (Fig. 1b) presumably reflects the progressive Cd detoxification by metallothionein-like proteins produced in cultivar Zibo-3 (Giguere et al., 2006). In addition, Cd associated with the HDP and organelle fractions increased in the 5 mg/L Cd treated samples compared to the 2 mg/L Cd treated samples, which could explain the increased Cd toxicity in the Zibo-9 samples. However, with the increased Cd concentration there was decreased Cd storage in the HSP fraction of the young leaves (Fig. 1b) this may be due to binding capacities being exceeded. Cadmium was subsequently bound to other fractions, which has the potential to cause toxicity in both of the castor cultivars (Rainbow, 2002).

These subcellular Cd distributions were grouped into MSF (organelle+HDP) and BDM (HSP+MRG), which represent ecotoxicologically important compartments (Wang and Wang, 2011). During Cd uptake, the BDM-Cd fraction may serve as the primary mechanism of detoxification. However, the system may become overwhelmed and Cd may bind to more sensitive cellular components (MSF), resulting in toxicity during a period of higher Cd exposure (Wallace et al., 2000). Wang and Wang (2008) suggested that the MSF-Cd best predicted Cd toxicity in marine diatoms under different environmental conditions. Our results provide some clear evidence for the involvement of the BDM-Cd fractions in effective Cd detoxification in old leaves from Zibo-3. Without the effective protection provided in the BDM fractions, the old leaves from Zibo-9, along the increased MSF-Cd was responsible for increased Cd sensitivity, in higher Cd exposure (Wang and Wang, 2011). At a 2 mg/L Cd dose, the Cd bound to the BDM fractions in the old leaves from Zibo-9 was higher than in Zibo-3, and the MSF-Cd in the old leaves of Zibo-9 was lower than in older Zibo-3 leaves (Fig. 2b), suggesting Zibo-9 had a higher tolerance than Zibo-3. However, in 5 mg/L Cd exposures, the MSF-Cd distribution in old leaves from Zibo-9 compared to those from Zibo-3 (Fig. 2b) indicated that Zibo-9 was more vulnerable to Cd toxicity than Zibo-3 was. Hence, the BDM-Cd fractions or the MSF-Cd in old leaves associated with TI, indicating the Cd tolerance of different castor cultivars.

4.3. Antioxidant enzymes

The SOD, CAT, and POD are important components of antioxidant defense mechanisms in plants. SOD is a metalloenzyme that catalyzes superoxide radicals in H_2O_2 and O_2, protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments (Del Rio et al., 2002). CAT and POD are the most important enzymes in the regulation of intracellular H_2O_2 levels (Blokhina et al., 2003). Some researchers have indicated the activity of antioxidative enzymes to be elevated in plant tissues under Cd stress (Elobeid et al., 2012; He et al., 2013a; Yu et al., 2013). However, here, the activity of antioxidant enzymes (SOD, CAT, and POD) was inhibited or unchanged after various Cd treatments (Fig. 3). In the leaves, decreased SOD, CAT, and POD activity in both castor cultivars after Cd treatment (Fig. 3) may be attributed to the lack of an effective antioxidant enzyme system (Liu et al., 2007) or the excessive production of oxygen free radicals (Maksymiec and Krupa, 2006; Li et al., 2011a). Nouairi et al. (2009) showed that SOD and CAT activity in the leaves of *Brassica napus* decreased approximately 40% and 37%, respectively, to that of the control, and GPX activity was drastically reduced ($\sim$57%) in the leaves of *Brassica juncea* plants, at a 50 μM Cd dose. Li et al. (2011b) reported SOD and CAT activity decreased when wheat

seedlings (*Triticum aestivum* L. Yangline 15) were exposed to Cd, as compared to control plants. He et al. (2011) found antioxidant enzymes (SOD, POD, CAT) in *Populus* × *canescens* were influenced by Cd exposure time and tissue (root, wood, bark, leaf). He et al. (2013b) indicated the activity of antioxidant enzymes (SOD, POD, CAT) in *Populus* × *canescens* were unchanged or reduced under Cd stress. In most cases, the activity of anti-oxidative enzymes was reduced after Cd exposure which did not detoxify free Cd ions in plant cells (He et al., 2015). These results also suggest antioxidant enzymes (SOD, CAT, and POD) are not involved in Cd detoxification in neither young nor old leaves of castor cultivars.

5. Conclusions

Castor Cd tolerance was dependent on the level of Cd exposure and the cultivars themselves. The order of Cd accumulation in castor was found to be root > stem > young leaf > old leaf. Moreover, Cd concentrations in different tissues (roots, stems, old leaves, and young leaves) were directly related to Cd exposure levels and the cultivars themselves. Cd tolerance and toxicity in castor can be feasibly explained by subcellular partitioning. Cd primarily accumulated in the heat stable protein (HSP) and cellular debris fractions, followed by the Cd-rich granule (MRG), heat denatured protein (HDP) and organelle fractions. With increasing Cd concentrations, the decreased distribution of the detoxified fractions (BDM) and the increased distribution of Cd-sensitive fractions (MSF), in young leaves, may be responsible for increased Cd toxicity. The BDM-Cd fractions or MSF-Cd in old leaves associated with Cd tolerance of different cultivars of castor. The antioxidant enzymes (SOD, CAT and POD) were not involve in Cd detoxification on the leaves. In summary, castor is not a Cd phytoextraction plant, but it can be used as a Cd phytostabilization plant participating in Cd stabilization and these results also suggest the importance of understanding the Cd detoxification mechanism in castor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.06.003.

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