

RESPONSE OF ANTIOXIDANT ENZYMES IN *POPULUS × CANESCENS* UNDER CADMIUM STRESS

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Abstract

Hydroponic experiments were conducted to study the tolerance of *Populus canescens* were exposed to different levels of Cd²⁺ (0, 10, 30, 50, and 70 μM/L) as compared to the control. After 28-day (s) of Cd²⁺ exposure it was found that the biomass of all tissues in *P. × canescens* was unaffected by Cd²⁺ exposure, Cd²⁺ concentration 70 μM/L. To investigate the possible effects of Cd ions in the generation of oxidative stress, we detected the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) in the different tissues of *Populus canescens*, it was found that 1) oxidative stress in *P. canescens* took place at different concentrations of Cd ions and with increasing of exposure time as evidenced by the malondialdehyde (MDA) and H₂O₂ concentrations; and 2) antioxidant enzymes activities were also stimulated by Cd treatment and were associated with changes in ascorbate; Among all tissues, significant increases of GR activities and significant changes in the ascorbate (ASC) and glutathione (GSH) pool were observed, which suggest that Cd²⁺ can be added to the list of stresses. The results in this study suggest that the growth of *P. canescens* in the presence of Cd metal showed a concentration-dependent oxidative stress situation in the different tissues, as a result of the inhibition of the antioxidant systems.

Introduction

Cadmium (Cd) has been an occupationally and environmentally important toxic element that is present in air, soil, sediment and water. In humans, non-occupational exposure has been primarily due to diet and smoking (Ahmad *et al.*, 2010; Shafi *et al.*, 2010; Dai *et al.*, 2011a; Dai *et al.*, 2012a). Cd accumulates in the human body and has a long biological half-life of two to three decades, which usually lead to the illnesses including lungs, liver, kidneys, bone, cardiovascular system and the immune system illnesses (Schützendübel & Polle, 2002; Fowler, 2009; Dai *et al.*, 2011a; Kabir *et al.*, 2011). In plants, it causes a reduction in photosynthesis, water and nutrient uptake (Sanità di Toppi & Gabbrielli, 1999; Supalkova *et al.*, 2009; Raziuddin *et al.*, 2011). Cadmium is toxic to plant cells, even at low concentrations (Dai *et al.*, 2012b). Leaf concentrations greater than 5-10 μg Cd/g dry matter (DM) are toxic to most plants (White & Brown, 2010), although some ecotypes of a few plant species have adapted to grow on soils with high Cd concentrations and can tolerate leaf concentrations in excess of 100 μg Cd/g DM (Broadley *et al.*, 2001; Verbruggen *et al.*, 2009). Moreover, Cd²⁺ has been a pollutant that accumulates in soil as a result of industrial processes or intensive usage of fertilizers in agriculture (Nriagu & Pacyna, 1988). The technique is environmentally friendly, potentially cheap and visually unobtrusive, and offers the possibility of bio-recovery of metals. The main limiting factors for efficient phytoextraction of metals are: (1) bioavailability of the trace elements; (2) uptake and translocation within the plant; and (3) metal phytotoxicity (Seth *et al.*, 2012).

Redox homeostasis and heavy metal (HM) ion homeostasis are closely intertwined, and high concentrations of HM produce stress responses in plants such as oxidative stress (White & Brown, 2010). Dalton *et*

al., (1986) have discovered that accumulation trends of reactive oxygen species (ROS) could give rise to oxidative stress leading to cell damage, mutation, and/or even death. Changes in ROS levels were observed in many Cd treated plants (Chao *et al.*, 2010). Among all ROS, H₂O₂ is relatively long living molecule that can diffuse some distance from its site of production and can penetrate the membrane structures. The antioxidant protection in plant cells is complex and highly compartmentalized. Protection against ROS and peroxidation reactions is provided by antioxidant system including antioxidant enzymes such as superoxide dismutase (SOD; EC1.15.1.1). This is a family of metalloenzymes that catalyze the dismutation of superoxide radical (O₂^{•-}) to hydrogen peroxide (H₂O₂) (Tarchoune *et al.*, 2010), catalase (CAT; EC 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.11) and non-enzyme antioxidants, such as glutathione and ascorbate.

H₂O₂ is a strong nucleophilic oxidizing agent and the oxidation of SH-group is one major mode of its toxicity. In plant cells, the ascorbate-glutathione cycle represents an alternative and more effective detoxification mechanism against H₂O₂ both in the chloroplasts and the cytosol (Chao *et al.*, 2010). The reduction of H₂O₂ by ascorbate could occur directly or it is catalyzed by APX. Then, the oxidized form of ascorbate could be reduced enzymatically by dehydroascorbate reductase (DHAR; EC 1.8.5.1) using glutathione (GSH) as a substrate, which in turn is reduced by glutathione reductase (GR; EC 1.6.4.2) in the presence of NAD (P) H. However, different plant species exhibited various antioxidative responses and degrees of tolerance to oxidative stress.

Good candidates for reducing metal concentrations in contaminated soils are species of poplar and willow, given their ability to accumulate and/or tolerate high levels of heavy metals due to their extensive root systems, fast growth rates, easy propagation and high biomass production (Dai *et al.*, 2011a) Nevertheless, a further insight on metal

resistance and tolerance mechanisms in poplar is necessary when considering this energy plant for growth in contaminated sites (Lux *et al.*, 2011; Dai *et al.*, 2012a).

The aim of this work was to investigate the capacity of *Populus × canescens* to uptake and detoxification mechanisms of Cd in all tissues (tolerance) in glasshouse experiments. we have chosen *Populus canescens*, a hybrid of *Populus tremula* × *Populus alba*. The *P. × canescens* was exposed to five different levels of cadmium (Cd) (0-control, 10, 30, 50, and 70 µM CdSO₄) for up to 28 days in the nutrient solution and used for analyses of Cd concentrations, ROS and antioxidants in different tissues along the whole transport route including root, wood, leaf and bark.

Material and Methods

Experimental setup: The experiment was conducted in an orchard at Northwest A&F University, Yangling (34°20'N, 108°24'E), P.R. China. Plantlets of *P. × canescens* (*P. tremula* × *P. alba*) were multiplied by micropagation (Dai *et al.*, 2012a). Caulicles of *P. × canescens* were grown for 3-week-old in an osmotic solution and used for inoculation. Each seedling was inoculated in 20mL of liquid rooting medium (1/2 MS, 0.05 mg/L NAA, and 4 mg/L AgNO₃) and allowed to grow for 3-week-old in a growth room (21°C, 50%-60% RH, 16 h of light per day, 150 µmol photons m⁻² s⁻¹ photosynthetic active radiation at plant length). After 4 weeks the rooted plantlets were transferred to an aerated Hoagland nutrient solution in a growth room with the same environmental condition as in the climate chamber. The nutrient solution was exchanged every 3 days. After 12-week-old cultivation, the plants were treated with 10, 30, 50, and 70µM CdSO₄ by adding CdSO₄ into the nutrient solution and the plants served as the controls, pH of the solution was adjusted to 6.5±0.1 with NaOH or HCl as required.

Sampling and Measurements: The seedlings were harvested after 28 days of exposure, the roots were immersed in the 5mM CaCl₂ for 15 min, and then the whole plants were rinsed with deionized water. Plant and length root were measured. Root surface area and lengths were measure with a Delta-T root scanner system (Dynamax) on subsample of fine roots to obtain a conversion factor between root dry weight and length. Preparing samples for the root scanner was time consuming for direct measurement of all root areas and lengths. The leaf and shoot (bark and wood) were divided and dried in an air oven at 80°C until a constant weight to obtain dry weight. The effects of Cd treatment on along with root, shoot (bark and wood), and leaf dry weights were assessed using two-way ANOVA.

Measurement of malondialdehyde (MDA) and H₂O₂ content: Oxidative damage to lipids was estimated as the content of the total 2-thiobarbituric acid (TBA) reactive substance and expressed as equivalents of malondialdehyde (MDA) as described by Dai *et al.*, (2011a,b). H₂O₂ concentrations were measured according to Dai *et al.*, (2012). The absorbance rate at 410nm was measured and the H₂O₂ concentration was calculated according to a standard curve.

Assay of antioxidant enzymatic activity: The activity of SOD (EC 1.15.1.1) was determined according to Dai *et al.*, (2012). One unit of SOD was defined as the amount of enzyme that caused a 50% decrease in the SOD-inhibited nitrobluetetrazolium reduction at 550nm. The activity of CAT (EC 1.11.1.6) after Dai *et al.*, (2011a,b), APX (EC 1.11.1.11) after Griffith (1980) and GR (EC 1.6.4.2) after the method of Ma *et al.*, (2011) were determined.

Non enzymatic antioxidant analysis-Ascorbate and total ascorbate (ASC+DHA): Ascorbate (ASC) and dehydroascorbic acid (DHA) contents were determined according to the method of Law *et al.*, (1983) and Ma *et al.*, (2011). Total ascorbate was determined through the reduction of DHA to ASC by 0.97 mM dithiothreitol (DTT) and the DHA concentration was determined by estimating the difference between total ascorbate and AsA values. A standard curve covering the range 0-25µmol AsA was used.

Glutathione: GSH and total glutathione (GSH+GSSG) were assayed according to Griffith (1980) and Ma *et al.*, (2011).GSSG was determined from the difference between GSH+GSSG and GSH.

Statistical analysis: A completely randomized design, incorporating six replicates, was used for each time point. Data were subjected to analysis of variance (ANOVA) to examine the effects of time, treatment, and organs. Statistical analysis was conducted using STATISTICA 5.1 software (Statsoft Inc., United States of America). Separation of means was carried out using Fisher's LSD test at p<0.05 and p<0.01 significance level.

Results

Populus×canescens growth: To analyse the toxic effects of Cd on plant growth, biomass of plant and root length was recorded (Fig. 1). The biomass of plant and root length in *P. × canescens* was unaffected by different levels of Cd²⁺ exposure. Continuous accumulations of biomass of plant and root length were found with increases in exposure time.

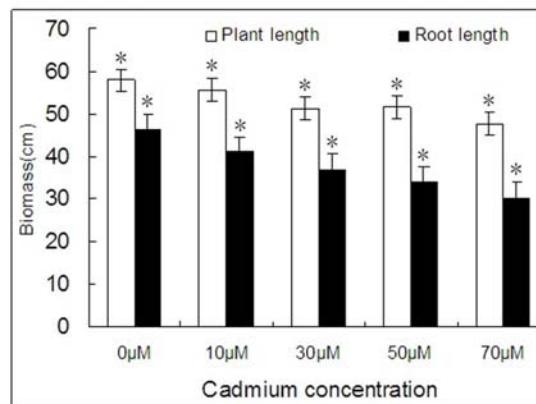


Fig. 1. Biomass of *P. × canescens* exposed to 0(control), 10, 30, 50, and 70 µM CdSO₄/L for 28day. Data were presented as mean ± standard error (n = 6). *Represents the significance level at p<0.05; **represents the significance level at p<0.01 when compared with control.

Determination of lipid peroxidation (MDA) and H₂O₂ content: After 28-days cadmium exposure, the contents of H₂O₂ and MDA significantly increased when the Cd²⁺ stress increased to 50 and 70 μM, compared to control (non-Cd), the MDA concentration in *P. × canescens* leaf (bark, wood and root) increased after 0, 10, 30, 50 and 70 μM Cd treatment respectively (Table 1). Exposure to 10 to 70 μM Cd also resulted in an increase in the H₂O₂ content, compared to control, respectively ($p<0.05$). Among of all tissues, the content of H₂O₂ and MDA were significant highest level in root, followed by wood, bark and leaf.

Enzymes: Components of ROS scavenging system (i.e. enzymatic antioxidants; SOD, CAT, APX and GR) were studied in *P. × canescens* plants treated with increasing Cd concentration. SOD, CAT and APX, which are involved in H₂O₂ removal, generally exhibited decreases in activities in the presence of Cd²⁺ compared with controls regardless of the tissue. Exposures to realistic Cd concentrations in *P. × canescens* leaf, bark, wood, and root (Table 2). Compared to the control, enzymes activities increased with the addition of Cd²⁺ in different tissues, there were significant increase in the activities of SOD, CAT and APX were observed after exposure to lower levels of Cd²⁺ (10 μM Cd), the high levels of Cd²⁺ (50 and 70 μM Cd) treatments, enzymes activities were decreased in different tissues, respectively ($p<0.01$). In these tissues, all the Cd treatments significantly increased the activities

of GR. Among all tissues, the activities of SOD, CAT, APX and GR were significantly at highest level in leaf, followed by wood, bark and root. *Populus canescens* have developed well-established detoxification mechanisms to cope with this metal-induced oxidative challenge. Moreover, it is observed that hyper-accumulators exhibit a stronger antioxidative capability than their non-accumulator relatives.

Metabolites: It was demonstrated that Cd toxicity of *P. canescens* was accompanied by a decrease in the contents of total ascorbate (ASC +DHA), ascorbate (ASC) and in the ratios of ASC/DHA in all tissues (Figs. 2A, C, D). It was found that ASC was markedly decreased to a level at high concentration (70 μM) of Cd stress, with a corresponding increase in the level of reduced dehydroascorbate acid (DHA) indicating that DHA content was significantly enhanced probably due to a decrease in ASC synthesis. Among all tissues, the contents of ASC, DHA, ASC /DHA and ASC +DHA were significant highest level in leaf, followed by root, wood and bark. ($p<0.01$).

During the whole period of cadmium exposure, In Figs. 3 observed a significant decrease of reduced GSH in higher levels of Cd-treated plants, while Cd²⁺ treatment increased the accumulation of GSSG, keeping the GSH/GSSG ratio and GSH+GSSG lower than in control plants, respectively ($p<0.01$).

Table 1. Changes in MDA and H₂O₂ content in *P. x canescens* after 28 days at 0 (control), 10, 30, 50 and 70 μM CdSO₄ exposure.

	Organ	Cd treatment (μM L ⁻¹)				
		0	20	30	50	70
MDA _{μmol/gFW}	leaf	0.55 ± 0.03**	0.61 ± 0.04**	0.81 ± 0.09**	1.13 ± 0.04**	1.28 ± 0.09**
	bark	0.56 ± 0.09*	0.63 ± 0.05**	0.90 ± 0.03**	1.11 ± 0.05**	1.30 ± 0.06*
	stem	0.51 ± 0.06*	0.92 ± 0.05*	1.21 ± 0.09**	1.58 ± 0.05**	1.99 ± 0.10*
	root	0.71 ± 0.08*	1.39 ± 0.09**	1.43 ± 0.09**	2.10 ± 0.10*	2.71 ± 0.07*
H ₂ O ₂ _{μmol/gFW}	leaf	0.78 ± 0.03**	0.85 ± 0.04**	1.19 ± 0.03**	1.27 ± 0.03**	1.37 ± 0.08**
	bark	0.81 ± 0.02**	0.83 ± 0.04**	1.06 ± 0.09**	1.32 ± 0.04**	1.46 ± 0.09**
	stem	0.83 ± 0.02*	1.01 ± 0.03*	1.20 ± 0.08**	1.43 ± 0.08**	1.62 ± 0.10*
	root	0.89 ± 0.03*	1.02 ± 0.04*	1.39 ± 0.05**	1.70 ± 0.03*	1.81 ± 0.14*
Organ	Primary effects			Interactions		
	Metal	Time		M×T		
ANOVA p values						
Leaves		0.000	.004		0.008	
bark		0.000	.001		0.006	
stem		0.000	.000		0.003	
root		0.000	.005		0.017	

Each point represents the mean of six biological replicates ± S.E. Mean values of studied parameters were marked with * or **, including control treatment, * represents the significance level at $p<0.05$, while ** represents the significance level at $p<0.01$ when

compared with control

Table 2. Enzyme activity (U/mg FW) in leaf material at 28 days for hybrid poplar (*P. × canescens*) exposed to 0 (control), 10, 30, 50, or 70 µM Cd.

Enzyme activity (Units/Mg Protein/min)	Organ	Cd treatment				
		0µM	10µM	30µM	50µM	70µM
SOD	leaf	276.6 ± 20.3*	281.9 ± 30.5*	247.9 ± 30.5**	190.4 ± 56.6**	167.6 ± 46.7
	bark	241.1 ± 29.9**	269.9 ± 33.3**	207.6 ± 43.1**	106.3 ± 13.5	90.5 ± 9.8**
	wood	208.0 ± 18.1*	261.8 ± 20.9**	129.7 ± 14.3**	80.4 ± 11.5**	72.3 ± 9.7*
	root	109.9 ± 17.1*	129.1 ± 10.0**	78.3 ± 10.6**	75.8 ± 10.1**	60.8 ± 3.7*
CAT	leaf	16.3 ± 5.7**	22.7 ± 12.4**	20.7 ± 1.1*	17.4 ± 3.7*	11.6 ± 5.7**
	bark	11.9 ± 6.7**	23.8 ± 5.6**	21.6 ± 9.4**	15.7 ± 12.4**	9.4 ± 6.7**
	wood	10.4 ± 6.3**	13.3 ± 8.1**	10.3 ± 16.1*	8.8 ± 5.6**	6.9 ± 2.3*
	root	6.3 ± 3.8*	12.9 ± 3.7*	9.8 ± 9.4**	7.5 ± 1.1**	6.2 ± 0.8*
APX	leaf	133.3 ± 3.5**	175.0 ± 3.2**	104.1 ± 3.6**	91.6 ± 5.5**	70.8 ± 3.5**
	bark	91.7 ± 6.7**	145.8 ± 7.1**	54.1 ± 8.9**	62.5 ± 3.2**	37.5 ± 0.7**
	wood	83.3 ± 0.4*	116.7 ± 2.3*	63.6 ± 3.6**	58.3 ± 0.7**	33.3 ± 0.4*
	root	76.5 ± 1.9*	88.2 ± 1.5**	51.6 ± 1.9**	39.1 ± 1.3*	25.0 ± 1.2*
GR	leaf	9.6 ± 0.09*	10.6 ± 0.12*	13.9 ± 0.38**	14.3 ± 0.15**	15.5 ± 0.3*
	bark	7.3 ± 0.5**	8.6 ± 0.62**	10.1 ± 0.2**	12.5 ± 0.3*	13.2 ± 0.2*
	wood	8.1 ± 0.2*	7.6 ± 0.3*	9.1 ± 0.5*	9.9 ± 0.2*	10.3 ± 0.2*
	root	5.3 ± 0.2*	5.8 ± 0.2**	6.6 ± 0.3**	7.8 ± 0.1**	9.6 ± 0.2*
Enzyme activity	Primary effects			Interactions		
	Metal	Organ		M×O		
ANOVA p values						
SOD	.000	.000		.000		
CAT	.004	.000		.012		
APX	.012	.006		.039		
GR	.000	.000		.000		

Each point represents the mean of six biological replicates ± S.E. Data were presented as mean ± standard error (n = 6). *Represents the significance level at p<0.05; **represents the significance level at p<0.01 when compared with the control

Discussion

Cadmium is a non-redox metal unable to produce ROS Fenton and/or Haber-Weiss reactions. However, several lines of evidence have revealed that oxidative stress is a major component of Cd phytotoxicity (Chao *et al.*, 2010; Shah *et al.*, 2001; Smeets *et al.*, 2005; Potters *et al.*, 2010). In this study, to our knowledge, this is the first report on using this technique in a dicotyledonous plant. In previous studies, a similar method to evaluate the tolerance to Cd in plants is to measure the survival rate in heavily toxic substrates or the reduction in growth rate, and the impairment of main physiological functions (Belimov *et al.*, 2003). We observed that increases in

Cd²⁺ accumulation in the above ground tissue (leaf, bark, wood) and root after exposure for 28 days suggest that it may take a long time for *P. canescens* to reach saturation of Cd²⁺; biomass of roots, wood, bark and leaves was recorded, the biomass accumulation of all tissues of *Populus × canescens* exposed to Cd with increases in exposure time (Fig. 1 and suggests that poplars still grow under current experimental conditions, these data imply that *P. × canescens* has a great potential for Cd²⁺ tolerance.

One of the important targets of Cd²⁺ at the cellular level might be the plasma membrane. This is supported by the interference of Cd²⁺ in membrane lipids which is caused by the increased production of highly ROS. These

observations, and others showing an increase in MDA and accumulated Cd, indicate that *P.*× *canescens* experience

substantial oxidative damage when exposed to high levels Cd²⁺ for 28 days (Figs. 2b).

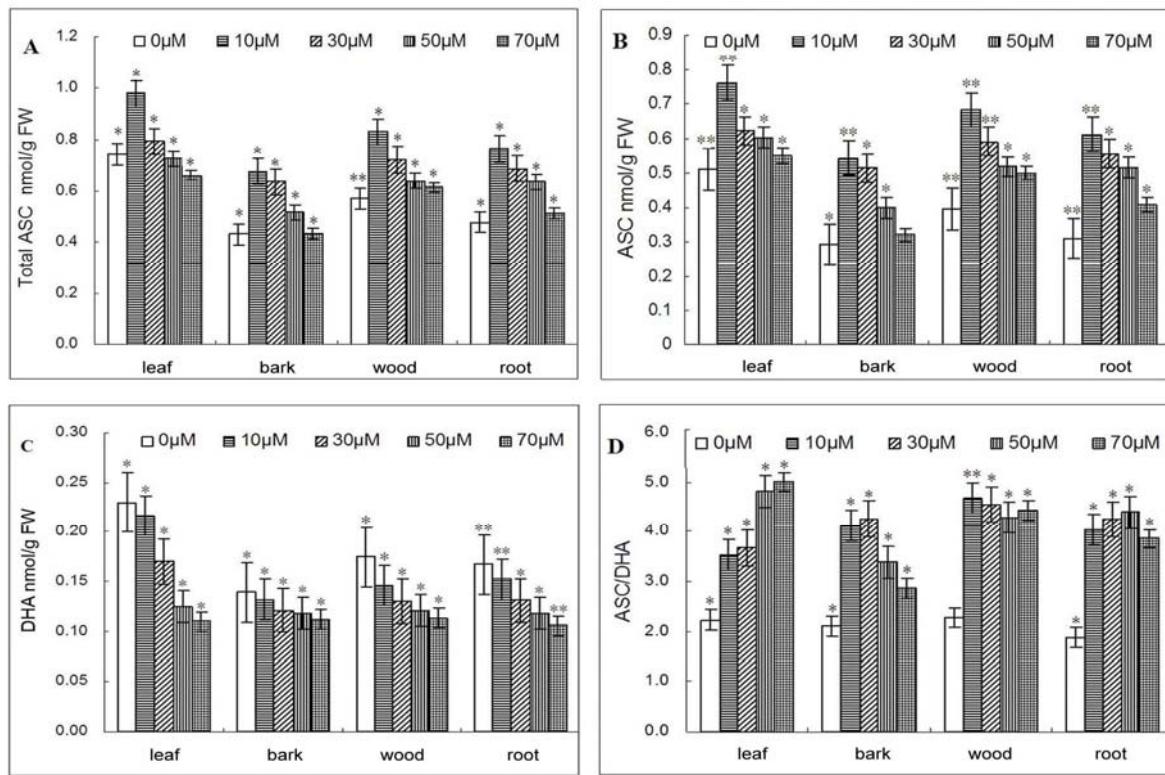


Fig. 2. A: Total ASC (nmol/g FW); B: ASC (nmol/g FW); C: DHA (nmol/g FW); and D: ASC/DHA (nmol/g FW) content in leaf, bark, wood, root of hybrid poplar (*P. × canescens*) were exposed to different levels of CdSO₄: 0 (control), 10, 30, 50, and 70 μM Cd/L compared to the control at 28 days in all organs of *P. × canescens*.

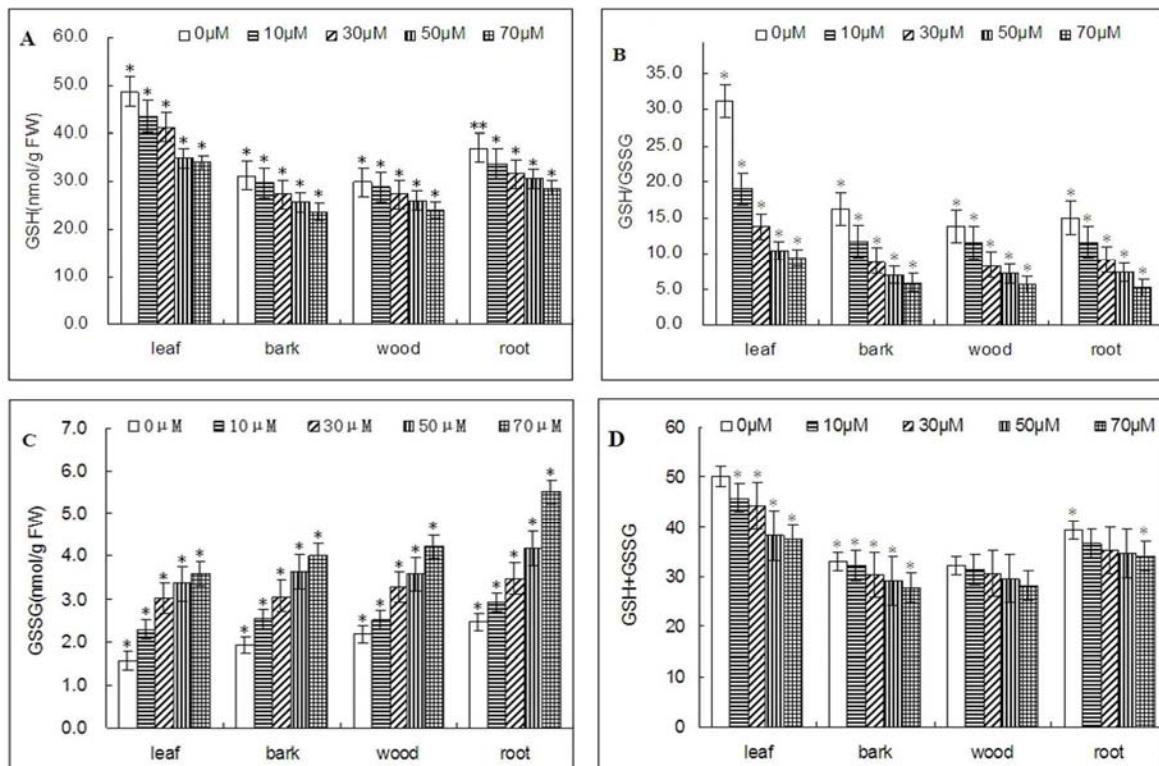


Fig. 3. A: GSH (nmol/g FW); B: GSH+GSSG; C: GSSG (nmol/g FW) and D: GSH/GSSG content in leaf, bark, wood, root of hybrid poplar (*P. × canescens*) were exposed to different levels of CdSO₄: 0 (control), 10, 30, 50, and 70 μM Cd/L compared to the control at 28 days in all organs of *P. × canescens*.

Major ROS-scavenging enzymes in wood plant include SOD, APX and CAT (Polle, 2001; Dai *et al.*, 2011a). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide (Chao *et al.*, 2010). The different affinities of APX and CAT for H₂O₂ suggest that they belong to two different classes of H₂O₂-scavenging enzymes: APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress. Furthermore, results of the present study clearly indicate that Cd induced an increase in H₂O₂ content in *P. canescens* all tissues (Fig. 2a), which coincided with the increase of ROS-scavenging enzymes such as APX, CAT or SOD, indicating that the plants respond to Cd stress by activation of the ASC-GSH defence network at both transcriptional and enzymatic level.

The role of ASC as an efficient scavenger for oxidative compounds is well-known. Furthermore, the effectiveness of ASC-GSH regenerating enzyme system comprising DHAR and GR, and the maintenance of ASC, DHA, GSH and GSSG pools may contribute to controlling Cd-caused oxidative stress in plants (Paradiso *et al.*, 2008). The cellular concentration of ASC, in fact, determined by the rate of its synthesis and decay. DHA is rapidly hydrolyzed into 2, 3-diketogulanic acid if not reduced by DHAR. Besides, Anjum *et al.*, (2010) reported Cd-induced increase in DHA with a corresponding increase in MDHAR activity and confirmed that this metabolite was chiefly formed by enzymatic action and not by non-enzymatic disproportionation which is in coincidence with results of (Paradiso *et al.*, 2008). In addition, Anjum *et al.*, (2010) reported Cd-induced decrease in GSH pool in Cd-treated moongbean cultivars and suggested that the depletion of GSH pool due to Cd stress in spite of higher GR activity may indicate the mechanism of antioxidant defense through enhanced oxidation of GSH to GSSG by DHAR thus yielding AsA which was later utilized by APX for the detoxification of H₂O₂. In fact, GSH functions as an antioxidant by scavenging ROS, resulting in the oxidation of GSH to GSSG. It is well-established that not only the pool of GSH but also GSH/GSSG ratio is important to maintain the redox status of the cell (Foyer *et al.*, 1994; Paradiso *et al.*, 2008). In this study was found that the pool of GSH and also the ratio of GSH/GSSG (more, mainly due to higher lower of Cd²⁺-induced decline in GSH pool) were significantly reduced(Fig..3). Furthermore, the reduced GSH/GSSG redox state of glutathione under Cd stress also indicated that maximum metabolic load was exerted to maintain redox buffer status of the cells, suggesting a leading role of GSH in an adaptive response to Cd stress and the maintenance of redox status in physiological conditions to a greater extent in Cd-tolerant *P. canescens*.

Conclusion

One important finding observed in this study, the cellular redox status that seems to be affected by cadmium and oxidative stress could be an important mechanism of cadmium toxicity. Initially, among of all

tissues, the root activated by antioxidative defence mechanisms of *P. canescens* establish a redox balance at an environmentally realistic and cadmium concentration. These results provide an indication of the way in which low levels of cadmium might inhibit root emergence and growth. Our data furthermore suggested that the increase in H₂O₂ production in *P. canescens* may be related to the fact that the antioxidant system was not be able to overcome the toxicity caused by higher levels of Cd²⁺. This resulted in negative effects such as lipid peroxidation which affected membrane protein oxidation and brought about a decrease in the growth of *P. ×canescens*. These results demonstrate that the toxic effects of Cd²⁺ are harmful for plant development and affect the quality of plant productivity.

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