

Adaptive response of poplar (*Populus nigra* L.) after prolonged Cd exposure period

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Abstract An outdoor pot experiment was designed to study the changes of growth parameters, accumulation, and distribution of Cd in poplar (*Populus nigra* L.) during a prolonged exposure period (growing period of 17 months including three harvest points), allowing the consideration of time effects and prolonged adaptation to Cd stress. Simultaneously, changes to the antioxidant system in roots and leaves were monitored. It was demonstrated that poplar could adapt to the Cd-contaminated soils after prolonged exposure. Total Cd accumulation in the aerial parts of poplar, due to high biomass production and acceptable Cd accumulation parameters, implies that the tested poplar species could be a good candidate for Cd phytoextraction application as well as could be used as phytostabilizer of Cd in heavily polluted soil. Furthermore, the activity of the antioxidant machinery displays both a tissue- and exposure-specific response pattern to different Cd treatments, indicating that strict regulation of the antioxidant defense system is required for the adaptive response of poplar. In addition, this report highlights the importance of prolonged exposure studies of physiological responses of plants,

especially for long-life-cycle woody species under heavy metal stress, since some misleading conclusions could be reached after shorter time periods.

Keywords Antioxidative enzymes · Cadmium · Heavy metal stress · Phytoremediation · *Populus nigra* · Prolonged exposure period

Introduction

The management of environmental contaminants such as heavy metals has been an ongoing issue for the past two decades. Among heavy metals, cadmium (Cd) is considered as one of the most toxic to prokaryotic and eukaryotic organisms (Godt et al. 2006; Satarug et al. 2003). Therefore, appropriate and effective remediation processes for Cd from soil and water is required. One of the interesting remediation method is phytoremediation processes, i.e., the naturally ability of plants to accumulate heavy metals. Phytoremediation has been proposed as a low-impact in situ cleanup procedure due to its minimal environmental disruption and easy large-scale applicability (Salt et al. 1995). Plants ideal for phytoremediation should have the following properties: fast growth accompanied with high biomass yield, a deep root system, easy to harvest and can tolerate and accumulate a range of heavy metals in their harvestable parts (Bhargava et al. 2012; Salt et al. 1995). There are more than 400 known hyperaccumulator plants, mainly herbaceous species (Li et al. 2012), which are able to uptake and retain Cd within the shoot at a minimum concentration of 100 mg kg⁻¹ dry weight without suffering phytotoxicity (Baker and Brooks 1989). However, most hyperaccumulators are characterized by slow biomass growth, leading to small accumulation quantities of Cd, making these plants unsuitable for commercial application. Consequently, the search for novel plant species with

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higher biomass yield coupled with metal tolerance and accumulation has become a phytoremediation research strategy. In the last decades, fast-growing trees with high biomass production, such as *Populus* species, have been proposed as an alternative choice for phytoremediation processes (Bhargava et al. 2012; Pulford and Watson 2003). Furthermore, a huge variation in Cd tolerance among different poplar species as well as among different clones of the same species are demonstrated, and seeking of poplar species with greater Cd tolerance is still an ongoing interest (He et al. 2011; Induri et al. 2012; Gaudet et al. 2011; Pietrini et al. 2010; Wu et al. 2010).

Plant accumulation of Cd from soil depends on a large number of factors. The bioavailability of metals and various physiological processes in plants (e.g., plant growth stage, exposure time, and Cd concentrations in soil) significantly affect Cd toxicity, adsorption, and distribution in the plant (Bhargava et al. 2012; Lux et al. 2011; Benavides et al. 2005). However, Cd is easily taken up by plant roots, resulting in Cd accumulation in each part of the plant and consequently causing various symptoms of toxicity, such as inhibition of plant growth, chlorosis, necrosis, perturbation in the chloroplast ultrastructure, photosynthesis rate, and the uptake and distribution of macronutrients and micronutrients. Furthermore, Cd generates oxidative stress through the overproduction of reactive oxygen species (ROS), which can lead to cell death due to membrane lipid peroxidation, protein oxidation, enzyme inhibition, and damage of nucleic acid (Benavides et al. 2005). To ameliorate this oxidative stress, plants employ the antioxidative machinery by alternations in activities of antioxidative enzymes (e.g., superoxide dismutase SOD, ascorbate peroxidase APX, catalase CAT, glutathione reductase GR, guaiacol peroxidase GPX, and glutathione *S*-transferase GST) and the level of low molecular weight antioxidants (ascorbic acid, reduced glutathione, carotenoids, phenolics) (Hossain et al. 2012; Benavides et al. 2005; Noctor and Foyer 1998; Siedlecka and Krupa 2002). Maintaining the balance between ROS and the antioxidative system is crucial for plant adaptation and survival under stressful conditions such as heavy metals.

Furthermore, the majority of our knowledge about Cd accumulation and its impacts on the antioxidative system in different plant species has been determined for young plants grown in hydroponic systems. Less is known about specific Cd toxicity in soil and about the uptake and translocation process changes during prolonged exposure. From the above, an outdoor pot experiment was designed in order to study more realistic changes of growth parameters, accumulation, and distribution of Cd in poplar (*Populus nigra* L.) during prolonged exposure (total of 17 months growth) with three harvest points, allowing the consideration of prolonged adaptations to Cd stress. Simultaneously, changes to the antioxidant system in roots and leaves were monitored. Based on the

results presented here, and in the available literature, the possible antioxidant defense system for adaptive response of tested poplar species after prolonged Cd exposure is discussed.

Materials and methods

Plant material and experimental design

Cuttings of poplar (*P. nigra* L. var. *pyramidalis* (Rozier) Spach) were collected and grown at the Croatian Forest Research Institute. For experiment purposes, rooted 1-year cuttings were used (Toogood, 1999). To simulate field conditions, the pot experiment was conducted outdoors from 15 May 2011 to 15 September 2012 at the experimental station of the Croatian Forest Research Institute, Jastrebarsko, Croatia (N 45°40'18.71", E 15°39'4.27"). Meteorological data for the 17-month growing period were as follows: total rainfall 1,285.1 mm, total sunshine 3,188.5 h, and average air temperature 12.81 °C. The soil used in this study was Universal Substrate, Durpeta, Lithuania, made from eco-friendly peat, enriched with nutritional supplements and microelements (pH (H₂O) 5.5–6.5, N 140–200 mg L⁻¹, P₂O₅ 120–300 mg L⁻¹, K₂O 150–250 mg L⁻¹, total organic 92–96 %). The soil was placed in containers and spiked with an appropriate solution of CdCl₂ H₂O (Sigma-Aldrich, Germany) in order to obtain Cd concentrations of 0, 10, 25, and 50 mg Cd kg⁻¹ soil and then left to equilibrate outdoors under a waterproof tarpaulin for 2 months. Soil samples were placed into 10-L plastic pots and single cuttings of poplar in 25 pots per treatment group, i.e., control group with 0 mg Cd kg⁻¹ soil, Cd10 with 10 mg Cd kg⁻¹ soil, Cd25 group with 25 mg Cd kg⁻¹ soil, and Cd50 group with 50 mg Cd kg⁻¹ soil, were planted. For analysis, six plants were harvested from each treatment group at the end of each of three growing periods, i.e., growing period 1 (GP1): 0–2 months of plant growth, growing period 2 (GP2): 2–5 months of plant growth, and growing period 3 (GP3): 5–17 months of plant growth, and plant biomass was recorded for each growing period. During the experiment, no growth restriction due to limited pot volume was observed. The relative growth rate (RGR) was the RGR of all plant parts and was calculated as follows:

$$\text{RGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \quad (1)$$

where *W* is the dry biomass at the beginning (*W*₁) and at the end (*W*₂) of each period, and *t*₂–*t*₁ is the duration of the period (duration of GP1: 61 days; duration of GP2: 92 days; duration of GP3: 260 days corresponding to 1 year of growing period without the dormant season of 3.5 months).

Chemical analysis of plant material and soil

Harvested plants were separated into roots, stems, and leaves. They were washed twice, first with tap water to remove the soil and then with deionized water. Plant samples were oven-dried at 80 °C for 24 h (UNECE, 2010a). Dry samples were ground to obtain a homogeneous powder in a metal-free mill (Ika-Werke, M20, Germany). Collected soil samples were air-dried, ground using a mortar and pestle, and passed through a mesh sieve (fractions < 2 mm) (UNECE, 2010b). Approximately 0.3 g of sub-samples was digested using a mixture of 30 % H₂O₂ (1 mL) and 65 % HNO₃ (5 mL) for plants and a mixture of 65 % HNO₃ (3 mL) and 37 % HCl (9 mL) for soil in a closed high-pressure microwave system (Anton-Paar, Multiwave 3000, Germany). Sub-samples were analyzed for Cd using flame atomic absorption spectroscopy (Perkin-Elmer, Aanalyst 600, USA).

Evaluation of Cd phytoextraction ability of poplar

In order to evaluate poplar Cd phytoextraction ability, poplar cadmium accumulation, the bioconcentration factor (BCF), translocation factors from concentration (TF) and from accumulation (TF'), and Cd removal capacities were calculated at the end of experiment. The BCF was calculated as the ratio of the Cd concentration in plant shoots to the Cd concentration in soil as follows:

$$\text{BCF} = \frac{C_{\text{shoots}}}{C_{\text{soil}}} \quad (2)$$

where C_{shoot} is the concentration in plant shoots and C_{soil} is the concentration in soil (Wu et al. 2010).

The translocation factor from concentration was calculated as the ratio of the Cd concentration in plant shoots to the Cd concentration in plant roots as follows:

$$\text{TF} = \frac{C_{\text{shoot}}}{C_{\text{root}}} \quad (3)$$

where C_{root} is the concentration in plant root (Wu et al. 2010).

The translocation factor from accumulation (TF') was calculated as the ratio of the Cd accumulation content in plant shoots to the Cd accumulation content in plant roots as follows:

$$\text{TF}' = \frac{C_{\text{shoot}} * W_{\text{shoot}}}{C_{\text{root}} * W_{\text{root}}} \quad (4)$$

where W_{shoot} is the dry biomass of shoots at the end of experiment and W_{root} is the dry mass of soil (Wu et al. 2010).

Cd removal capacities (%Cd removal) were calculated as the ratio of the Cd accumulation content in plant shoots to the Cd content in soil as follows:

$$\% \text{ Cd removal} = \left(\frac{C_{\text{shoot}} * W_{\text{shoot}}}{C_{\text{soil}} * W_{\text{soil}}} \right) * 100 \quad (5)$$

where W_{soil} is the dry mass of soil (Li et al. 2012).

Evaluation of lipid peroxidation

The levels of lipid peroxidation in the leaves and roots of poplar were evaluated by measuring malondialdehyde (MDA) using the thiobarbituric acid method (Heath and Packer 1968). Fresh tissue (100 mg) was homogenized in 1.5 mL of 0.25 % thiobarbituric acid (TBA) in 10 % trichloroacetic acid (TCA). The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 15,000g for 10 min, the absorbance of the supernatant was read at 532 nm and correction for unspecific turbidity was conducted by subtracting the absorbance at 600 nm. A total of 0.25 % TBA in 10 % TCA served as the blank. The MDA content was calculated according to its extinction coefficient of 155 mM⁻¹ cm⁻¹ and expressed as nmol g⁻¹ FW.

Antioxidant enzyme extraction and assay

For enzyme analysis, fresh leaves and roots were homogenized with 0.1 g hydrated PVPP (polyvinylpolypyrrolidone) in 100 mM potassium phosphate (K₂HPO₄/KH₂PO₄) buffer, pH 7.0 solution, that included 1 mM EDTA using a pre-chilled mortar and pestle. The homogenates were centrifuged at 15,000g for 30 min at 4 °C. The supernatant was used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Lowry (1951) using bovine serum albumin (Sigma Aldrich) as a standard.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate (KPO₄) buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA, and a suitable aliquot of enzyme extract. Test tubes were shaken and placed 30 cm below a light source consisting of a 15-W fluorescent lamp. The increase in absorbance due to formazan formation was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50 %. The activity of SOD was expressed as unit mg⁻¹ protein.

Catalase (CAT) activity was determined by decomposition of H₂O₂ and was measured spectrophotometrically by assessing the decrease in absorbance at 240 nm (Aebi 1984).

The reaction mixture contained 50 mM KPO₄ buffer (pH 7.0), 10 mM H₂O₂ and enzyme extract. Activity was calculated using the extinction coefficient ($\epsilon=40 \text{ mM}^{-1} \text{ cm}^{-1}$) and was expressed as mmol of H₂O₂ decomposed $\text{mg}^{-1} \text{ protein min}^{-1}$.

Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). The reaction mixture contained 0.5 mM ascorbate, 0.1 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0), and a suitable aliquot of enzyme extract. APX activity was determined by following the decrease in absorbance of ascorbate at 290 nm ($\epsilon=2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) and was expressed as mmol of ascorbate oxidized $\text{mg}^{-1} \text{ protein min}^{-1}$.

Guaiacol peroxidase (GPX) activity was measured following the method of Chance and Maehly (1955). The method is based on the monitoring of the H₂O₂ decomposition rate by GPX, with guaiacol as a hydrogen donor. The reaction mixture contained 50 mM phosphate buffer (pH 7), 5 mM H₂O₂, 18 mM guaiacol, and a suitable aliquot of enzyme extract. GPX activity was estimated by the increase in absorbance of oxiguaiacol at 470 nm ($\epsilon=26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and was expressed as nmol of guaiacol oxidized $\text{mg}^{-1} \text{ protein min}^{-1}$.

Glutathione reductase (GR) activity was measured according to Esterbauer and Grill (1978) by following the rate of NADPH oxidation. The assay mixture contained 50 mM NADPH, 10 mM oxidized glutathione (GSSG), 3 mM MgCl₂ in 0.1 M phosphate buffer (pH 7.8), and a suitable aliquot of enzyme extract. GR activity was estimated by the decrease in absorbance at 340 nm ($\epsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and was expressed as nmol of NADPH oxidized $\text{mg}^{-1} \text{ protein min}^{-1}$.

Glutathione *S*-transferase (GST) activity was measured following the method of Habig et al. (1974) by using 1-chloro-2,4-dinitrobenzene (CDNB) as an electrophilic substrate. The assay mixture contained 100 mM phosphate buffer (pH 6.5), 1 mM glutathione (GSH), 1 mM CDBN, and a suitable aliquot of enzyme extract. GST activity was estimated by the increase in absorbance at 340 nm ($\epsilon=9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and was expressed as nmol of the amount of conjugate formed $\text{mg}^{-1} \text{ protein min}^{-1}$.

Statistical analysis

All experimental results were statistically analyzed using the program Statistica 9.1. The results presented are the mean $\pm$ SD. Data in the text, tables, and figures were expressed as mean $\pm$ standard deviation ($\pm$ SD), and error bars in the figures indicate standard deviation. Homogenized tissues from control treatments were used as control samples for antioxidant enzyme assays. Control samples were included in measurement for all growing periods. Differences between means were analyzed by ANOVA test followed by post hoc Tukey's test. A significant difference was considered at the level of $P<0.05$.

Significant differences between the measurements of “check” sample were not observed. Therefore, we believe that data are accurate and that samples simply differed due to growing stage and/or conditions.

Results and discussion

Plant growth and Cd accumulation

Cd treatment caused significant growth reductions in all poplar organs which resulted in significant decreases ($p<0.05$) in plant biomass in comparison with control plants (Table 1). The only exception was for root biomass at the end of experiment where no significant changes were recorded for the Cd10 and Cd25 treatments, while an increase of root biomass was observed in the Cd50 treatment. However, during the experiment, symptoms of metal toxicity, such as necrotic spots on leaves and browning of roots, were not detected. The relative growth rate (RGR) showed an interesting trend. During GP1, the RGR was reduced for all Cd treatment, which corresponds to biomass reduction, while a reduced RGR was observed for shoots only during the GP2. A similar RGR trend was observed for treated and control plants during GP3, where significant increases of the entire plant RGR was observed in the Cd50 treatment due to the increased root biomass.

In addition, growth stimulation of roots rather than shoots was reported in several studies, which corresponds to the present results. Allocation of biomass to the root might be indicative of plant Cd tolerance since plants translocate their resources in the root as a first line of detoxification (Elobeid et al. 2012; Domínguez et al. 2011; Nikolić et al. 2008). In the literature, growth inhibition of poplar species exposed to Cd has been frequently reported, although the severity of Cd stress symptoms depends largely on the capacity of plants to tolerate this heavy metal (Dai et al. 2013; He et al. 2011; Induri et al. 2012; Gaudet et al. 2011; Pietrini et al. 2010; Wu et al. 2010; Nikolić et al. 2008). Since the Cd tolerance of plants can be measured by their variation in biomass and adaptive response, biomass production is the primary tolerance indicator by which the plant can cope with Cd contamination. Thus, despite biomass reductions, the RGR values obtained here indicate that during prolonged exposure, the plant could adapt and respond to Cd contamination. These findings are in accordance to previous studies on poplar plant which suggest that poplars may well adapt to Cd after prolonged exposure by minimizing the negative effect on growth (Dai et al. 2013; He et al. 2011; Kieffer et al. 2009).

The Cd accumulation capabilities of poplar are presented in Fig. 1. In general, the concentration in roots was typically higher than in other organs (roots > stems $\geq$ leaves), and the increase in Cd concentration in plants was consistent with its

Table 1 Growth of poplar (*P. nigra* var. *Italica*) treated with different cadmium concentrations for 2, 5, and 17 months

GP	Cd treatment (mg kg ⁻¹ soil)	dry weight (g)				Relative growth rate (mg g ⁻¹ per day)		
		Entire plant	Leaves	Stem	Roots	Entire plant	Shoot	Root
0	0	3.2±0.2	0.6±0.1	0.7±0.2	1.9±0.3	—	—	—
1	0	6.2a±0.3	1.7a±0.2	3.2a±0.2	1.3a±0.2	15.5a±2.4	10.8a±1.2	11.7a±0.7
	10	4.6b±0.3	1.0b±0.0	2.8ab±0.2	0.8b±0.1	7.9c±2.0	6.1b±0.8	6.4b±1.0
	25	4.8b±0.2	1.1b±0.2	2.7b±0.2	1.1ab±0.1	11.1b±0.8	7.3b±0.5	7.3b±0.5
	50	3.9b±1.3	0.8b±0.1	2.4b±0.2	0.7b±0.1	5.4c±1.2	3.6c±1.3	3.9c±1.0
	50	13.6a±1.2	2.9a±0.2	5.2a±0.5	5.2a±3.6	15.3a±1.1	5.9a±1.0	8.5a±0.9
2	0	9.1b±0.5	1.8b±0.1	3.4b±0.6	3.9b±0.4	17.0a±1.8	3.7b±0.4	7.5a±0.5
	10	9.1b±0.4	1.7b±0.1	3.6b±0.3	3.8b±0.4	14.4a±0.6	3.9b±0.3	7.1a±0.4
	25	7.2c±0.5	1.5b±0.2	2.8b±0.3	2.9c±0.6	15.4a±0.9	3.4b±0.9	6.6a±0.8
	50	20.1a±1.	3.6a±0.2	8.8a±0.9	7.7b±0.7	1.4b±0.4	0.9a±0.3	1.2b±0.2
	50	16.0b±0.7	2.3b±0.2	7.4b±0.8	6.3b±0.5	2.4b±0.2	1.2a±0.2	1.5b±0.3
3	0	15.7b±0.7	2.0c±0.2	6.8c±1.1	6.9b±0.6	2.2b±0.2	0.7a±0.2	1.3b±0.2
	10	17.5b±0.9	1.6c±0.1	6.0c±0.9	9.9a±0.8	4.7a±0.4	1.1a±0.3	2.8a±0.4
	25							
	50							
	50							

Mean values ($n=6$) ± SD in each column followed by non-identical lowercase letters (a–c) indicate significant difference ($P<0.05$) among each growing period in comparison to control plants as determined by Tukey's HSD test. In the calculation of relative growth rate for GP3, dormant season of 3.5 months was excluded

GP growing period (0, 1, 2, and 3 correspond to growing period of 0, 0–2, 2–5, and 5–17 months of the plant growth, respectively)

concentration in soil. Exceptions were found for the Cd25 and Cd50 treatment after GP1 when similar concentrations were detected in leaves and stem. The Cd concentration in leaves showed no significant differences ($p<0.05$) between GP1 and GP2, indicating that the transport system to leaves was in steady-state conditions. This observation is in accordance with the proposed plant tolerance mechanism, indicating that Cd ions are mainly retained in the roots and stems and only small amounts are transported to the leaves, likely providing protection to photosynthetic organs (Lux et al. 2011; Benavides et al. 2005). Unlike the leaf tissues, the Cd concentration in stems

and roots increased over time, which was more drastic under the Cd50 treatment. Also, the Cd concentration in roots and stems was similar for the Cd10 and Cd25 treatments at the end of the experiment. Cd concentrations in stems and roots for Cd50 reached 36.46 ± 3.08 and 70.91 ± 2.72 $\mu\text{g kg}^{-1}$ dw, respectively.

In order to evaluate the cadmium phytoextraction ability in poplar cadmium accumulation, the BCF, TF, and TF', and Cd removal capacities (%Cd removal) were calculated at the end of experiment (Table 2). Total Cd accumulation in poplar significantly increased with the increase in Cd concentration in soil and

Fig. 1 Cadmium content in different plant parts of poplar treated with different cadmium concentrations for 2, 5 and 17 months: GP1, GP2, and GP3, respectively. Mean values ($n=3$) ± SD in each column followed by different lowercase letters are not significantly different ($P<0.05$) within the specific plant organ as measured by Tukey's HSD test. Cd10, Cd25, Cd50—10, 25, and 50 mg Cd kg⁻¹ soil, respectively

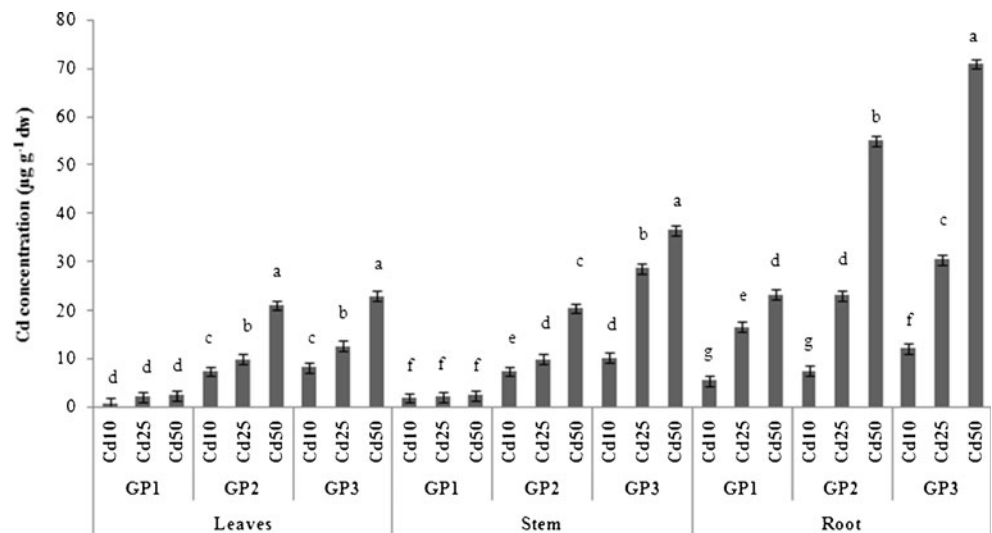


Table 2 Cadmium accumulation in different plant parts, bioconcentration factor, translocation factor from concentration and from accumulation, and %Cd removal capacities determined at the end of the experiment

Cd treatment (mg kg ⁻¹ soil)	Cd content (μg per plant)				BCF	TF	TF	%Cd removal
	Leaves	Stem	Roots	Total				
10	17.46b±1.22	98.22c±2.54	56.52c±1.47	172.21c±2.30	1.10a±0.02	0.88a±0.03	1.88a±0.10	7.49a±0.25
25	21.38b±2.42	222.40b±7.66	170.09b±6.84	413.87b±14.63	1.06a±0.01	0.85a±0.02	1.34b±0.08	6.95ab±0.15
50	30.91a±5.16	257.26a±12.27	606.61a±14.92	894.77a±33.35	1.02a±0.01	0.48b±0.01	0.53c±0.06	6.70b±0.22

Mean values ($n=6$) ± SD in each column followed by non-identical lower-case letters (a–c) indicate significant difference ($P<0.05$) among treatments as determined by Tukey's HSD test

ranged from 172.21 to 894.77 μg per plant. For the Cd10 and Cd25 treatments, cadmium accumulation in different plant parts were in the order stem > root > leaves, while under Cd50 treatment the order was roots > stem > leaves, indicating that heavily contaminated soil such as 50 mg kg⁻¹ soil dw inhibits Cd transfer to aboveground tissues. Moreover, the BCF was greater than 1 and independent of Cd content in the soil, which defines this poplar as a cadmium hyperaccumulator (Bhargava et al. 2012). On the other hand, TF was lower than 1, which does not meet the hyperaccumulator criteria. However, TF' was greater than 1, which is considered a better indicator due to the calculation based on biomass production (Wu et al. 2010). TF and TF' were drastically decreased for the Cd50 treatment, which was in accordance with the observation that poplar primarily accumulates cadmium in its root in heavily contaminated soil and is therefore not appropriate for the phytoremediation process. Another interesting parameter indicating Cd uptake potential is %Cd removal capacity. In the work of Li et al. (2012), a Cd removal capacity > 5 % was selected as the threshold value that represents the plant's capacity to remove 50 % of the total Cd in soil within 15 years, with the presumption that the Cd removal percentage would not change over time. Also, based on the data presented in the literature, Li et al. (2012), calculated %Cd removal capacities for eight woody species, which put this tested species among the best with a %Cd removal capacity of around 7, indicating that the tested poplar is a promising candidate for large-sale Cd phytoremediation. Only three other woody species (*Salix × smithiana*, *Salix dasyclados*, and *P. nigra × maximoviczii*) were found to be capable of extraction of >5 % of total soil Cd in pots (Fuksová et al. 2009; Komárek et al. 2008; Vysloužilová et al. 2003).

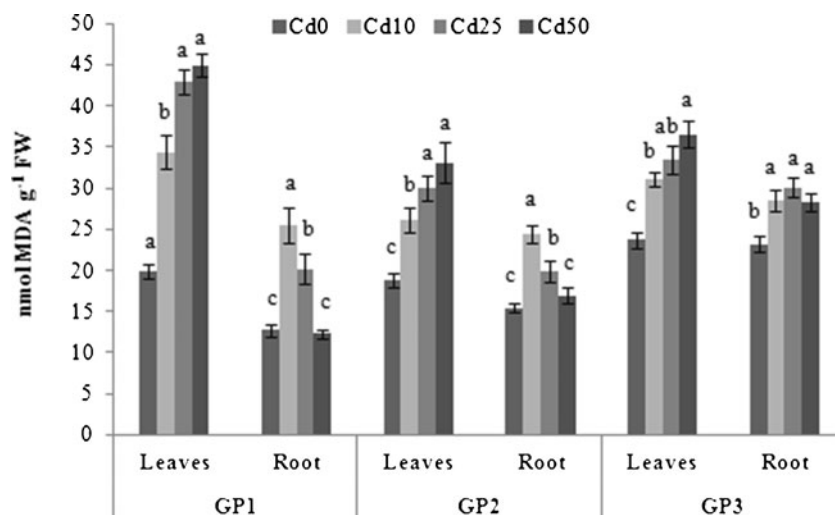
Generally, an ideal plant for phytoextraction application is described as a high biomass-producing species with the ability to concentrate Cd to high levels within their above-ground biomass. So far, no plant has been described to meet all these criteria, and the tested poplar plant is also not ideal. However, total Cd accumulation in the aerial parts of poplar, due to the high biomass production, is by far greater than those accumulated by herbaceous hyperaccumulators, indicating that a fast-growing plant has higher remediation efficiency compared to

slow-growing hyperaccumulators (Liu et al. 2009; Wu et al. 2010). In addition, data concerning plant adaptive biomass production during prolonged periods and data for Cd accumulation imply that the tested poplar species could be a good candidate for Cd phytoextraction application as well as could be used as phytostabilizer of Cd in heavily polluted soil.

Cd-induced oxidative stress and evaluation of the role of antioxidant machinery in the adaptive response of poplar

The Cd-induced oxidative stress is related to the level of Cd accumulation in plant tissue, the duration of treatment, and other parameters such as plant species or plant age (Benavides et al. 2005). The present experiment, designed to follow prolonged poplar performance, showed significant differences in the accumulation rates of Cd between tissues, as well as adaptive biomass production after prolonged exposure. Therefore, the question arose as to how the antioxidant machinery is coordinated with the adaptive response of poplar after prolonged Cd exposure. Firstly, in order to estimate the extent of oxidative stress, the MDA content was measured as an indicator of membrane lipid oxidation as a consequence of enhanced levels of ROS under Cd stress (Singh et al. 2006) (Fig. 2). In general, MDA contents were increased in tissues, root, and leaves, although tissue-specific responses due to differences in Cd content and exposure time were observed. For example, the MDA content in leaves showed an increase with increasing Cd content in soil throughout the experiment, although the increased MDA content decreased with exposure time (increases of about 55.85, 43.38, and 35.15 % was observed for the Cd50 treatment after GP1, GP2, and GP3, respectively). On the other hand, during GP1 and GP2, the MDA content in roots was increased for treatments up to Cd25, while for the treatment Cd50, the MDA levels were similar to the control. Also, the root MDA content was higher for Cd10 treatments. In addition, after GP3, the root MDA contents were enhanced by about 20 %, irrespective of the Cd treatments. Similar to leaves, MDA content decreased with exposure time, which is in correlation with the reduced growth under prolonged exposure.

Fig. 2 Changes in MDA content in leaves and root of poplar treated with different cadmium concentrations for 2, 5, and 17 months: GP1, GP2, and GP3, respectively. Mean values ($n=3$) $\pm$ SD in each column followed by different *lowercase letters* are not significantly different ($P<0.05$) within the specific plant organ for each treatment as measured by Tukey's HSD test. Cd10, Cd25, Cd50—10, 25, and 50 mg Cd kg⁻¹ soil, respectively



Previously, MDA accumulation has been detected in various plant species, both in roots and leaves (Ge et al. 2012; Singh et al. 2006; Wang et al. 2008). Patterns similar to those presented here for roots were also found, indicating that minor lipid peroxidation in roots treated with the highest Cd concentrations may be due to more efficient ROS scavenging (Mohamed et al. 2012; Verma et al. 2008). Furthermore, activities of the antioxidant machinery display tissue- and exposure-specific response patterns to different Cd treatments (Fig. 3). In order to evaluate the accuracy of the data, control samples (homogenized tissues from control treatments) were included in all growing period measurements, and no significant differences between these measurements were observed. For example, the activity of SOD in leaves, as the cells' first line of defense against ROS, was increased throughout the experiment, and the highest values were obtained for Cd50 treatment. Also, during GP1 and GP2, increased SOD activity was in relation with the Cd content in soil, although no significant differences were found for the Cd10 and Cd25 treatment. At the end of the experiment, SOD activity was increased for all treatments with no significant differences between them. In roots, SOD activity showed a different pattern than in leaves. After GP1, the SOD activity decreased for the Cd10 and Cd25 treatments, while a drastic increase of activity was noted for the Cd50 treatment. At GP2, no changes were observed for Cd10 treatment, while increased enzyme activity was detected for Cd25 and Cd50. A similar response was found at the end of experiment, where the strongest induction of SOD activity was detected, especially for the Cd50 treatment. Also, the root activity of SOD was enhanced when Cd concentration in the root exceeded 20 $\mu\text{g kg}^{-1}$ dw. That concentration could be the critical value that causes serious damage in roots and consequently activation of SOD activity. Since overproduction of H_2O_2 , as a result of SOD activity, is still harmful, the plant has to activate further

mechanisms such as the activity of CAT, GPX, and the ascorbate–glutathione cycle to overcome the stress. CAT is one of the key enzymes in the scavenging of H_2O_2 to water and oxygen, and it is often associated with the elimination of high H_2O_2 content during stress. On the other hand, APX and GPX control slight changes in H_2O_2 content from the metabolic pathway rather than oxidative stress (Sharma et al. 2012). In the present experiment, CAT activity of leaves showed similar patterns to SOD activity. Differences were noted only at the end of experiment where a slight increase of CAT activity was recorded for the Cd10 treatment, while treatments with higher Cd concentrations in soil did not cause significant changes in CAT activity. Furthermore, APX and GPX activity showed parallel regulation in leaves. After GP1, both peroxidases showed decreased activity under the Cd10 and Cd25 treatments, while increased activity was recorded for the Cd50 treatment. Prolonged exposure induced APX and GPX activity, which was more obvious at the end of the experiment. When comparing CAT and peroxidase activity, an interesting pattern that is possibly related to Cd content in leaves was revealed. At the beginning of experiment, when Cd accumulation in leaves is in the dynamic stage, CAT activity is a major mechanism for ameliorating stress, though this is not evidently insufficient as growth retardation and MDA content was highest at this stage. Afterward, with an increase in Cd concentration in leaves, the activity of all enzymes involved in H_2O_2 scavenging increased and resulted in better plant performance. At the end of the experiment, when Cd transport to leaves was in a steady state and no inhibition of relative growth rate was noted, only the activity of peroxidases was increased.

Moreover, the increased GR activity in leaves through the experiment suggests that the ascorbate–glutathione cycle could be responsible for the adaptive response of poplar leaves under Cd exposure. A similar mode of H_2O_2 regulation was

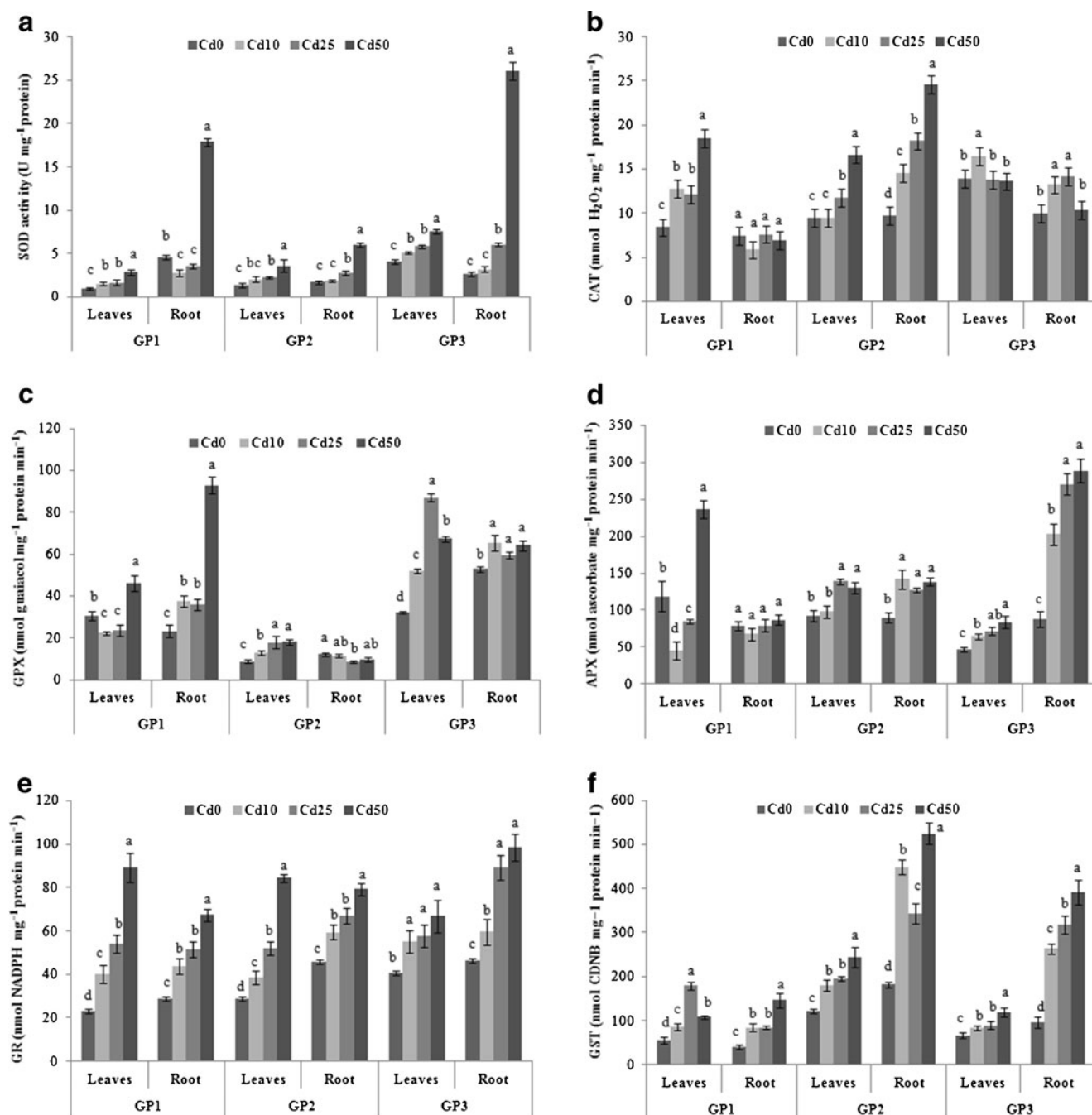


Fig. 3 Enzymatic activities of SOD (a), CAT (b), GPX (c), APX (d), GR (e), and GST (f) in leaves and roots of poplar treated with different cadmium concentrations for 2, 5, and 17 months: GP1, GP2, and GP3, respectively. Mean values ($n=3$) $\pm$ SD in each column followed by

different lowercase letters are not significantly different ($P<0.05$) within the specific plant organ for each treatment as measured by Tukey's HSD test. Cd10, Cd25, Cd50—10, 25, and 50 mg Cd kg⁻¹ soil, respectively

previously described for several plant species, though mainly associated with adult plants and/or a longer exposure to metal stress (Nehnevajova et al. 2012; Lanza de Sáe Melo Marques and Soares 2011; Słomka et al. 2008). Furthermore, the root response pattern differed from leaves, suggesting strong tissue-specific detoxification mechanisms, which was also previously confirmed in a study of proteomic changes in response to Cd

treatment in poplar (Kieffer et al. 2009). At GP1, the activity of CAT and APX was not significantly different, while enhanced activity of GPX (especially for Cd50 treatment) was observed. Enhanced activity of GPX was in good correlation with decreased MDA content in roots after GP1, indicating that stress in roots, at least concerning lipid peroxidation, was well ameliorated with GPX activity. Also, this response in enzyme activity

could be in relation with the previously reported statement that as long as the stress is not too strong for the plant's defense capacity, the main response to heavy metals is an increase in SOD and GPX activities (Siedlecka and Krupa, 2002). Nevertheless, during the prolonged exposure time, Cd accumulation in roots increased and poplar triggered other H_2O_2 -scavenging enzymes to cope with metal stress. Thus, CAT and APX showed enhanced activities during GP2 and GP3, with the exception of CAT activity for Cd50 treatment at the end of the experiment. GPX activity at GP2 decreased while a slight induction of GPX was noticed at the end of the experiment. These data suggest that roots, as the organ continuously exposed to metal pressures, require the activation of all available defense mechanisms to be capable of surviving such stress. Moreover, growth stimulation of roots was noticed in the Cd50 treatment with the highest Cd concentration ($70.91 \pm 2.72 \mu\text{g kg}^{-1} \text{ dw}$), which was quite surprising. A reasonable explanation might be that the high Cd content triggers a signal related to enhanced root development. In fact, recently H_2O_2 was proposed as a vital signalling molecule that controls root development during Cd stress (Li et al. 2009, Li et al. 2007; Zhao et al. 2012). Zhao et al. (2012) reported that H_2O_2 affected the expression of key genes in the auxin signalling pathway, an important phytohormone for root development, as well as for some cell cycle genes, thereby enhancing root growth. Furthermore, two enzymes related with glutathione metabolism were analyzed in this study: GR and GST. GR catalyzes the last rate-limiting step of the ascorbate–glutathione cycle. This enzyme is important for maintaining the high ratio of reduced GSH to GSSG required for the regeneration of ascorbate as a critical point for the functioning of the glutathione–ascorbate cycle (Noctor and Foyer 1998). Furthermore, GSH is involved in other mechanisms for reducing metal toxicity. GSH is a precursor of phytochelatin that can chelate metals and might be conjugated with the degradation product arising from oxidative stress. The latter reaction is catalysed by GST (Hossain et al. 2012; Benavides et al. 2005). However, during the entire experiment, the activities of GR and GST were induced in both plant tissues. For GR activity, a similar response was found in roots and leaves, while GST activity was higher in roots. GST activity was also time-dependent, with the highest activity at the end of experiment. These results suggest that the regulation of glutathione metabolism is involved in the adaptive response in the whole plant.

Generally, adaptive plant responses to metal stress indicate that a fine orchestration of physiological changes, including the balance between produced ROS and the oxidative system, is crucial for plant survival and adaptation (Hossain et al. 2012). Since the antioxidant enzyme activity was reported to be dependent on the plant species, duration of treatment, and experimental conditions, the broad variation without a unique response of antioxidant enzymes is not surprising (Benavides et al. 2005). Furthermore, the majority of our knowledge about Cd impacts on antioxidant enzymes in poplar species and in other plant

species has been determined for young plants grown in hydroponic systems, without sufficient consideration of exposure duration and Cd concentration (Ge et al. 2012; He et al. 2013, He et al. 2011; Domínguez et al. 2011; Nikolić et al. 2008). Thus, it is quite difficult to compare the present results with the literature since poplar was grown in an outdoor pot experiment for longer than a year, though it was reported that only well-coordinated physiological changes such as enhanced antioxidant system in poplar species exposed to Cd lead to greater Cd tolerance (He et al. 2013). However, based on the literature data and the data presented here, we propose the antioxidant defense system for adaptive response of the tested poplar species after prolonged Cd exposure. The ascorbate–glutathione cycle seems to be sufficient for the adaptive response in leaves, while in roots under constant Cd pressure, the activation of all measured antioxidant enzymes was found. Moreover, the regulation of glutathione metabolisms appears to be responsible for adaptive response of the entire poplar plant.

Conclusions

This study demonstrated that tested poplar (*P. nigra* L.) can adapt to Cd-contaminated soils after prolonged exposure. Total Cd accumulation in the aerial parts of poplar due to high biomass production along with Cd accumulation parameters implies that the tested poplar species could be a good candidate for phytoextraction application and could be used as phytostabilizer of Cd in heavily polluted soil. Furthermore, activities of the antioxidant machinery display tissue- and exposure-specific response patterns to different Cd treatments, indicating that strict regulation of the antioxidant defense system is required for survival of the poplar plant under Cd stress. In addition, this report highlights the importance of prolonged time studies of physiological response of plants under heavy metal stress, especially for long-life-cycle woody species since some misleading conclusions could be drawn following short study periods.

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