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Net cadmium flux and accumulation reveal tissue-specific oxidative stress and detoxification in *Populus* × *canescens*

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To characterize the dynamics of Cd^{2+} flux in the rhizosphere and to study cadmium (Cd) plant-internal partitioning in roots, wood, bark and leaves in relation to energy metabolism, reactive oxygen species (ROS) formation and antioxidants, *Populus* × *canescens* plantlets were exposed to either 0 or 50 μ*M* $CdSO_4$ for up to 20 days in the nutrient solution. A strong net Cd^{2+} influx in root apex was observed after Cd exposure for 24 h, even if net Cd^{2+} influx decreased gradually in roots. A large amount of Cd was accumulated in roots. Cd ions were uploaded via the xylem to leaves and further transported to the phloem where significant accumulation was detected. Cd accumulation led to decreased photosynthetic carbon assimilation but not to the depletion in soluble carbohydrates. Increased levels of ROS were present in all tissues, except the bark of Cd-exposed poplars. To combat Cd-induced superoxide and hydrogen peroxide, *P.* × *canescens* appeared to rely mainly on the formation of soluble phenolics as these compounds showed the highest accumulation in the bark and the lowest in wood. Other potential radical scavengers such as proline, sugar alcohols and antioxidant enzymes showed tissue- and exposure time-specific responses to Cd. These results indicate a complex pattern of internal Cd allocation in *P.* × *canescens* resulting in higher ROS stress in wood than in bark and intermediate responses in roots and leaves, probably because of differential capacities of these tissues for the production of protective phenolic compounds.

Introduction

Cadmium (Cd) is a widespread heavy metal in the environment largely because of anthropogenic activities, such as mining, urban traffic and production of phosphate fertilizers (Schützendübel and Polle 2002). It constitutes a serious threat to human health because it is absorbed by plants and eventually enters the human body through the food chain (Peralta-Videa et al. 2009). Furthermore, high Cd concentrations in soil are phytotoxic (Schützendübel and Polle 2002). Therefore, remediation of Cd polluted soils is required and the use of plants (phytoremediation), which can absorb and sequestrate large quantities of this toxic metal, has been suggested (Merkle 2006). Some herbaceous Cd hyperaccumulators such as *Thlaspi caerulescens*

Abbreviations – AAO, ascorbate oxidase; APX, ascorbate peroxidase; CAT, catalase; GPX, guaiacol peroxidase; NMT, Non-invasive Micro-test Technique; ROS, reactive oxygen species; SOD, superoxide dismutase.

and *Arabidopsis halleri* have been identified but the quantities of Cd that can be accumulated by these plants are small because of their low biomass (Clemens 2006, Milner and Kochian 2008). Instead, the use of fast growing tree species with deep rooting systems such as *Populus* has been proposed (Merkle 2006).

It is well documented that poplars as well as other plant species retain large quantities of Cd in their root system, most likely bound to the cell walls (Polle and Schützendübel 2003). Although Cd entry to root cells is the first key process for phytoremediation, only two reports have currently described the dynamics of Cd^{2+} flux along root length in monocots by employment of ion-selective microelectrodes (Farrell et al. 2005, Pineros et al. 1998). Up to now, little is known about the dynamics of Cd^{2+} flux in the rhizosphere of dicotyledonous plants. Once taken up into the apoplast, Cd ions enter the root cells, are further uploaded into the xylem and are transported to the leaves with the transpiration stream (Clemens 2006). There is evidence that Cd can also be re-distributed via phloem transport (Mendoza-Cozatl et al. 2008). Therefore, the question arises whether the bark can serve as a significant deposit for Cd.

Negative effects of Cd on plant performance have frequently been studied (Clemens 2006, Verbruggen et al. 2009). In poplars as in other species, Cd exposure results in reduced chlorophyll concentrations and decreases in photosynthesis (Durand et al. 2010a, Kieffer et al. 2009a). The products of carbon assimilation such as soluble sugars and sugar alcohols are altered and Cd-exposed poplars and protein levels of photosynthetic enzymes are decreased (Kieffer et al. 2008, 2009a, Lorenc-Plucinska and Stobrawa 2005). In the cambial zone and in roots, proteome analyses revealed significant decreases in the abundance of proteins involved in energy metabolism (Durand et al. 2010b, Kieffer et al. 2009b). These results suggested a higher sensitivity of these tissues to Cd than that of leaves. However, physiological evidence in support of this idea is still lacking.

The mechanisms of Cd toxicity in plants have been explored in the last decades (Clemens 2006). One of the main reasons for Cd toxicity in plants is the induction of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) that elicit oxidative stress (Gratao et al. 2005). Concomitant increases in O_2^- and H_2O_2 in the presence of free Fe and Cu may furthermore result in increased formation of hydroxyl radicals ($OH^\bullet$) (Sharma and Dietz 2009). Induction of ROS in plants exposed to Cd disturbs the redox balance in cells. To cope with Cd-induced oxidative stress, antioxidant defences need to be activated. Plants employ non-enzymatic and enzymatic systems to protect cells from oxidative injury. An array of metabolites is involved in non-enzymatic scavenging of ROS. With regard to ameliorate Cd stress, proline is especially relevant as it reacts rapidly with $OH^\bullet$ (Sharma and Dietz 2009). However, phenolic compounds are also potent ROS scavengers and increase in Cd-exposed roots (Schützendübel and Polle 2002, Schützendübel et al. 2001). Enzymatic antioxidants largely consist of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). Depending on plant species, dosage and exposure duration, the responses of the antioxidant systems to Cd are variable (Clemens 2006, Sharma and Dietz 2009). In poplar roots short-term exposure to Cd results initially in increases but subsequently in decreases of antioxidant defence systems, which are accompanied by elevated H_2O_2 formation and growth reduction (Schützendübel et al. 2002). Redox homeostasis and avoidance of oxidative stress are determinants of Cd phytotoxicity (Sharma and Dietz 2009). But a comprehensive analysis of antioxidant defences at the whole-plant level in relation to Cd accumulation is lacking.

Because poplars are attractive species for phytoremediation, we have chosen *Populus* × *canescens*, a hybrid of *Populus tremula* × *Populus alba*, to investigate the dynamics of Cd^{2+} flux in the rhizosphere and to study Cd plant-internal partitioning in roots, wood, bark and leaves in relation to energy metabolism, ROS formation and antioxidants. For this purpose, the poplars were exposed to 50 μ*M* $CdSO_4$ for up to 20 days in the nutrient solution and used for analyses of Cd concentrations, soluble carbohydrates, ROS and antioxidants in different tissues along the whole transport route including roots, wood, leaves and bark.

Materials and methods

Cultivation of plants and Cd exposure

Plantlets of *P.* × *canescens* (*P. tremula* × *P. alba*) were produced by micropropagation (Leple et al. 1992) and cultivated in a climate chamber (day/night temperature, 25/18°C; relative air humidity, 50–60%; light per day, 14 h and photosynthetic photon flux, 150 μmol m^{-2} s^{-1}). 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 10-week cultivation, half of the plants were treated with 50 μ*M* $CdSO_4$ by adding $CdSO_4$ into the nutrient solution and the other half of the plants served as the controls.

Measurement of net Cd^{2+} flux in roots

To monitor net Cd^{2+} flux in roots of *P.* × *canescens* exposed to 50 μ*M* $CdSO_4$, white fine roots from 14-week-old plants were selected. The net Cd^{2+} flux was measured non-invasively by using the Non-invasive Micro-test Technique (the NMT system BIO-IM; Younger USA, LLC, Amherst, MA) at the company (Xuyue Science & Technology Co., Ltd. Beijing, China). The NMT system and its application in ion flux detection were described in detail (Farrell et al. 2005, Ma et al. 2010, Pineros et al. 1998, Xu et al. 2006). Briefly, the ion-selective microelectrode with an external tip (ca. 2–4 μm in diameter) was manufactured and silanized with tributylchlorosilane and the tip was backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, St Louis, MO). Prior to the net Cd^{2+} flux measurement, the microelectrode was calibrated in 10 and 100 μ*M* Cd^{2+} and only electrodes with Nernstian slopes more than 25 mV per 10 times concentration difference were used.

Three fine roots per plant (six plants in total) were used for this analysis. The white fine roots excised from the plants exposed to 50 μ*M* $CdSO_4$ for 24 h were immediately transferred to a Petri dish containing 10 ml of measuring solution (0.05 m*M* $CdSO_4$, 0.05 m*M* KCl, 0.25 m*M* NaCl, 0.15 m*M* MES and 0.1 m*M* Na_2SO_4, pH 6.0). To determine the appropriate point for measurement along the root tip, a preliminary experiment was carried out with an initial measurement at the root tip followed by 300 μm walk steps (Fig. 1A). Gradients of Cd^{2+} near to the root surface (ca. 2–5 μm) were measured by moving the Cd^{2+}-selective microelectrode between two positions (with a distance of 30 μm) in perpendicular direction to the root surface. The recording rate for Cd^{2+} flux was 10 readings per 64 s. The Cd^{2+} flux was recorded for a period of 4 min. Acquisition of root images and processing of Cd^{2+} flux data were performed with IM-FLUX software attached to the NMT system.

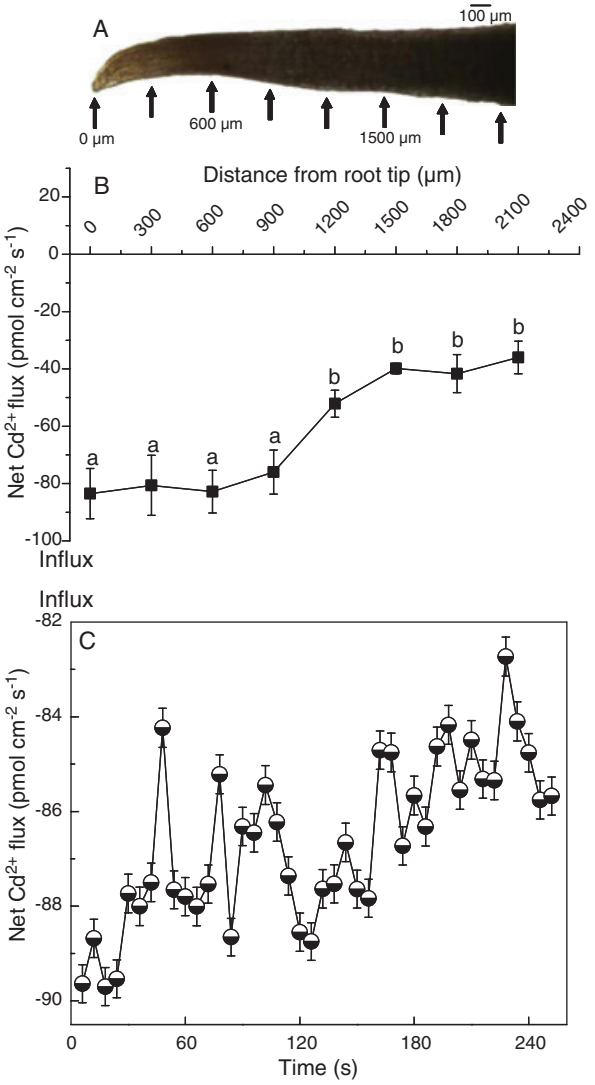

Fig. 1. Root tip (A), net Cd^{2+} flux along root tip (B) and the average of net Cd^{2+} flux in 4 min in the region of 450–750 μm from root tip (C) of *P.* × *canescens* after exposure to 50 μ*M* $CdSO_4$ for 1 day. Data indicate means ± SE (n = 6). The negative value indicates an influx. Different letters on the error bars indicate significant difference between the measured points. Net Cd^{2+} flux in root was measured non-invasively by using NMT (for details see the text).

Gas exchange measurement and harvest

Before each harvest, three mature leaves (leaf plastochron index = 7–9) of each plant were selected for gas exchange measurements. Net photosynthetic rate (*A*), stomatal conductance (g_s) and transpiration rate (*E*) were determined using a portable photosynthesis system (LiCor-6400; LiCor Inc., Lincoln, NE) with an attached LED light source (6400–02). The measurements were carried out from 8:00 to 11:00 h with a light intensity of 1000 μmol photon m^{-2} s^{-1}. The air flow through the sample chamber was set at 500 μmol s^{-1}, the leaf temperature was 25 ± 0.8°C and the CO_2 concentration in the sample chamber was 400 μmol mol^{-1}.

After Cd exposure for 1, 10 and 20 day(s), six plants were $CdSO_4$ treated and six control plants were harvested per date. The plants were separated into roots, wood, bark and leaves. Samples were wrapped with tinfoil and immediately frozen in liquid nitrogen. Frozen samples were ground into fine powder in liquid nitrogen with a mortar and a pestle and stored at −80°C for further analysis. Fresh powder (ca. 30 mg) from each tissue per

plant was dried at 60°C to determine the fresh-to-dry mass ratio which was used to calculate the biomass of each tissue.

Analysis of Cd, foliar chlorophyll and carotenoid

To analyse Cd in different tissues, fine powder (ca. 100 mg) from roots, wood, bark and leaves was digested in a mixture (7 ml concentrated HNO_3 and 1 ml concentrated $HClO_4$) at 170°C as described (Schützendübel et al. 2001). Subsequently, Cd was determined by flame atomic absorbance spectrometry (Hitachi 180-80, Japan). Standard curve was prepared using a series of diluted solutions of a commercially available standard (National criterion solutions, National Analysis Centre, Beijing, China).

To determine chlorophyll and carotenoid contents in leaves, fine powder of fresh leaves (ca. 40 mg) was extracted for 24 h in 5 ml of 80% acetone in darkness. The contents of chlorophyll a, chlorophyll b and carotenoids in the extracts were determined by a spectrophotometer (UV-3802, Unico Instruments Co. Ltd., Shanghai, China) at 663, 646 and 470 nm, respectively (Wellburn 1994).

Analysis of soluble sugars and sugar alcohols

Soluble sugars and sugar alcohols were determined by GC-MS as described previously (Hu et al. 2005, Luo et al. 2009a, 2009b). Briefly, fine powder (ca. 50 mg) of fresh tissues was extracted in 500 µl of an extraction solution (methanol:chloroform:water, 12:5:3, v/v/v). Subsequently, the extracted compounds were acetylation derivatized and then separated in a DB-17 capillary column (30 mm × 0.25 mm × 0.25 µm; J & W Scientific, Folsom, California, CA) attached to a Finnigan Trace GC ultra and quantified with a Finnigan Trace GC ultra-Trace DSQ GC-MS system (Thermo Electron Corporation, Austin, TX). Ribitol was used as the internal standard in the analysis. Mannitol, galactose, sorbitol, myo-inositol, fructose, glucose and sucrose were used as standards to identify and quantify the concentrations of soluble sugars and sugar alcohols.

Determination of O_2^- and H_2O_2

The O_2^- production rate in plant materials was analysed as described by Lei et al. (2006). The fine powder of fresh tissues (ca. 100 mg) was homogenized in 2 ml of 50 m*M* potassium phosphate buffer (pH 7.8) and centrifuged (10 000 *g*, 4°C, 10 min). One millilitre of the supernatant was mixed with 0.9 ml of 50 m*M* potassium phosphate buffer (pH 7.8) and 0.1 ml of 10 m*M* hydroxylamine hydrochloride. Subsequently, the reaction mixture was incubated at 25°C for 20 min before adding 1 ml of 17 m*M* p-aminobenzene sulphonic acid and 1 ml of 7 m*M* α-naphthylamine. After further incubation (25°C, 20 min), the absorbance of the mixture was recorded spectrophotometrically at 530 nm.

The concentration of H_2O_2 in plant materials was analysed after Lei et al. (2007). The fine powder of fresh tissues (ca. 60 mg) was extracted in 2 ml of acetocaustin and centrifuged (10 000 *g*, 4°C, 10 min). The supernatant was collected. After adding 0.1 ml of 20% $TiCl_4$ and 0.2 ml of 25% aqueous ammonia to the supernatant, the mixture was immediately centrifuged again (10 000 *g*, 4°C, 10 min). Subsequently, the supernatant was discarded and the pellet was dissolved in 3 ml of 1 *M* H_2SO_4. The absorbance was recorded spectrophotometrically at 410 nm.

Determination of free proline and soluble phenolics

Free proline was determined according to Tamas et al. (2008). In brief, frozen plant powder (ca. 100 mg) was extracted in 1.5 ml of 3% sulfosalicylic acid in a water bath at 100°C for 15 min. After centrifugation of the homogenate (12 000 *g*, 25°C, 10 min), the supernatant was collected. Subsequently, 1 ml of the supernatant was mixed with 1 ml glacial acetic acid and 1 ml ninhydrin reagent (2.5 g ninhydrin in 60 ml glacial acetic acid and 40 ml of 2 *M* phosphoric acid). The mixture was incubated at 98°C for 30 min. The absorbance of the mixture was recorded spectrophotometrically at 518 nm after cooling to the room temperature. The standard curve was generated using a serial of diluted solutions of L-proline (Amresco Inc., Solon, OH).

Soluble phenolics in plant materials were determined as reported previously (Luo et al. 2008). The frozen powder (ca. 60 mg) was extracted in 1.5 ml of 50% methanol and incubated in an ultrasonic bath for 1 h at 40°C. After centrifugation (4500 *g*, 4°C, 10 min), the supernatant was collected. The pellet was extracted again as mentioned above and the supernatant was collected and combined with the previous one. The soluble phenolics in supernatant were determined spectrophotometrically at 765 nm by using the Folin–Ciocalteus reagent. Standard curve was prepared using a serial of diluted solutions of catechin (Sigma, St Louis, MO).

Assay of enzyme activities

Soluble proteins in plant materials were analysed as described before (Luo et al. 2008). Frozen plant powder

(ca. 200 mg) was homogenized in 4 ml of cold extraction buffer [100 m*M* potassium phosphate (pH 7.8), 200 mg polyvinylpolypyrrolidone and 0.5% (v/v) Triton X-100]. The mixture was incubated for 15 min on ice and centrifuged (15 000 *g*, 4°C, 30 min). The supernatant was eluted through Sephadex G-25 columns (PD-10 column, Pharmacia, Freiburg, Germany). The soluble proteins in the eluent were determined according to the Bradford method, using bovine serum albumin (Interchim, Montluçon, France) as the standard. The eluent was also used for the assays of enzyme activities.

The activity of SOD (EC 1.15.1.1) was determined according to Morina et al. (2010). One unit of SOD was defined as the amount of enzyme that caused a 50% decrease in the SOD-inhibited nitrobluetetrazolium reduction at 550 nm. The activity of GPX (EC 1.11.1.7) was measured as described (Sun et al. 2009). Activities of CAT (EC 1.11.1.6) after Ma and Cheng (2003), APX (EC 1.11.1.11) after Polle et al. (1990) and ascorbate oxidase (AAO; EC 1.10.3.3) after the method of Tamas et al. (2006) were determined.

Statistical analysis

All statistical tests were performed with STATGRAPHICS (STN, St Louis, MO). For net Cd^{2+} flux in roots, the effect of distance from root tip was analysed by one-way ANOVA. For photosynthetic parameters and biomass, two-way ANOVAs were applied with $CdSO_4$ (Cd) and time course (time) as two factors. For other experimental variables, three-way ANOVAs were applied with $CdSO_4$ (Cd), tissue and time course (time) as three factors. When the interaction was significant, a posteriori comparison of means was made. To reduce the chance of type I errors, all *P* values of these multi-comparisons were corrected by the Tukey-HSD method. Data were tested for normality prior to the statistical analysis. Differences between means were considered significant when the *P*-value of the ANOVA *F*-test was less than 0.05.

Results

Net Cd^{2+} flux in roots and biomass

To detect spatial dynamics of Cd^{2+} movement along root tips in *P.* × *canescens* after Cd exposure for 24 h, net Cd^{2+} flux was analysed by a NMT (Fig. 1B). At the root tips of *P.* × *canescens*, net Cd^{2+} flux displayed an influx, ranging from 35.9 ± 5.7 to 83.5 ± 8.8 pmol cm^{-2} s^{-1}, depending on the distance from root tips (Fig. 1B). The net influx of Cd^{2+} decreased markedly after 900 µm from root tips (Fig. 1B). To further analyse temporal dynamics of Cd^{2+} flux, the average of net Cd^{2+} influx at the distance of 450, 600 and 750 µm from root tips was monitored (Fig. 1C). Cd^{2+} influx in the region of 450–750 µm from root tips varied from 82.7 ± 0.4 to 89.7 ± 0.4 pmol cm^{-2} s^{-1}, which displayed a decrease trend with increases in exposure time.

To analyse the toxic effects of Cd on plant growth, biomass of roots, wood, bark and leaves was recorded (Appendix S1). The biomass of all tissues in *P.* × *canescens* was not affected by Cd exposure. Continuous accumulations of biomass of all tissues were found with increases in exposure time.

Cd accumulation in different tissues of *P.* × *canescens* in relation to gas exchange and energy metabolism

Exposure of *P.* × *canescens* to 50 µ*M* $CdSO_4$ for 1, 10 and 20 day(s) led to significant increases in Cd concentrations in the following order: roots > bark > wood = leaves [$P_{(tissue)} < 0.0001$, Fig. 2, Appendix S2]. The dynamics of this accumulation differed between different tissues. After a strong increment in roots within 24 h, subsequent Cd incorporation was slow and reached about twice the level after 20 days than that observed after 1 day (Fig. 2A). In leaves, Cd increased gradually and reached about sevenfold higher concentrations after 20 days than that after 1 day (Fig. 2D). In contrast to leaves and roots, Cd accumulation in bark and wood occurred only within the first 10 days of exposure (Fig. 2B, C). Because the Cd concentrations in leaves continued to increase between 10 and 20 days of Cd exposure, xylem uptake and unloading processes and probably also those in the bark must have been active during this period of time.

To obtain further evidence for the functionality of *P.* × *canescens* during the Cd-exposure period, gas exchange and carbohydrates as indicators for energy metabolism were determined. The CO_2 assimilation rates were significantly inhibited by 37 and 19% in plants exposed to $CdSO_4$ for 1 and 20 day(s), respectively, in comparison with those of controls (Table 1). This indicates that net photosynthetic rates were partially recovered with increasing time of Cd exposure. Similarly, initial strong reductions of stomatal conductance and transpiration rates recovered partly after 20 days of Cd exposure (Table 1). Mean pigment concentrations of 10.0 ± 0.6 mg $chlorophyll_{(a+b)}g^{-1}$ dry mass and 1.5 ± 0.1 mg carotenoids declined only slightly by 17 and 15%, respectively, in response to Cd (Table 1).

In line with a relatively high extent of sustained photosynthetic carbon assimilation, Cd exposure did not result in a general depletion of carbohydrates or sugar alcohols (Fig. 3, Appendixes S3 and S4). At most

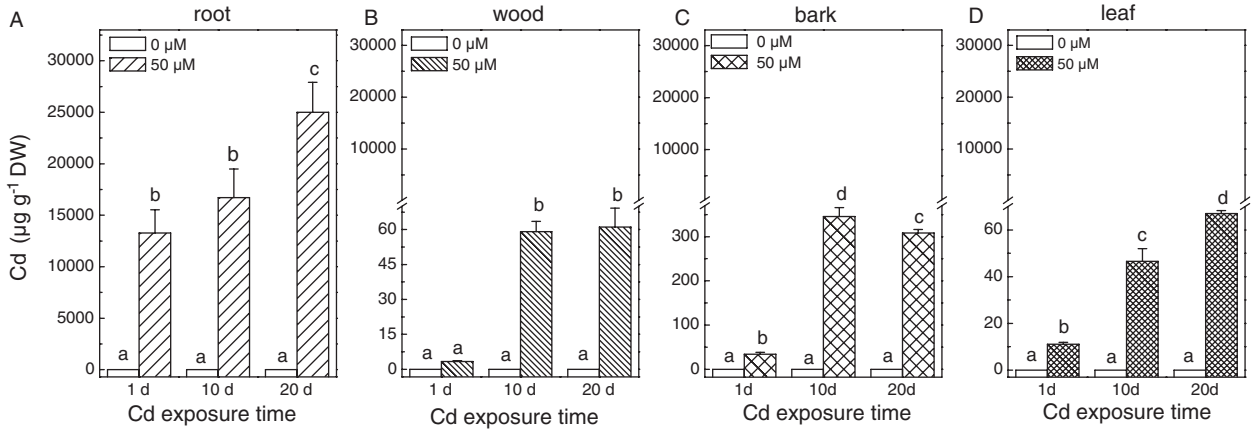

Fig. 2. Cd in roots, wood, bark and leaves of *P.* × *canescens* exposed to 0 or 50 µ*M* $CdSO_4$ for 1, 10 or 20 day(s). Bars indicate means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant difference between the treatments. The results of statistical analysis were shown in Appendix S2.

time points, the primary soluble carbohydrates glucose, fructose and sucrose were increased in leaves, bark and roots of Cd-treated *P.* × *canescens* (Fig. 3). The increment was highest for sucrose, the transport form of sugars. Only wood showed moderate decreases by 19 and 21% for glucose and sucrose, respectively (Fig. 3). Sugar alcohols have many functions in plants and play roles, e.g. in membrane lipid biosynthesis (inositol) and oxidant detoxification (Ayako et al. 2008). Sorbitol, mannitol and galactose were minor compounds. Although they showed some significant changes, no consistent responses to Cd were observed (Appendixes S3 and S4). Inositol was generally present in about 5- to 10-fold higher concentrations than the sum of galactose, mannitol and sorbitol, and showed significant but minute increases across different time points only in roots (Fig. 3, Appendixes S3 and S4). Overall, these observations suggest that carbohydrates for energy supply and biosynthetic processes would be available, whereas no consistent changes in response to Cd concentrations or accumulation rates were observed for the minor carbohydrate-based compounds across different poplar tissues.

Cd-induced oxidative stress and detoxification systems

Because the concentrations and accumulation rates of Cd showed significant differences between the tissues, we wondered if this behaviour resulted in tissue-specific differences in oxidative stress. In roots, which showed the fastest and strongest Cd accumulation, O_2^- concentrations were significantly induced only after Cd exposure for 10 and 20 days compared to those of control plants (Fig. 4A, Appendix S5). Higher H_2O_2 concentrations in roots than those in controls also

Table 1. CO_2 assimilation rate (*A*, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (*E*, mmol H_2O m^{-2} s^{-1}) and photosynthetic pigments (mg g^{-1}dry weight) in leaves of *P.* × *canescens* exposed to 0 or 50 µ*M* $CdSO_4$ for 1, 10 or 20 day(s). Data indicate means ± SE (n = 6). Different letters behind the values in the same column indicate significant difference between the treatments. *P* values of the ANOVAS of $CdSO_4$(Cd), time and their interactions were also shown. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; *****P* ≤ 0.0001. ns, not significant; Chl a, chlorophyll a; Chl b, chlorophyll b; Chl (a + b), sum of chlorophyll a and b; Car, carotenoid.

Cd µ*M*	Time (days)	*A*	g_s	*E*	Chl a	Chl b	Chl (a + b)	Car
0	1	3.61 ± 0.20 b	0.100 ± 0.005 d	2.00 ± 0.04 bc	7.53 ± 0.46 b	2.65 ± 0.25 bc	10.18 ± 0.70 b	1.63 ± 0.10 b
	10	3.44 ± 0.19 b	0.079 ± 0.004 cd	2.14 ± 0.04 bc	7.08 ± 0.27 ab	2.47 ± 0.04 ab	9.55 ± 0.29 ab	1.51 ± 0.11 b
	20	4.30 ± 0.27 c	0.098 ± 0.005 d	2.62 ± 0.05 c	7.35 ± 0.58 b	3.04 ± 0.31 c	0.38 ± 0.95 b	1.38 ± 0.01 ab
50	1	2.29 ± 0.14 a	0.040 ± 0.004 a	1.01 ± 0.04 a	6.42 ± 0.48 ab	2.27 ± 0.08 ab	8.69 ± 0.49 ab	1.36 ± 0.17 ab
	10	3.80 ± 0.13 bc	0.055 ± 0.004 ab	1.66 ± 0.03 b	6.38 ± 0.43 ab	2.22 ± 0.14 ab	8.59 ± 0.58 ab	1.35 ± 0.07 ab
	20	3.49 ± 0.31 b	0.067 ± 0.005 bc	1.64 ± 0.04 b	5.73 ± 0.45 a	2.09 ± 0.06 a	7.83 ± 0.51 a	1.14 ± 0.12 a
P values	Cd	**	****	****	**	**	**	*
	Time	***	ns	**	ns	ns	ns	ns
	Cd × time	***	*	ns	ns	ns	ns	ns

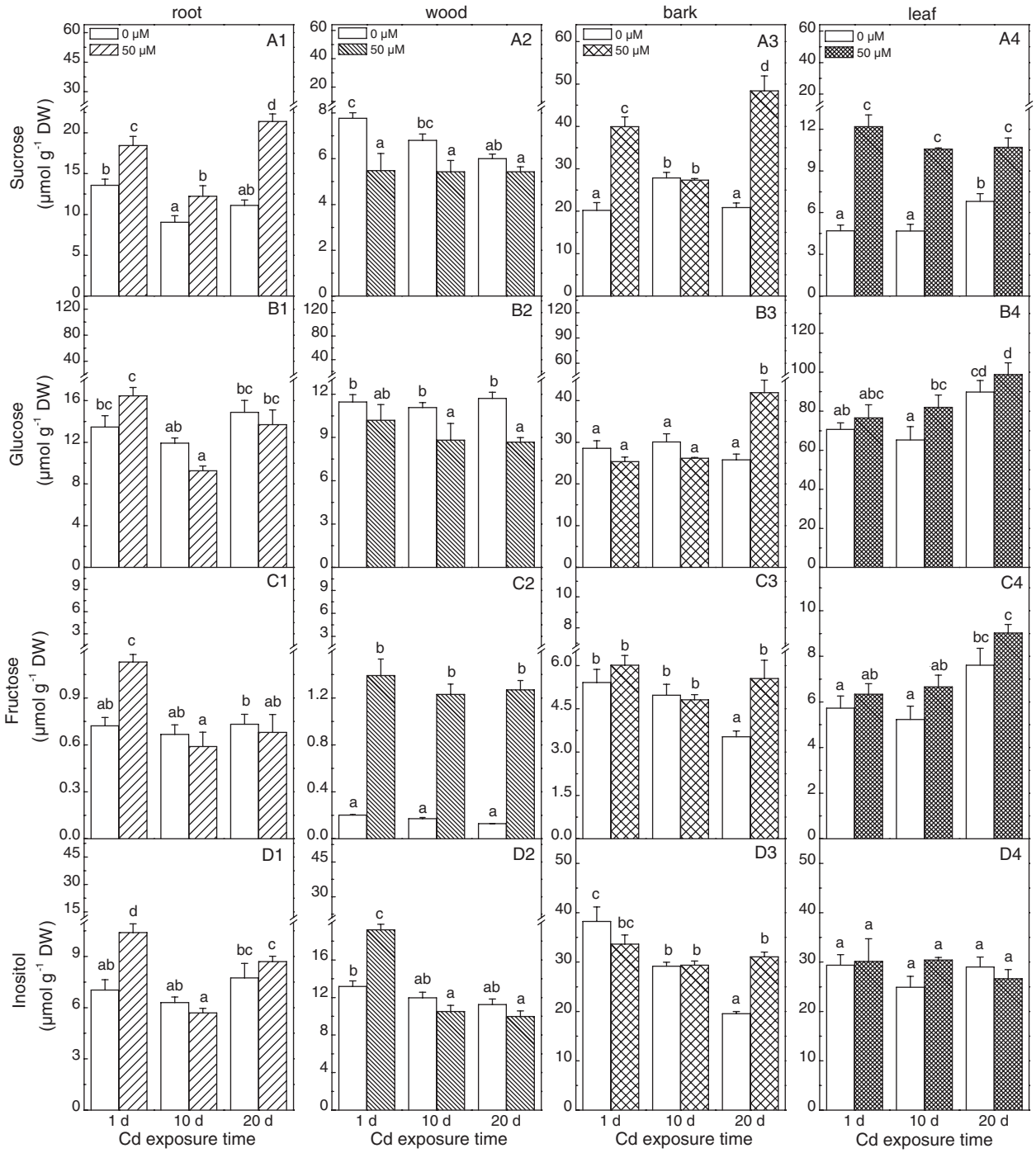

Fig. 3. Soluble sugars and sugar alcohols in roots, wood, bark and leaves of *P.* × *canescens* exposed to 0 or 50 μ*M* $CdSO_4$ for 1, 10 or 20 day(s). Bars indicate means ± SE (n = 6). Different letters on the bars for the same sugar or sugar alcohol indicate significant difference between the treatments. The results of statistical analysis were shown in Appendix S4.

occurred after 20 days of Cd exposure (Fig. 4E). Leaves and wood reacted more sensitively to Cd treatment than roots although they showed the least increments in Cd (Figs 2 and 4). In both tissues, the O_2^- production rate was increased already after 24 h and subsequently decreased with prolonged Cd incubation time to levels similar to those in controls or even less (Fig. 4B, D). These tissues also showed earlier increases in H_2O_2 than roots and

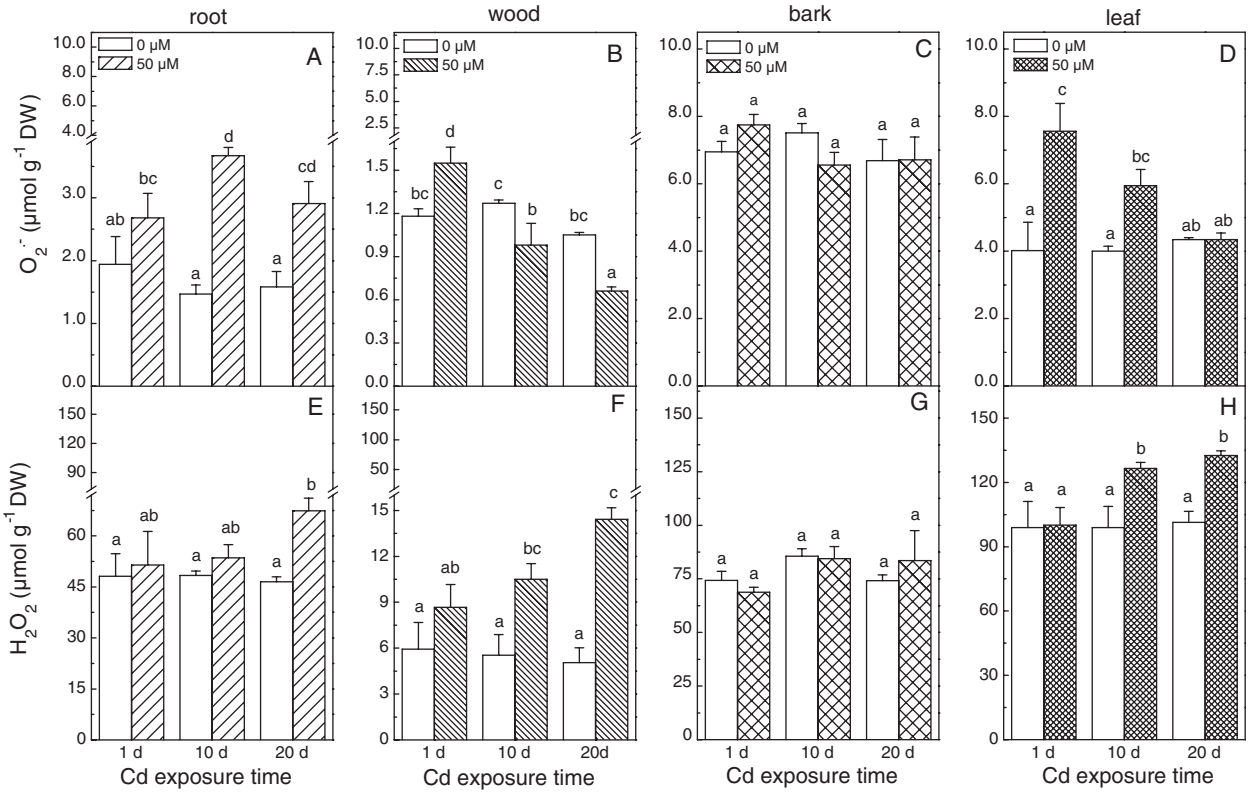

Fig. 4. O_2^- and H_2O_2 in roots, wood, bark and leaves of *P.* × *canescens* exposed to 0 or 50 μ*M* $CdSO_4$ for 1, 10 or 20 day(s). Bars indicate means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant difference between the treatments. The results of statistical analysis were shown in Appendix S5.

the strongest increment was observed in wood after 20 days of Cd exposure (Fig. 4F). Although bark showed a strong accumulation of Cd during the first 10 days, neither O_2^- production nor H_2O_2 concentrations were increased in this tissue in comparison with untreated controls (Fig. 4C, G).

Because these results suggest that tissues with moderate Cd accumulation experienced stronger oxidative stress than tissues with strong Cd accumulation, we studied the stress responses of antioxidative systems. Proline and free phenolics involved the non-enzymatic scavenging of $OH^\bullet$ and of O_2^- and H_2O_2, respectively. The concentrations of free proline were moderately increased in roots and bark of *P.* × *canescens* after $CdSO_4$ exposure for 10 and 20 days and leaves after 20 days in comparison with those in unstressed plants, respectively (Fig. 5, Appendix S6). More pronounced Cd-induced increases were found in these tissues for free phenolics, especially in bark and roots (Fig. 5E, G, H). In wood, Cd exposure did neither influence proline nor phenolic concentrations compared with those in controls (Fig. 5B, F).

Wood and leaves showed a rapid induction of SOD activities within the first day of Cd exposure (Fig. 6, Appendix S7), whereas the induction was delayed in roots (Fig. 6). In these tissues, SOD activities declined with prolonged Cd exposure but more rapidly in wood than in roots or leaves (Fig. 6). In contrast to these Cd responses, SOD activities were initially decreased in bark and increased subsequently to a maximum after 20 days of Cd exposure (Fig. 6).

GPX, CAT and APX, which are involved in H_2O_2 removal, generally exhibited decreases in activities in the presence of Cd compared with controls regardless of the tissue (Fig. 6, Appendix S7). However, the time course and the extent of the suppression differed. The activities of GPX showed a decline between 1 and 20 day(s). Stimulation of GPX above control levels was only observed in wood after 1 day of Cd exposure (Fig. 6). CAT activities were increased after 1 day of Cd exposure in roots and leaves, and declined thereafter more strongly in leaves than in roots (Fig. 6). CAT activities in wood were most sensitive and declined already strongly with 1 day of Cd treatment (Fig. 6). Similarly, in bark, reductions in CAT activities were

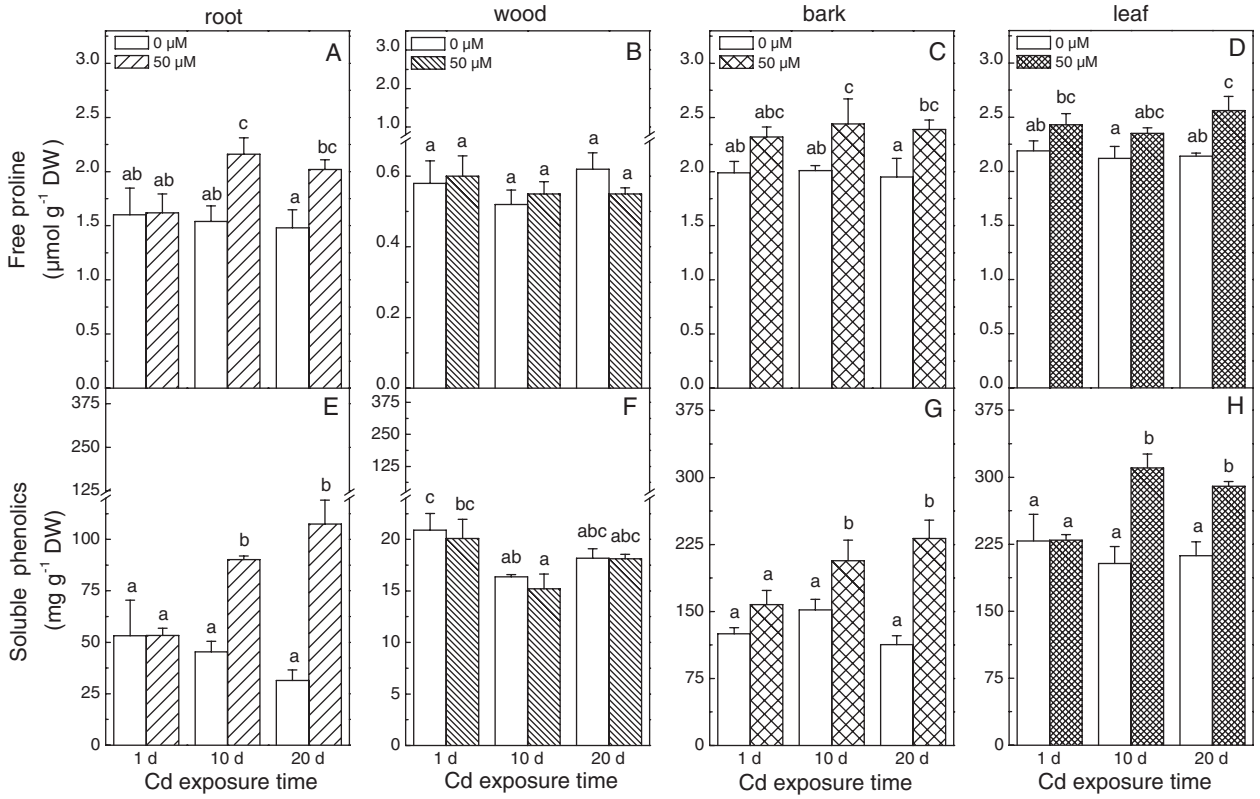

Fig. 5. Free proline and soluble phenolics in roots, wood, bark and leaves of *P.* × *canescens* exposed to 0 or 50 μ*M* $CdSO_4$ for 1, 10 or 20 day(s). Bars indicate means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant difference between the treatments. The results of statistical analysis were shown in Appendix S6.

found in response to Cd (Fig. 6). APX activities declined during Cd exposure in roots and leaves, were unaffected in wood and were retained at a reduced level in bark throughout the duration of Cd exposure (Fig. 6). The activities of AAO were also suppressed in response to Cd treatment in wood and bark more rapidly than in leaves and roots (Fig. 6, Appendix S7).

Discussion

Strong net Cd^{2+} influx in roots indicates a great potential for Cd enrichment

In this study, the spatial and temporal kinetics of the net Cd^{2+} flux were examined in the roots of *P.* × *canescens* by a NMT that has a high sensitivity to Cd^{2+} movement (Fig. 1B). To our knowledge, this is the first report on using this technique in a dicotyledonous plant. In previous studies, a similar method was applied to detect Cd^{2+} flux along roots of monocotyledonous plants (Farrell et al. 2005, Pineros et al. 1998). The Cd^{2+} influx in roots of wheat (*Triticum aestivum* cv Grandin) exposed to 50 μ*M* Cd^{2+} was greatest (0.28–0.35 pmol Cd^{2+} cm^{-2} s^{-1}) in the region of 0.6–1.2 mm from the root tip (Pineros et al. 1998). Similar positional effects were observed along roots of other herbaceous plants exposed to Cd stress (Farrell et al. 2005, Pineros et al. 1998). In roots of *P.* × *canescens*, however, the Cd^{2+} influx was the greatest in the apex region (0–0.9 mm from root tip) (Fig. 1B). These data indicate that the spatial patterns of the net Cd^{2+} flux along roots are probably different between monocots and dicotyledonous plants. However, the reason remains unclear for this spatial difference in net Cd^{2+} flux between monocots and dicotyledonous plants. In further experiments, it will be important to examine whether there is a difference in root anatomy or localization of Cd^{2+} uptake system between these plant groups. Furthermore, the net Cd^{2+} influx into roots of *P.* × *canescens* was more than 100 times higher than that observed in wheat exposed to the same Cd^{2+} concentration. This finding suggests that poplar roots still absorb large quantities of Cd^{2+} after Cd exposure for 24 h and underlines that the sink for Cd is much larger in *P.* × *canescens* than that in wheat. Continuous

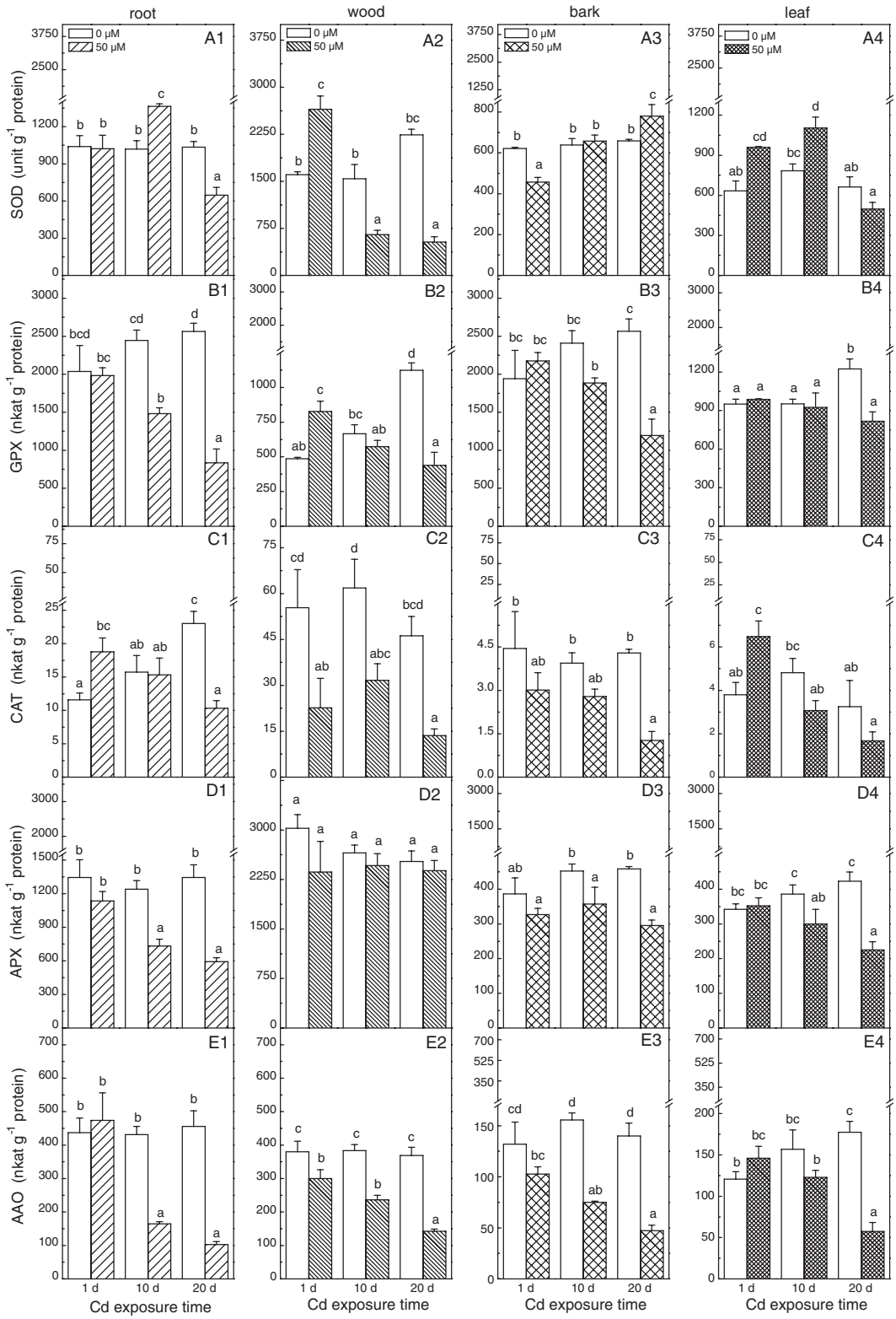

Fig. 6. Activities of SOD, GPX, CAT, APX and AAO in roots, wood, bark and leaves of *P.* × *canescens* exposed to 0 or 50 μ*M* $CdSO_4$ for 1, 10 or 20 day(s). Bars indicate means ± SE (n = 6). Different letters on the bars for the same tissue indicate significant difference between the treatments. The results of statistical analysis were shown in Appendix S7.

increases in Cd accumulation in leaves and roots after exposure for 20 days (Fig. 2) suggest that it may take a long time for *P.* × *canescens* to reach saturation of net Cd^{2+} influx. The biomass accumulation of all tissues of *P.* × *canescens* exposed to Cd with increases in exposure time (Appendix S1) suggests that poplars still grow under current experimental conditions. Overall, these data imply that *P.* × *canescens* has a great potential for Cd enrichment.

Cd is markedly accumulated in bark but does not reduce carbohydrate availability in *P.* × *canescens*

The strong net Cd^{2+} influx in roots of *P.* × *canescens* during Cd exposure is corroborated by marked Cd accumulation in different tissues of *P.* × *canescens* (Fig. 2). Most Cd ions that enter roots are probably bound to root cell walls (Clemens 2006, Douchiche et al. 2010) or sequestrated in vacuoles (Clemens 2006), while only a fraction is transported to the aerial tissues. The continuous increases in Cd accumulation in roots and leaves of *P.* × *canescens* after Cd exposure for 20 days suggest that there is still further potential for Cd enrichment in these tissues. Cd concentrations in the bark of *P.* × *canescens* after Cd exposure for 10 and 20 days are far above 100 mg kg^{-1} dry weight which is a widely accepted threshold for Cd hyperaccumulation (Milner and Kochian 2008), indicating that bark is a major sink for this element among aboveground tissues. It remains intriguing in which form and how Cd is enriched in bark. Cd is mainly uploaded in the free ionic form from roots via the xylem to the leaves (Ueno et al. 2008), but foliar Cd can be further transported in complex forms of Cd-phytochelatins and Cd-glutathione in the phloem of herbaceous plants (Mendoza-Cozatl et al. 2008). Thus, Cd in the bark of *P.* × *canescens* may exist in the forms of Cd-phytochelatins and Cd-glutathione complexes.

As in previous studies, Cd accumulation in leaves caused a reduction but not complete failure of photosynthesis (Fig. 3, Table 1). For example, poplars grown in Cd-treated soil (ca. 20 μ*M* Cd) for approximately 2 months showed a significant downregulation of photosynthesis that was ascribed to decreases in photosynthesis-related proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll a/b-binding proteins (Durand et al. 2010a, 2010b). In addition, Cd disturbed stomatal regulation by interference with Ca signalling (Perfus-Barbeoch et al. 2002). Therefore, the observed decreases in stomatal conductance and decreases in photosynthetic pigments likely contribute to the partial inhibition of carbon assimilation in *P.* × *canescens* (Table 1). Maintenance of transpiration and carbon fixation, though at reduced levels in Cd stressed *P.* × *canescens*, indicates the active functioning of xylem transport and phloem fuelling which are important processes for Cd uptake and distribution in aerial tissues. In contrast to decreased photosynthesis, an accumulation of carbohydrates, particularly of sucrose in roots, bark and leaves of *P.* × *canescens* exposed to Cd was found. This finding suggests that photosynthates are less demanded under Cd stress, although the growth of poplars was uninhibited (Appendix S1). Accumulation of carbohydrates in leaves has previously been reported for various plant species under Cd stress (Devi et al. 2007, Kieffer et al. 2009a). The accumulated carbohydrates in Cd-stressed plants may function as osmoprotectants and radical scavengers (Ayako et al. 2008).

Cd exposure results in a shift in ROS and antioxidants balance along the Cd transport route

Current data suggest that Cd is absorbed in roots, uploaded to leaves via the xylem and further transported into the bark through phloem loading in *P.* × *canescens*. Differential responses of ROS and antioxidants balance to Cd accumulation were observed along the Cd transport route (Figs 4–6).

Accumulation of O_2^- and H_2O_2 in roots and leaves of Cd-treated *P.* × *canescens* indicate that Cd exposure led to oxidative stress as in a number of previous studies (Garnier et al. 2006, Rodriguez-Serrano et al. 2006, 2009, Romero-Puertas et al. 2004, Schützendübel and Polle 2002, Schützendübel et al. 2001, 2002). Our data suggest that initially O_2^- production prevailed, whereas H_2O_2 accumulation mainly occurred in the tissues after extended Cd exposure and was probably a consequence of an increasing inhibition of H_2O_2 scavenging enzymes such as GPX, APX and CAT (Fig. 6). For example, wood showed the most severe inhibition of CAT activities and the strongest H_2O_2 accumulation. In contrast to wood, which accumulated comparatively little Cd, the highest Cd concentrations were detected in the bark of *P.* × *canescens* (Fig. 2), while the levels of O_2^- and H_2O_2 remained unchanged (Fig. 4). This points to a better protection of bark than that of wood from Cd toxicity, possibly because Cd may exist mainly in complex forms without ROS induction and/or the presence of a more effective ROS scavenging system than in wood.

Because the antioxidative enzymes were only transiently increased in the different poplar tissues, non-enzymatic defences might have been essential for ROS scavenging. Free proline and soluble phenolics are known non-enzymatic antioxidants (Sharma and Dietz 2006, 2009). Proline can react with $OH^{\bullet}$ forming a

stable radical (Sharma and Dietz 2006) and quench the singlet oxygen (Alia and Matysik 2001). However, proline accumulation in *P.* × *canescens* was modest, not supporting an important role in poplar for scavenging Cd-induced ROS. Similarly, our data do not support significant contributions of sugar alcohols to ROS scavenging because their overall concentrations did not increase in response to Cd stress in poplar. Furthermore, phenolics are potent Cd chelators and radical scavengers (Kovacik and Klejdus 2008, Kupper et al. 2004). Accumulation of soluble phenolics was recorded in plants under Cd stress (Gratao et al. 2005, Kovacik and Klejdus 2008, Uraguchi et al. 2006, Yaakoubi et al. 2010, Zornoza et al. 2010). Our data suggest that in *Populus* phenolics may be particularly important as ROS scavengers because their concentrations increased most strongly in the tissues with the strongest Cd accumulation, i.e. bark and roots. The constitutive concentrations of phenolics were high in leaves and increased to some extent in response to Cd, whereas in wood, which showed little Cd but strong ROS accumulation, no increases in free phenolics occurred. Recently, we used *Populus deltoides* with low phenolics and *P.* × *canescens* with relatively high phenolics contents to examine their Cd tolerance in a field experiment and the results indicate that high phenolics in *P.* × *canescens* play a pivotal role in scavenging ROS and Cd tolerance (He and Luo, unpublished data). Taken together, phenolics in *P.* × *canescens* may play a role in scavenging ROS under Cd exposure.

In conclusion, the Cd^{2+} influx in roots of *P.* × *canescens* exhibited spatially and temporally dynamic patterns. A large amount of Cd was accumulated in the roots of *P.* × *canescens.* Bark was the major sink for Cd in aerial tissues. Although Cd accumulation led to decreased photosynthesis, carbohydrates, which are required for the production of reductants to sustain antioxidative processes, were not depleted. Increased levels of ROS were present in all tissues, except the bark of Cd-exposed poplars, whereas the production of induced soluble phenolics was the highest in the bark and the lowest in wood under Cd exposure. Antioxidant enzyme activities displayed tissue and exposure time-specific response patterns to Cd exposure. These data indicate that phenolics in *P.* × *canescens* probably play a role in scavenging ROS under Cd exposure. It will be worthwhile to screen for poplar cultivars and species with high ability for production of phenolics and test these plants for their Cd tolerance under laboratory and field conditions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Biomass of *P.* × *canescens*.

Appendix S2. Statistical results of Cd concentrations in *P.* × *canescens*.

Appendix S3. The minor sugars and sugar alcohols of *P.* × *canescens*.

Appendix S4. Statistical results of soluble sugars and sugar alcohols in *P.* × *canescens*.

Appendix S5. Statistical results of $O_2^{\cdot-}$ and H_2O_2 in *P.* × *canescens*.

Appendix S6. Statistical results of proline and soluble phenolics in *P.* × *canescens*.

Appendix S7. Statistical results of enzyme activities in *P.* × *canescens*.

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