Tolerance, toxicity and transport of Cd and Zn in Populus trichocarpa

Original Article

Tolerance, toxicity and transport of Cd and Zn in *Populus trichocarpa*

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Abstract

Metal inputs to terrestrial ecosystems are of great concern due their toxicity to biota, especially for elements with no biological function such as cadmium. Fast-growing trees such as poplars may have potential in phytoremediation schemes. We assessed accumulation, metal partitioning, gene expression (*Pt-HMA4*) and overall tolerance to, and interaction between, cadmium (Cd) and zinc (Zn) in *Populus trichocarpa* ‘Trichobel’. We predicted that Zn would have an antagonistic effect in Cd accumulation and anticipated some level of tolerance to these metals. Poplars were grown in sandy substrate under different metal applications, ranging from 1 to 243 mg kg^{-1} Cd; or 30 to 7,290 mg kg^{-1} Zn; and also two combined treatments: 27 mg kg^{-1} Cd with 90 or 270 mg kg^{-1} Zn. Growth parameters and metal contents in shoots and roots were determined. Transcriptional levels of the *Pt-HMA4* gene were assessed in roots and leaves. *P. trichocarpa* showed a surprisingly high tolerance to Cd, with root biomass being affected only at the highest doses applied. Metals accumulated mainly in roots (up to 6,537 mg kg^{-1} Cd and 21,500 mg kg^{-1} Zn), root-to-shoot translocation peaked at the 9 mg kg^{-1} dose for Cd (41%) and 90 mg kg^{-1} for Zn (40%). At high Cd/Zn applications, expression of *Pt-HMA4* in roots decreased significantly. Contrary to the initial presumption, Zn addition increased Cd uptake, reaching hyperaccumulator-like concentrations in shoots ($\geq$ 100 mg kg^{-1} Cd). Differential root-to-shoot partitioning has a major role in Cd tolerance in *P. trichocarpa*; partly by down-regulating the *Pt-HMA4* gene in roots. Zn addition promoted high Cd uptake without any detriment to plant growth. *P. trichocarpa* was tolerant to extreme Cd concentrations, offering a great potential to be used in phytoremediation techniques for stabilization/extraction of Cd from soils contaminated by both Cd and Zn.

Key-words: gene expression, heavy metal, heavy metal transporter, metal partitioning, phytoremediation, phytotoxicity, poplar.

1. Introduction

Cadmium (Cd) is one of the most hazardous metals in the environment, ranked seventh in toxicity by the Agency for Toxic Substance and Disease Registry (ATSDR, 2017), it lacks any known biological function, being toxic to humans and other organisms at relatively low concentrations (Alloway 2013) and has a high mobility in soils (Lei *et al*., 2010). Cd is frequently found in zinc (Zn) bearing minerals (Alloway 2013) and, due to their similar geochemical characteristics Zn is often associated with Cd in soils (Kabata-Pendias and Pendias 2001), although not as toxic, high concentrations of Zn can be extremely harmful to biota. Plant exposure to Cd often leads to phytotoxicity depending on the concentration, plant genotype, soil characteristics and exposure time (Das *et al*., 1997; Benavides *et al*., 2005) mainly due to the fact that Cd has a chemical similarity to other essential elements, such as Ca, Fe and particularly Zn (Clemens, 2006; Verbruggen *et al*., 2009). Growth impairment, biomass decrease, foliar necrosis and chlorosis are typical effects from Cd toxicity in plants (He *et al*., 2017; Tran and Popova, 2013; Pál *et al*., 2006). Similar to Cd, Zn toxicity effects in plants include growth inhibition, leaf chlorosis and necrosis, oxidative stress, inhibition of protein functions and impairment of photosynthesis (Todeschini *et al*., 2011; Hasan *et al*., 2017). Cd^{2+} and Zn^{2+} are long known for being competing ions in the soil matrix due to their chemical similarities and same uptake pathways in plants (Clemens, 2006; Kirkham, 2006) in which Zn is often responsible for decreasing Cd uptake and even considered as a soil amendment to reduce Cd concentration in edible crops (Green *et al*., 2003; Garg and Kaur, 2013). However, it has been reported recently that Zn not always impact Cd accumulation (Green *et al*., 2017).

Phytoremediation is the use of plants and their associated microorganisms for environmental decontamination (Pilon-Smits, 2005), from which phytoextraction is considered to be useful for inorganic contaminants (Marmiroli *et al*., 2006). It is an *in situ* technique that preserves soil structure and microbial activity, offering protection against erosion (Pulford and

Watson, 2003; Guerra *et al.*, 2011). Poplars (*Populus* sp.) are trees widely considered for phytoextraction of several metals, such as Cd, Zn, Pb and Cu (Castiglione *et al*., 2009; Zacchini *et al*., 2009; Guerra *et al*., 2011; Dai *et al*., 2013; Luo *et al.*, 2016), mainly due to their biomass production, deep root systems (Bhargava *et al*., 2012), tolerance to high metal concentrations and fast growth (Robinson *et al*., 2009). *Populus* species can also rapidly invade disturbed sites, reproduce asexually (Sebastiani *et al*., 2004; Hamberg *et al*., 2011) and are not a source of food for farm animals, reducing the risk of heavy metals entering the human food chain (Shim *et al*., 2013).

Metal tolerance and partitioning in plants are important features to be considered in phyoextraction (Luo *et al*., 2016), in which root-to-shoot translocation of Cd is regarded as a major factor in determining its toxicity thresholds in poplar (Durand *et al*., 2011). Several transmembrane proteins are involved in cation efflux from the cytoplasm, from which HMA4 (Heavy Metal ATPase 4), a common metal transporter from the P-type ATPase family, is known to play a role in the xylem-loading of metals (Hanikenne *et al*., 2008; Luo *et al.*, 2016), affecting transport and accumulation in poplar (Adams *et al*., 2011). The *HMA4* gene is considered to be key in Zn and Cd hyperaccumulation and also tolerance, which was previously verified in *Arabidopsis thaliana* (Mills *et al*., 2005), *Noccaea caerulescens* (Lochlainn *et al*., 2011) and transgenic *Nicotiana tabacum* plants (Grispen *et al*., 2011).

Populus trichocarpa (black cottonwood) is considered a model tree species (Bradshaw *et al*., 2000), with its genome already fully sequenced (Tuskan *et al*., 2006). However, little is known about heavy metal accumulation, toxicity and translocation in *P. trichocarpa*, most studies being mainly focused on other species from the *Populus* genus. The objectives of this work were to investigate (1) the effects of different concentrations of Cd and Zn on *P. trichocarpa*, (2) the accumulation and distribution of Cd and Zn within the plant and their effects on the expression of the metal transporter *Pt-HMA4*, and (3) the interactive effects between Cd and Zn in terms of phytotoxicity and metal distribution. We predicted that Zn could prevent Cd uptake, consequently alleviating toxicity effects and that tolerance is associated with different metal translocation patterns, influenced by the expression of *Pt-HMA4*.

2. Materials and Methods

2.1 *Plant material and pre-growth*

Cuttings (15 cm, two nodes) of *Populus trichocarpa* 'Trichobel' clones were rooted in sand for four weeks, and fertilised three times with 10 mL of a modified Long Ashton's solution (macronutrients: $(NH_4)_2SO_4$ (4 mM), K_2SO_4 (2 mM), $CaCl_2{\cdot}2H_2O$ (3 mM), $MgSO_4 \cdot 7H_2O$ (1.5 mM), $NaNO_3$(8 mM), FeEDTA (0.1 mM); micronutrients: H_3BO_3 (2.86 mg l^{-1}), $MnCl_2{\cdot}4H_2O$ (1.81 mg l^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg l^{-1}), $NaMoO_4{\cdot}2H_2O$ (0.025 mg l^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg l^{-1})), according to Kariman *et al*., (2014) and 1 mL of a solution with KH_2PO_4 (1 mM). This clone is an intraspecific hybrid of *Populus trichocarpa* Torrey & A. Gray ex Hook (Burgess *et al*., 2005).

All rooted cuttings were transplanted to plastic pots (without holes in the bottom) filled with 1 kg of substrate: 50 g vermiculite, 50 g peat moss and 900 g of sand (pH 6.9); one cutting per pot. Water holding capacity was maintained at 70% (300 mL of distilled water). The experiment was carried out in the glasshouse of the University of Reading, between December 2015 and February 2016. The temperature average recorded in the glasshouse during this period was 24.5^{o}C (± 2.4), and artificial light was provided (18h/day). Poplar cuttings were obtained from AF Hill & Son, Redditch, UK and were kept refrigerated at 4^{o}C until the experiment.

2.2 *Treatments and Experimental Design*

The experiment was designed in randomized blocks, cuttings with similar sizes were assigned to one of the flour blocks. After one week, the final fertilisation was applied and all cuttings had their expanded leaves counted and stems measured from the node sprouting to the apex; a sample from the substrate was also taken for further analysis. All pots were spiked with either Cd or Zn solutions on the following day. Cd was added via $CdCl_2$ stock solutions to make up six different concentrations in the pot substrate: 1, 3, 9, 27, 81 and 243 mg kg^{-1} Cd; Zn was added via $ZnSO_4$ stock solutions, making up six different concentrations in the substrate: 30, 90, 270, 810, 2430 and 7290 mg kg^{-1} Zn. Two further treatments included both Cd and Zn: 27 mg kg^{-1} Cd + 90 mg kg^{-1} Zn (Cd_{27} + Zn_{90}); and 27 mg kg^{-1} Cd + 270 mg kg^{-1} Zn (Cd_{27} + Zn_{270}). Control had water only instead of the metal solutions, and all pots contained only one poplar cutting. Metals were added in a single dose and each treatment had four replicates arranged in blocks.

Two weeks before harvest, all plants had leaves analysed for stomatal conductance (gs, in mol m^{-2} s^{-1}) and transpiration rate (mmol m^{-2} s^{-1}) using a portable infrared gas analyser (LC*i* Portable Photosynthesis System). Plants were assessed in the glasshouse near solar noon, under constant lighting. The two youngest expanded leaves of each plant were measured, except for the two highest Zn treatments (2430 and 7290 mg kg^{-1}), which had too many dead leaves for analysis.

2.3 *Harvest and Phytotoxicity assessment*

After exposure to the toxic metals for five weeks, all plants had their living expanded leaves counted and stems measured (before and after exposure to metals). Visual toxicity symptoms recorded using the method described by Kariman *et al*., (2016), in which leaf areas with symptoms such as discoloration, chlorosis or necrosis were ranked into 6 classes (0 to 5), in which 0 represents no toxicity symptoms, 1 is up to 20% of symptomatic leaf tissue area

(SLTA), 2 from 20 to 40%, 3 from 40 to 60%, 4 from 60 to 80% and 5 for symptomatic area greater than 80%. Two mature leaves were assessed for each plant, and the final scoring was the average between those leaves.

Plants were then harvested and separated into roots, stems and leaves (initial cuttings were not included in any analyses). Roots were washed thoroughly with tap water and immersed in a 0.05 mM $CaCl_2$ solution for 30 minutes to remove any surface adhering metals (Marmiroli *et al*., 2013), roots were rinsed with deionized water and scanned using the software WinRhizo®, to determine the root length, diameter, root tips, surface area and volume. All plant parts were dried separately in an oven at 70^{o}C for seven days, then dry weight (DW) was determined. Soil was air dried, sieved (2 mm) and soil pH was determined in a water-soil suspension (2.5:1) shook for 15 min at 120 rpm (Rowell, 1994).

2.4 *Acid Digestion and Metal Determination*

Dried samples were ground and 50 mg of plant material was digested for 8 hours in 5 mL of 70% HNO_3 (≥69% TraceSELECT® for trace analysis) in closed glass vessels in heating blocks at 110^{o}C (Huang *et al*., 2004). All digestions were performed in duplicates, and for quality control, a blank and a plant certified reference material (IAEA-359 cabbage leaves) were included. Digested extracts were then diluted in a solution of 2% HNO_3 + 5 ppb Rh, and filtered. The concentrations of Cd and Zn were determined by inductively coupled plasma mass spectrometry (Thermo Scientific™ iCAP™ Q ICP-MS), using rhodium as an internal standard.

2.5 *Bioconcentration Factor, Translocation Factor and Tolerance Index*

The bioconcentration factor (BCF), the translocation factor (*Tf*), and tolerance index (TI) are used as indices to assess the plant's capacity to accumulate, translocate (from roots to shoots) and tolerate heavy metals (Rafati *et al*., 2011). BCF is the ratio between the metal concentrations within the plant tissue and in the soil or substrate; *Tf* is the ratio between the metal concentrations in leaves and roots; and TI is the ratio between a parameter assess in heavy metal treated plants and the control (Saraswat and Rai, 2009; Zacchini *et al*., 2009; Rafati *et al*., 2011); see equations below, in which [M]: metal concentration; T: treated plants; C: control plants.

$$BCF = \frac{[M]plant}{[M]soil} \qquad (1)$$

$$Tf = \frac{[M]leaf}{[M]root} \times 100 \quad (2)$$

$$TI\ \% = \frac{T}{C} \times 100 \qquad (3)$$

2.6 *Pt-HMA4 expression in roots and leaves*

Poplar cuttings (15 cm) were grown inside a growth chamber (23^{o}C 16h/8h day/night) in a mixture of TerraGreen clay and sand (1:5, w/w), one cutting per pot (photosynthetic photon flux, 100 µmol m^{-2} s^{-1}). All plants were fertilised weekly for the first three weeks with 10 mL of a modified Long Ashton's solution, as described previously. Water holding capacity was always maintained at 70% with distilled water. After five weeks, pots were spiked daily with either 27 mg kg $^{-1}$ Cd (via $CdCl_2$ solution) or 100 mg kg^{-1} Zn (via $ZnSO_4$) for three days amounting to total doses of 81 mg kg^{-1} Cd for the Cd treatment and 300 mg kg^{-1} Zn for the Zn treatment; Controls received deionized water instead of Cd or Zn solutions. All treatments had three replicates.

Plants were harvested eight weeks after contamination. The 9th leaf of each plant (counting from the base of the stem) was sampled and immediately frozen in liquid nitrogen for RNA extraction. Roots were washed with tap water and random sections (2 cm from root tips) were sampled and frozen.

Total RNA was extracted from approximately 100 g of fresh weight material (leaves or roots) macerated in liquid nitrogen via TissueLyser II (Qiagen®). Extraction was performed by the CTAB method (Jaakola *et al*., 2001) and RNA pellets were purified with the RNeasy Plant Mini kit (Qiagen, UK), including a DNAse treatment (Qiagen, UK) for 20 min. cDNA synthesis was carried out using the SensiFAST cDNA synthesis kit (BIOLINE, UK) following the manufacturer's instructions.

Specific primers were designed for *Pt-HMA4*, accession: XM_006381101, (F: 5' ACCAACGTTCTTATGCTTATTGC 3' / R: 5' CACTGGCCTTGTGGCTT 3') and Ubiquitin (*UBQ*), accession: XM_006373777 (F: 5' AGATGGCAGAACTTTGGCTGA 3' / R: 5' CGCCAAAGCCATCAAAGAAC 3') with the Primer-BLAST tool (Ye *et al*., 2012). Nucleotide BLAST showed 71% between *Pt-HMA4* and *Arabidopsis thaliana* ATPase, *At-HMA4* (accession: NM_127468).

The qPCR reactions were performed in duplicates and at least twice for each sample using PowerUp™ SYBRGreen™ (Applied Biosystems, UK) with the following the parameters: 1 cycle of 2 min at 50°C followed by 2 min at 95°C (DNA polymerase activation), then 40 cycles of 95°C for 3 seconds (denaturation) and 60°C for 30 seconds (annealing/extension). The qPCR run, data collection and analyses were performed using StepOne™ Real-Time PCR System (Applied Biosystems). Results were analysed by the standard curve method, and gene expression was normalised using *UBQ* as the house keeping gene.

2.7 *Statistical Analyses*

Statistical analyses were performed for all parameters assessed using R software. Metal treatments were considered as categorical factors and therefore ANOVA was performed for each parameter assessed ($p < 0.05$). When significant differences were detected, a Tukey test ($p < 0.05$) was carried out to discriminate differences between treatments. Pearson correlation was also performed. Data was transformed when necessary (determined by Shapiro-Wilk normality test and Levene's test, $p < 0.05$) to attain normal distribution and homoscedasticity, in order to meet ANOVA and Pearson correlation assumptions (Zar, 2010). Transformation was carried out mainly by two equations: log(x) or x^2; root dry weight data from Zn treatments were transformed by $\sqrt[3]{x}$ after a BoxCox plot. Data that could not be transformed to attain normality (i.e. a few root morphology parameters), Kruskal-Wallis followed by a Dunn's test ($p < 0.05$) were performed. A non-parametric correlation test (Spearman) was done for 14 different variables to verify possible monotonic relationships and only significant r_s values ($p < 0.05$) were reported.

3. Results

3.1 *Growth, biomass production and transpiration rate*

Both Cd and Zn caused toxicity in *P. trichocarpa* plants after only five weeks of exposure, and the visual effects are evident in shoots and roots (Fig. 1 and 2), especially in Zn treatments.

P. trichocarpa exhibited a considerable tolerance to Cd toxicity, and negative effects were significantly different from control only at the extreme concentration of 243 mg kg^{-1}, except for leaf biomass, which was also affected at 81 mg kg^{-1} Cd. Nonetheless, the total biomass produced (leaves + stems + roots) was similar in all Cd treatments except for the highest dose of 243 mg kg^{-1} Cd (Table 1). Zn toxic effects were detected at the lowest dose applied, of 30 mg kg^{-1}, which reduced leaf and shoot biomass (Table 1), although root biomass was unaffected in this treatment. Zn concentrations from 30 to 270 mg kg^{-1} caused comparable toxicity in *P. trichocarpa*, as seen in the total plant biomass produced, but further toxicity was observed at higher concentrations.

Fig. 1 - Phytotoxic effects of Cd and Zn in *Populus trichocarpa* at different soil concentrations, after five weeks of exposure.

Fig. 2 - Root scans of *Populus trichocarpa* exposed to different Cd and Zn concentrations during five weeks. Images were used for length, area, volume and diameter analyses.

Table 1. Dry biomass production, resulting pH and translocation index (TI) of *P. trichocarpa* exposed to different Cd or Zn concentrations during five weeks.

Metal (mg kg^{-1})	Dry biomass (g)			Final pH	TI (%)	
	Leaves	Stems	Roots		Leaves	Roots
Cadmium						
Control	1.9 ± 0.1 a	0.9 ± 0.1 a	0.4 ± 0.1 a	6.3 a	100	100
1	1.9 ± 0.1 a	0.9 ± 0.1 a	0.4 ± 0.1 a	6.2 ab	107	102
3	1.7 ± 0.1 ab	0.7 ± 0.1 a	0.4 ± 0.1 a	6.2 ab	96	93
9	1.5 ± 0.1 ab	0.6 ± 0.1 a	0.3 ± 0.0 a	6.2 ab	78	79
27	1.7 ± 0.1 ab	0.8 ± 0.1 a	0.4 ± 0.0 a	6.1 ab	94	92
81	1.4 ± 0.1 b	0.6 ± 0.1 a	0.3 ± 0.0 a	6.2 ab	75	74
243	0.5 ± 0.1 c	0.2 ± 0.0 b	0.1 ± 0.0 b	6.0 b	9	28
Zinc						
Control	2.0 ± 0.0 a	0.9 ± 0.0 a	0.5 ± 0.0 a	6.3 a	100	100
30	1.6 ± 0.1 b	0.7 ± 0.1 b	0.4 ± 0.1 a	6.3 a	83	80
90	1.5 ± 0.0 b	0.6 ± 0.0 b	0.4 ± 0.0 a	6.3 a	86	76
270	1.4 ± 0.1 b	0.6 ± 0.0 b	0.3 ± 0.0 a	6.0 b	62	68
810	0.9 ± 0.1 c	0.3 ± 0.0 b	0.1 ± 0.0 b	5.4 c	22	47
2430	0.9 ± 0.1 c	0.2 ± 0.0 b	0.1 ± 0.0 b	5.1 d	11	47
7290	0.9 ± 0.1 c	0.2 ± 0.0 b	0.1 ± 0.0 b	4.8 d	12	46

Values are the mean ± SE (Cd treatments and pH, n = 4; Zn treatments; n = 3)

Significant differences among treatments (for each metal) are represented by different letters.

Initial pH: 6.9;

Cd treatments and pH values: Tukey test: $p < 0.05$;

Zn treatments: Dunn test, $p < 0.05$.

Standard errors for the Final pH were ≤ 0.1 for all treatments.

Foliar symptoms of phytotoxicity were more evident in Cd treatments than in Zn treatments, when compared to control at lower concentrations, 30 to 270 mg kg^{-1} Zn (Fig. 3). All treatments displayed marginal necrosis in the leaves assessed (older leaves), including the control, but chlorosis and discoloration were present only in Cd-treated plants. Although necrosis and chlorosis were both considered for the toxicity scoring, chlorosis were predominantly in Cd treatments. At the highest Zn concentrations (2430 and 7290 mg kg^{-1}) all leaves were scored as a 5, due to extensive foliar necrosis (Fig. 2).

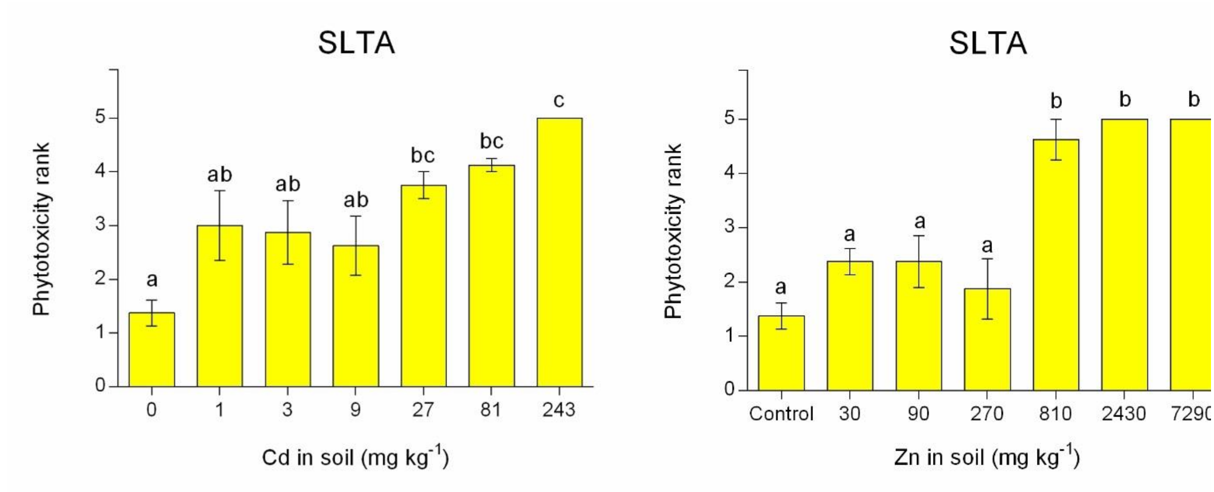

Fig. 3 - Toxicity ranks of *P. trichocarpa* exposed to different Cd and Zn concentrations. Symptomatic leaf tissue area (SLTA) was assessed visually and scored from 0 to 5 (each score represent 20% of leaf area). Significant differences are represented by different letters by Tukey test ($p < 0.05$) in Cd treatments and Dunn's test ($p < 0.05$) in Zn treatments.

Root scanning allowed the determination of root total length, area, volume and diameter for *P. trichocarpa* grown in different Cd and Zn concentrations. Results for root morphology parameters, leaf transpiration and stomatal conductance can be found in Table S1. Roots under Cd treatments displayed a similar response as the other parameters assessed, with evident toxicity effects only at the highest concentration of 243 mg kg^{-1}. In the case of Zn, length, area and volume reduction of roots was caused mainly at 810 mg kg^{-1} or higher concentrations. As for the analyses of stomatal conductance (gs) and transpiration rate (E), there were no significant differences among Zn treatments (Control – 810 mg kg^{-1}) or among Cd treatments, except for the highest concentration of 243 mg kg^{-1}, in which there was a reduction in the transpiration rate (E) in comparison to the control, from 2.65 to 0.48 mmol m^{-2} s^{-1}, and in stomatal conductance (gs), from 0.084 to 0.008 mol m^{-2} s^{-1} (Tukey test, p = 0.0009 and p = 0.0004, respectively).

3.2 *Cadmium and zinc uptake, accumulation and translocation*

Cd uptake in poplar roots increased almost exponentially and was at least 10 times the concentration applied in some treatments (1 to 9 mg kg^{-1} Cd) (Fig. 4). In leaves, an increasing uptake is observed only until the concentration of 9 mg kg^{-1} Cd, after which there is a plateau and Cd concentration is maintained around 50 mg kg^{-1} (Fig. 4). However, in the treatment with 243 mg kg^{-1}, Cd accumulation surpasses the plateau concentration in more than 10 times (from an average of 45 to 681 mg kg^{-1}). The bioconcentration factor (BCF) shows a decrease in Cd accumulation for both roots and leaves as concentrations in soil increases (Table 2), except at the highest concentration which had a BCF of 47.6 in poplar roots (tissue concentration of 6,537 mg kg^{-1} Cd), suggesting a loss of regulation in Cd uptake and excessive metal accumulation (Fig. 4). Overall the concentration of 9 mg kg^{-1} Cd appears to be the threshold in Cd translocation from roots to shoots (T*f* = 41%, the highest in this study), after which the ratio between root and leaf concentration was reduced almost by half (T*f* = 26% at 27 mg kg^{-1} Cd). At the applied dose of 81 mg kg^{-1} Cd, root biomass was not affected despite tissue concentrations reaching nearly 500 mg kg^{-1} Cd (Table 1 and Fig. 4). Cadmium concentration, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) can be found in Table S4.

Unlike with Cd, Zn content in roots did not differ significantly at lower soil concentrations ($\leq$ 90 mg kg^{-1}), increasing only after 270 mg kg^{-1} Zn (Fig. 4). Zn content in leaves was a direct result of the concentration applied, although only a slight increase was observed between treatments of 810 and 2430 mg kg^{-1} Zn.

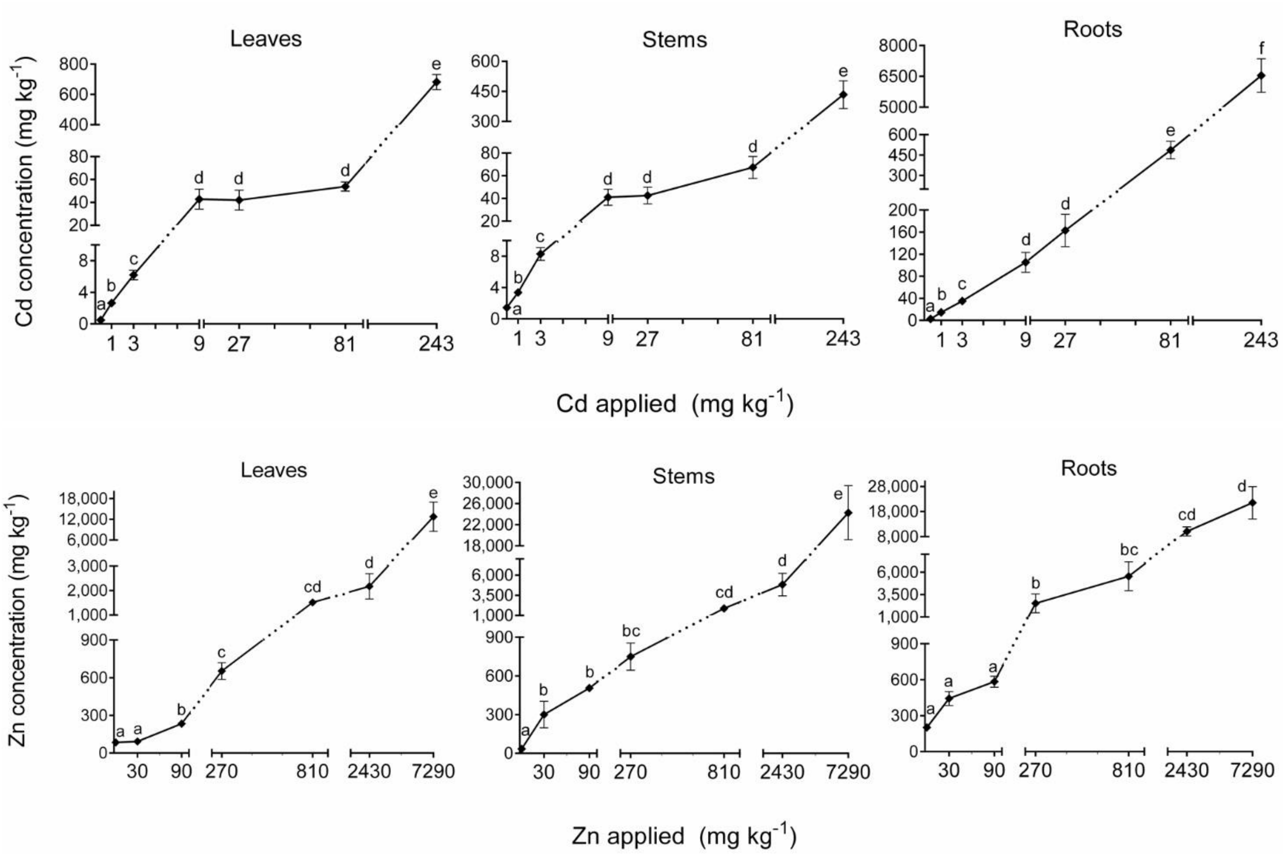

Fig. 4 - Cd and Zn concentrations (mg kg^{-1}) in leaves, stems and roots of *Populus trichocarpa* grown for five weeks in sandy substrate at different Cd or Zn doses. Error bars indicate standard error of the mean (n = 4). Different letters correspond to significant differences between doses applied (Cd: Tukey test, $p < 0.05$; Zn: Dunn's test, $p < 0.05$). To better visualise the complete data, x axis was set in log scale and breaks were added to both axes. Dotted lines between plotted data indicate the position of axis breaks. All values are presented in Tables S4 and S5.

Zn accumulation in roots varied across all treatments, and the highest BCF was found at 30 mg kg^{-1}, and lowest at 7290 mg kg^{-1} (Table 2), however at the latter, translocation of Zn from roots to leaves was the highest found in this study (Tf = 59%). Considering the tolerance indexes, 90 mg kg^{-1} Zn was the threshold for toxicity in both poplar roots and shoots (Table 1). Interestingly, this treatment showed a translocation factor of 40%, nearly the same factor found at the Cd threshold concentration of 9 mg kg^{-1}. Zinc concentration, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) can be found in Table S5.

Cd concentration in leaves and roots had an inverse relationship with all other variables.

Stomatal conductance (gs) and transpiration rates (E) had a lower correlation to almost all other parameters assessed (especially root parameters), however both variables were highly correlated ($r_s > 0.70$) to the number of leaves (NL) and shoot growth (SG) (Table S2). Overall Zn treatments had a similar correlation among all the parameters assessed to Cd treatments with almost no correlations between E and gs and other variables (Table S3).

Table 2. Total metal uptake, translocation factor (*Tf*: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* 'Trichobel' grown for five weeks under different Cd and Zn doses.

Cd (mg kg^{-1})	Cd uptake (µg plant^{-1})	T*f*	BCF	
			Leaf	Root
Control	3.2 ± 0.3	20	---	---
1	14.8 ± 3.1	18	2.6	14.4
3	31.0 ± 5.2	18	2.1	11.7
9	119 ± 11	41	4.8	11.7
27	167 ± 22	26	1.6	6.0
81	267 ± 52	11	0.7	6.0
243	629 ± 157	6	2.8	47.6

Zn (mg kg^{-1})	Zn uptake (mg plant^{-1})	T*f*	BCF	
			Leaf	Root
Control	0.3 ± 0.01	33	---	---
30	0.5 ± 0.1	21	3.1	14.8
90	0.9 ± 0.1	40	2.6	6.5
270	2.0 ± 0.2	26	2.4	9.3
810	2.5 ± 0.5	27	1.9	6.9
2,430	3.5 ± 1.1	22	0.9	4.2
7,290	17.9 ± 2.2	59	1.7	2.9

Values are the mean ± SE (Cd treatments, n = 4; Zn treatments; n = 3)
Tf = (leaf concentration / root concentration) × 100.
BCF = plant tissue concentration / dosage added.

3.3 *Expression of Pt-HMA4 under Cd and Zn stress*

Efficient amplifications of *Pt-HMA4* (POPTR_0006s07650g) were obtained from the designed primers (product length: 130 bp). In the control, *Pt-HMA4* expression was five times higher in roots than in leaves (t-test, p = 0.043), but this variation between tissues were not observed in contaminated treatments. Exposure to either Cd or Zn down-regulated *Pt-HMA4* expression in roots by 2.9-fold and 2.6-fold respectively (Fig. 5). No differences in transcript levels were found in leaves. Ubiquitin (*UBQ*) was used for normalisation of HMA4 results due to their homogeneous expression across treatments (Control, Cd and Zn): ANOVA, $p = 0.768$ (leaves) and $p = 0.781$ (roots).

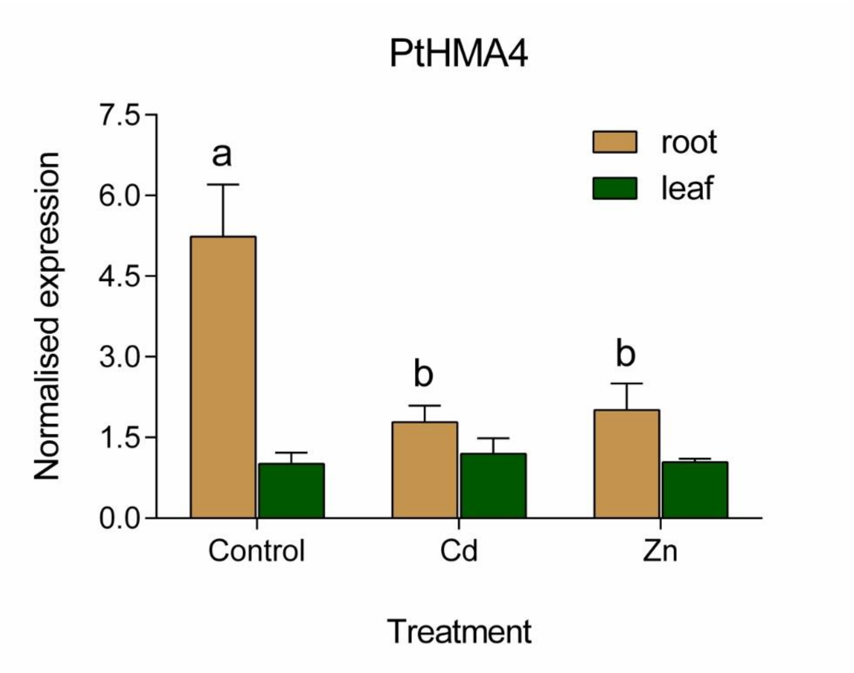

Fig. 5 - Transcript levels of the PtHMA4 gene in roots and leaves of *P. trichocarpa* after growing for eight weeks under Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) stress, and without any metal addition (Control). The mRNA levels were quantified by real-time qPCR and normalised in relation to Ubiquitin (UBQ) expression; which had similar expression across treatments: ANOVA, $p = 0.768$ (leaves) and $p = 0.781$ (roots);. Different letters represent significant differences among treatments, determined by Tukey test after ANOVA ($p = 0.0167$). There were no differences among treatments in leaf tissues.

3.4 *Cd and Zn interactions and uptake*

Biomass production in treatments with combined metal applications did not significantly change from the control nor their corresponded single metal treatments: 27 mg kg^{-1} Cd; or 90 and 270 mg kg^{-1} Zn. For instance, the Tolerance Index (TI) for total biomass was 100% for 27 + 90 mg kg^{-1} Cd Zn, and 83% for 27 + 270 mg kg^{-1} Cd Zn; percentages are related to the non-contaminated control. The same results were observed for root morphology, leaf transpiration and stomatal conductance (data not shown). Despite exhibiting the same tolerance patterns, Zn addition increased Cd uptake, for instance, leaf concentration was of 123 mg kg^{-1} in Cd_{27} + Zn_{90}, almost three times higher than the concentration found when Cd was added singly (Cd_{27}), of 42 mg kg^{-1} (Fig. 6). Stems and roots also presented higher Cd contents after Zn addition, regardless of Zn concentration. Zn uptake was not affected in the presence of Cd: leaf, stem and root concentrations were not different from when Zn was added separately (Zn_{90} and Zn_{270}) (Fig. 6).

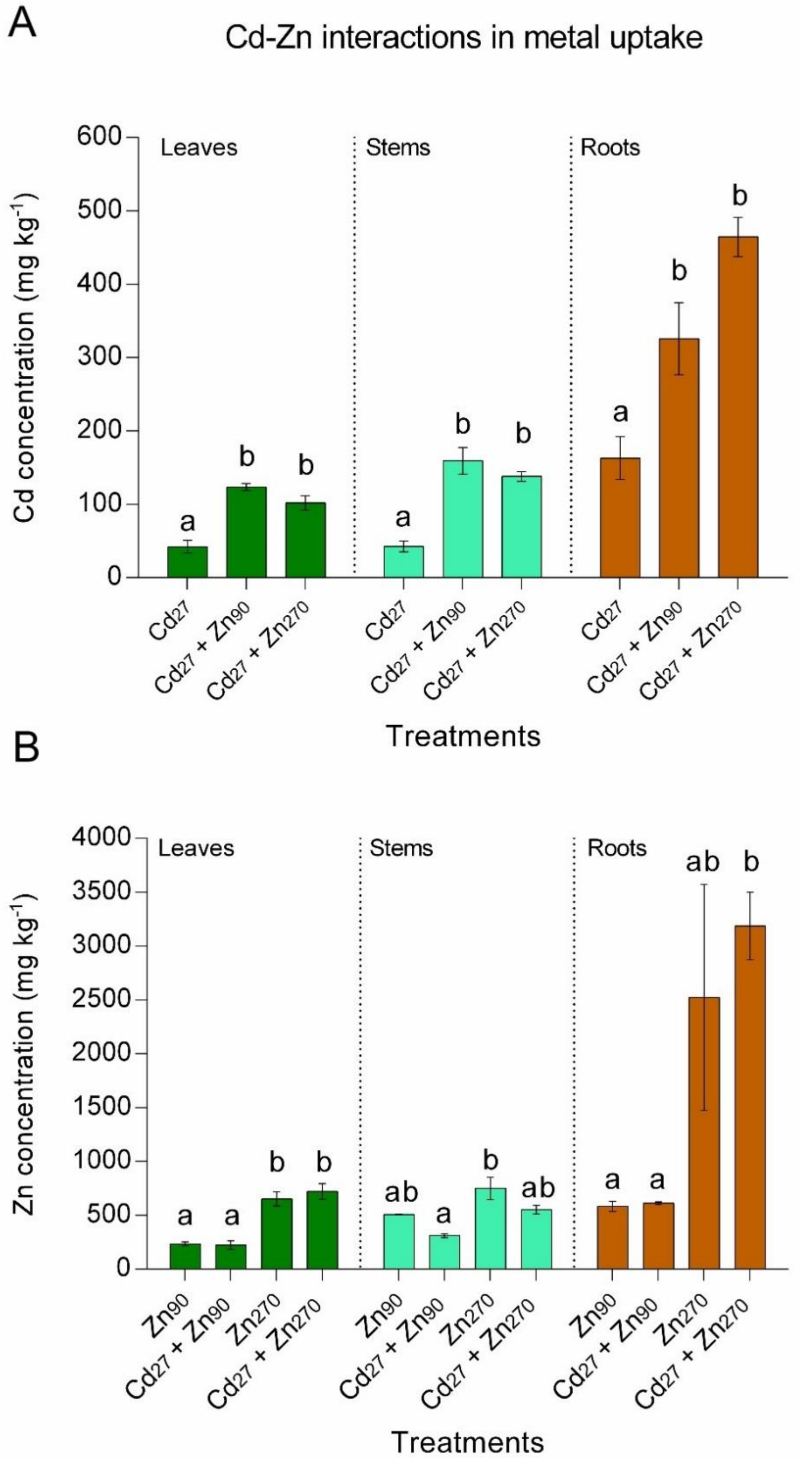

Fig. 6 - Concentrations of Cd (A) and Zn (B) in leaves, stems and roots of *Populus trichocarpa* exposed to different metal combinations: 27 mg kg^{-1} Cd, 90 or 270 mg kg^{-1} Zn. Different letters correspond to significant differences among treatments for the same plant tissue, Tukey test, $p < 0.05$ (A) and Dunn's test, $p < 0.05$ (B).

4. Discussion

4.1 *Cadmium accumulation, distribution and toxicity*

Exposure to Cd often leads to oxidative stress and phytotoxicity (Benavides *et al*., 2005) as a result of Cd replacing other essential elements (e.g. Ca, Fe, Mg and Zn) in enzymes, which usually lose their function (Clemens, 2006; Verbruggen *et al*., 2009; Kupper and Andresen, 2016). Growth impairment is a typical effect from Cd toxicity (Pal *et al*., 2006), biomass decrease in roots and shoots are commonly reported (Tran and Popova, 2013), as well as foliar chlorosis and necrosis (Das *et al*., 1997). In the current experiment, despite *P. trichocarpa* showing symptoms of toxicity in leaves under Cd exposure, particularly at soil concentrations higher than 27 mg kg^{-1} Cd, loss of biomass was not evident in most of the treatments. Only at the highest concentration did all roots, stems and leaves present obvious toxic effects, indicating a remarkable tolerance to Cd in comparison to other published studies (Table 3). According to Audet and Charest (2008), plants from the Brassicaceae family, known for their high tolerance to metals, tend to maintain a constant biomass allocation to roots despite exposure to higher metal concentrations in soils, similar to that observed for poplars exposed to Cd in the present study, suggesting that in both cases metal partitioning plays a larger role in tolerance than does biomass plasticity.

Tolerance index (TI) is a good measure to compare different studies regarding metal toxicity. In this work, the tolerance index ranged from 107 to 75% in leaves across all Cd treatments, excluding the highest Cd concentration, which displayed a conspicuous toxicity. These values are within the bounds reported for poplars exposed to Cd concentrations lower than 30 mg kg^{-1}: TI of 90 to 78% in *P x canescens* (Dai *et al.*, 2013) and 91% in *P. nigra* (Gaudet *et al*., 2011). The most important mechanism for Cd tolerance in plants is the metal chelation and compartmentalization into the vacuoles (Sharma *et al*., 2016), especially via the phytochelatin (PC) pathway (Clemens, 2006). Expression of genes encoding metallothioneins (metal chelation) and heat shock proteins (proper protein folding) due to Cd exposure were also associated with stress tolerance mechanisms in poplars (Hassinen *et al*., 2009; Hasan *et al.*, 2017).

Cadmium accumulated mainly in the roots, as it is reported in most studies on poplars (Dos Santos Utmazian *et al*., 2007; Zacchini *et al*., 2009; Di Lonardo *et al*., 2011) or other plant species (Obata and Umebayashi, 1997; Green and Tibbett, 2008; Lux *et al*., 2011); while stems and leaves had generally the same concentrations. Despite much higher Cd accumulation, the roots of *P. trichocarpa* were as tolerant as its aboveground parts for most treatments (TI of 102-74%).

Cd concentration generally increases in leaves as a result of increasing soil or nutrient solution concentrations (Di Lonardo *et al.*, 2011; Dai *et al.*, 2013; Jun and Ling, 2012). Interestingly, Cd contents in both leaves and stems did not significantly change among the treatments of 9, 27 and 81 mg kg^{-1} Cd, despite a significant increase in root concentration in the latter, exhibiting a plateau pattern in shoot accumulation. A similar pattern has been previously observed in *P. leucoides* (Jun and Ling 2012) and other plant species**,** such as barley (Green *et al*., 2006) and radish (Hamon *et al*., 1999), however this is generally uncommon in *Populus* species. This plateau concentration in shoots may be the main mechanism behind the tolerance observed even at high Cd doses. Root-to-leaf translocation decreased drastically from the treatments of 9 to 81 mg kg^{-1} Cd, which suggests two different strategies for this plant to cope with metal toxicity depending of the substrate concentration: one associated with hyperaccumulating plants (high translocation) and the other with woody plants (low translocation) at low and high Cd doses, respectively.

Table 3. Reports of Cd and Zn toxicity in poplar trees. For comparison, all units for the metal concentrations were converted to mg kg^{-1} for soils or other solid substrates, or mg L^{-1}, in the case of experiments using nutrient solution ('nutr. sol.') in hydroponic systems. The 'Phytotoxicity' column corresponds to the plant parameters most affected by metal toxicity. Lowest adverse observed effect concentration (LOAEC) shows the lowest Cd or Zn concentration to significantly cause toxicity (in some cases data was extracted from figures). The letter 'x' corresponds to cases in which no toxicity was detected.

Populus species	Growth substrate	Metal concentration	Phytotoxicity	LOAEC	Reference
P. alba	soil	3.53 Cd	x	x	1
	nutr. sol.	0 – 130 Zn; 0 – 30 Cd	root biomass	65 Zn	2
	soil	950 Zn + 1,300 Cu	overall biomass	950 Zn; 1,300 Cu	3
	soil	950 Zn + 1,300 Cu	x	x	4
	soil	300 Zn	overall biomass	300 Zn	5
	nutr. sol.	32 – 260 Zn	root length	130 Zn	6
	nutr. sol.	32 – 260 Zn	foliar symptoms	130 Zn	7
	soil	0 – 160 Cd	x	x	8
	soil	300 Zn	overall biomass	300 Zn	9
P. canescens	sand + peat moss	300 Zn	x	x	10
	sand + peat moss	50 Cd	shoot biomass	50 Cd	10
	soil	360 Cd	overall biomass	360 Cd	11
	soil	265 Zn	x	x	11
	soil	360 Cd	stem height, photosynthesis	360 Cd	12
	soil	0 – 2500 Zn	lethal	500 Zn	13
	nutr. sol.	5.6 Cd	chlorophyll	5.6 Cd	14
	nutr. sol.	1.12 – 7.8 Cd	overall biomass	7.8 Cd	15
P. deltoides	soil	8.14 Cd	photosynthesis	8.14 Cd	16
	soil + waste	10,300 Zn; 5.5 Cd	x	x	17
P. euramericana	soil	8.14 Cd	photosynthesis	8.14 Cd	16
	inert clay	0 – 650 Zn	overall biomass	327 Zn	18
	inert clay	0 – 650 Zn	overall biomass	327 Zn	19
	vermiculite	65 and 650 Zn	biomass, leaf area	65 Zn	20
	nutr. sol.	0, 0.1 and 11 Cd*	root biomass	0.1 Cd	21
	soil + waste	10,300 Zn; 5.5 Cd	x	x	17

Table 3**.** *Continued*.

Populus species	Growth medium	Metal concentration	Parameter affected	LOAEC	Reference
P. nigra	soil	1,760 Zn; 32.7 Cd	x	x	22
	soil	300 Zn	shoot height, root biomass	300 Zn	5
	nutr. sol.	5.6 Cd	leaf biomass	5.6 Cd	23
	nutr. sol.	5.6 Cd	overall biomass	5.6 Cd	24
	nutr. sol.	5.6 Cd	root length, leaf area	5.6 Cd	25
P. pyramidalis	soil	0 – 100 Cd	leaf biomass	25 Cd	26
P. tremula	soil	1,760 Zn; 32.7 Cd	x	x	22
	nutr. sol.	2.24 Cd	overall biomass	2.24 Cd	27
	nutr. sol.	2.24 Cd	shoot growth	2.24 Cd	28
	soil	3,000 Zn	x	x	29
P. trichocarpa	nutr. sol.	5.6 Cd	x	x	25
	sand + vermic.	0 – 243 Cd	leaf biomass	81 Cd	current study
	sand + vermic.	0 – 7,290 Zn	leaf, stem biomass	30 Zn	current study
Populus sp.	soil	60 – 486 Zn; 0.05 – 1.6 Cd	x	x	30

[1] Ciadamidaro *et al*., 2014; [2] Di Lonardo *et al*., 2011; [3] Cicatelli *et al*., 2010; [4] Cicatelli *et al*., 2012; [5] Lingua *et al*., 2008; [6] Castiglione *et al*., 2007; [7] Franchin *et al*., 2007; [8] Rafati *et al*., 2011; [9] Todeschini *et al*., 2011; [10] Durand *et al*., 2011; [11] Durand *et al*., 2010a; [12] Durand *et al*., 2010b; [13] Langer *et al*., 2009; [14] He *et al*., 2011; [15] Dai *et al*., 2013; [16] Pajevic *et al*., 2009; [17] Sebastiani *et al*., 2004; [18] Di Baccio *et al*., 2005; [19] Di Baccio *et al*., 2009; [20] Di Baccio *et al*., 2010; [21] Lukovic *et al*., 2012; [22] Dos Santos Utmazian and Wenzel 2007; [23] Gaudet *et al*., 2011; [24] Iori *et al*., 2016; [25] Zacchini *et al*., 2009; [26] Hu *et al*., 2014; [27] Kieffer *et al*., 2009; [28] Sergeant *et al*., 2014; [29] Brunner *et al*., 2008; [30] Laureysens *et al*., 2004. * - Cd solutions re-applied weekly for a total of six weeks.

At 9 mg kg^{-1} the high translocation of Cd to aboveground parts (T*f:* 41%) is considered to be a common mechanism of hyperaccumulators, in which the metal is detoxified by chelation, vacuole storage and rapidly translocation to shoots via the xylem (Tran and Popova, 2013). However, at 81 kg kg^{-1}, there is a much higher Cd uptake in roots, which is a reflection of the non-specific mechanisms by which Cd enters the plant system (Lux *et al.*, 2011), thus in order to avoid toxicity in the photosynthetic apparatus, there is a limited transport of Cd to the shoots (T*f*: 11%). Restricting root-to-shoot translocation is a strategy typical of woody species that may contribute to metal tolerance (Arduini *et al*., 1996) since the first important barrier against Cd toxicity is the immobilization in cell walls in roots (Sanita di Toppi and Gabbrielli, 1999). Lower translocation of Cd to shoots can be due to different mechanisms, such as down-regulation of transporter proteins (i. e. heavy metal ATPases and ABC transporters) responsible for Cd loading in the xylem or increasing production of metal chelators (Lux *et al.*, 2011). Lignification of cortical cells, sclerenchyma walls and vascular tissues can also be triggered by Cd (Luković *et al.*, 2012; Kupper and Andresen, 2016; Tylova *et al*., 2017), which may contribute to the thickening of the Casparian bands in the root apex (Schreiber *et al*., 1999; White, 2001) where high influx of Cd^{2+} occurs (He *et al*., 2011).

4.2 *Zn accumulation, distribution and toxicity*

Phytotoxic effects of Zn in plants is characterised by growth inhibition, leaf chlorosis and necrosis, oxidative stress, impairment of photosynthesis, degradation of mitochondria and chloroplasts (Todeschini *et al*., 2011) and, in general, Zn concentration in leaves above 300 mg kg^{-1} induces visible toxicity symptoms (Marschner, 1995). Although there were no differences from control in terms of foliar symptoms at lower Zn doses applied (≤ 270 mg kg^{-1} Zn), *P. trichocarpa* had significantly less leaf and stem biomasses even at the lowest dose of 30 mg kg^{-1}, considered to be a sub-lethal concentration (< 65 mg kg^{-1}) (Romeo *et al*., 2014). It should be noted that in our experiment the metal solutions were applied in a single pulse, in which a rapid uptake could have occurred in these plants immediately after contamination and may have impaired plant growth due to salinity or osmotic stress (Polle *et al*., 2013). Recent studies have classified poplars as being sensitive to moderately sensitive to salinity stress (Mirck and Zalesny, 2015). Moreover, high cation additions (≥ 270 mg kg^{-1} Zn; or 243 mg kg^{-1} Cd) significantly decreased the substrate pH, especially at extreme Zn concentrations (2430 and 7290 mg kg^{-1}) , thus it is evident that this acidification could have led to an acute toxicity by enhancing Zn^{2+} availability in the rhizosphere (Alloway, 2008).

Zn toxicity varies considerably among poplar species. Di Lonardo *et al*., (2011) found no effects from 130 mg L^{-1} on shoot biomass of three different *P. alba* varieties *in vitro*, although root biomass in one case decreased by 85% at only 65 mg L^{-1}. In our study, the shoots of *P. trichocarpa* were more sensitive to Zn than the roots, which only presented biomass loss at higher concentrations (≥ 810 mg kg^{-1} Zn). Root tolerance is an important feature in plants exposed to toxic metals, for it implies preservation of cell membranes selectivity properties, the initial step in uptake and xylem loading (Zacchini *et al.*, 2009). Roots accumulated more Zn than the leaves, which is in accordance to some studies in poplars (Dos Santos Utmazian and Wenzel, 2007; Romeo *et al*., 2014), although other poplar species have demonstrated significantly higher Zn contents in leaves (Lingua *et al*., 2008; Castiglione *et al*., 2009; Cicatelli *et al*., 2010; Todeschini *et al*., 2011).

Although Zn doses applied were 10 times higher than Cd, Zn translocation response (based on T*f* values) was somewhat analogous to the patterns seen in Cd-treated poplars. This suggests that *P. trichocarpa* adopts similar strategies for dealing with Cd and Zn toxicity by drastically decreasing metal translocation after a certain concentration threshold, in this case at 270 mg kg^{-1} Zn. Reducing Zn translocation as a protective effect was also seen in *P. alba* (Romeo *et al*., 2014) and *P. nigra* (Dos Santos Utmazian and Wenzel, 2007).

4.3 *Pt-HMA4 is down-regulated in roots under Cd and Zn stress*

The significant decrease in root-to-shoot translocation of Cd and Zn observed at the doses applied of 81 mg kg^{-1} Cd and 270 mg kg^{-1} Zn, led us to investigate if the ATPase HMA4, which plays a pivotal role in metal detoxification and long distance transport in plants (Luo *et al.*, 2016; Sarwar *et al*., 2017), could help explain such findings. *Pt-HMA4* was expressed highly in roots, similar to what has been observed for other members of the HMA family in poplar, specifically around xylem vessels (Migeon *et al*., 2010). In *A. halleri*, exposure to Zn clearly showed an abundance of HMA4 transcripts in the root xylem adjacent to the pericycle layer, which emphasises HMA4 involvement in xylem loading and justifies its high expression in root tissues (Hanikenne *et al*., 2008).

Both Cd and Zn amendments resulted in down-regulation of *Pt-HMA4* in poplar roots, which places this gene in the same subgroup of HMAs transporting Zn/Cd/Co/Pb as found in *A. thaliana* (*At-HMA1-4*) and *Oryza sativa* (*Os-HMA1-3*) (Takahashi *et al*., 2012). Transport proteins such as HMA, can contribute to Cd efflux to the apoplast, sequestration into the vacuoles and directly affect Cd uptake and localisation (Iori *et al*., 2016; Hasan *et al*., 2017). Similarly, at high levels of Zn, *P. nigra* down-regulated *Pt-HMA4* expression in just 48 hours (Adams *et al*., 2011), but in the present study we showed that after eight weeks of exposure to Cd or Zn the expression of *Pt-HMA4* was still much lower than uncontaminated control. Small variations in the expression of HMA4 in *A. thaliana* was demonstrated to have large effects in the Zn gradient in roots (Claus *et al*., 2013). Thus we can hypothesize that the regulation of *Pt-HMA4* expression under Cd and Zn stress is one of the mechanisms by which *P. trichocarpa* maintains the metal partitioning pattern observed previously, in which a drastic decrease in translocation occurs as metal concentration reaches its toxicity threshold.

4.4 *Cd and Zn interactions in poplar*

Decrease in Cd uptake in plants due to elevated Zn supply has been commonly shown and is often associated with competitive interactions during root uptake, in which Cd is believed to enter the plant via transport processes inherent to Zn (Marschner, 1995; Hart *et al*., 2002; Garg and Kaur, 2013). The opposite can also be observed, for instance in wheat, a decrease in Zn translocation was attributed to competition with high Cd concentrations in soil (Green *et al*., 2010). We predicted similar outcomes, in which Zn would be preferentially taken up by the roots, therefore reducing Cd accumulation in the plant. However Zn had the opposite effect in

P. trichocarpa under our experimental conditions and caused an overall increase in Cd uptake and accumulation.

A pH decline in the substrate due to high cationic concentration (Zn^{2+}) may have played an important part in increasing Cd uptake, which is known for the inverse relationship with soil pH (Chuan *et al*., 1996; Smolders and Mertens, 2013). But substrate pH was unaffected by the addition of 90 mg kg^{-1} Zn compared to when Cd was added singly (pH of 6.1 in both cases), yet it still lead to a significant increase in Cd concentrations in all plant parts: for instance Cd concentration in leaves increased from 42 mg kg^{-1} under single metal treatment to 123 mg kg^{-1} under the combined treatment. Similar effect was observed in *Nocceae caerulescens*, in which combined treatments of Zn (500 μM) and Cd (200 μM) in hydroponic cultures resulted in increasing Cd^{2+} influx into root tissues and higher accumulation in shoots (Papoyan *et al*., 2007), and this response has been associated with hyperaccumulator phenotypes (Lasat *et al*., 1998; Papoyan and Kochian, 2004). Moreover, the hyperaccumulator *Brassica juncea* had an increase in Cd uptake after Zn addition, leading also to a higher tolerance in comparison to plants exposed to Cd and Zn separately (Kutrowska *et al*., 2017). In field conditions, positive correlation between Zn and Cd accumulation in shoots was also reported in Cacao trees (Arévalo-Gardini et al. 2017). Such response might be related to an up-regulation of genes encoding some metal transporters in roots triggered by the exposure to Zn^{2+}, through which Cd^{2+} could have been actively transported. For instance, in *Salix caprea* the combined treatment of Cd and Zn induced the expression of transporters ZIP6 and HMA1 (Konlechner *et al*., 2013). Another reason for higher Cd uptake can be attributed to the direct competition between Zn and Cd for the soil adsorption sites (Lu and Xu 2009), for these elements have similar atomic characteristics and are both affected by electrostatic interactions (Moreira and Alleoni, 2010). Considering that the concentrations of Zn added were at least three times higher than Cd, it is likely that Zn caused a displacement of Cd into the solution, increasing its availability for plant uptake.

Metal accumulation in *P. trichocarpa* varied depending on external metal contents and also the plant's own regulatory system, which in some cases presented responses analogous to hyperaccumulator plants. Foliar concentration of 123 mg kg^{-1} Cd is not high compared to well established Cd-hyperaccumulators such as *N. caerulescens*, that can accumulate more than 3000 mg kg^{-1} DW (Papoyan *et al*., 2007). However Cd is naturally in plants at levels lower than 1 mg kg^{-1} (Reeves, 2006) and, according to Baker *et al*. (2000) and He *et al*. (2017), concentrations higher than 100 mg kg^{-1} Cd are exceptional and can be the threshold for recognizing a hyperaccumulator of Cd (0.01% of dry weight).

Zn addition lead to higher Cd accumulation in leaves and stems, but this did not result in higher toxicity, suggesting that Zn also had a protective effect. According to Cherif *et al.* (2011), Zn addition can restore and enhance the functional activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione reductase that are suppressed by Cd toxicity. Concentration around 65 mg L^{-1} Zn improved the photoprotective and antioxidant responses (α-Tocopherol and reduced glutathione) in two poplar clones in hydroponics (Fernandez-Martinez *et al*., 2014). Overall, Zn can protect cells from damaging reactions caused by reactive oxygen species (ROS) and compete with Cd for binding sites in enzymes (-SH groups) and membrane proteins (Cakmak, 2000; Cherif *et al*., 2011).

5. Conclusions

Cadmium and zinc toxicity affected growth and metal allocation in *Populus trichocarpa* 'Trichobel', in which Cd transport appears to be strongly regulated to some extent (≤ 81 mg kg^{-1}). Although shoot concentrations were not as high as found in extreme hyperaccumulator plants, this variety of poplar has an exceptional tolerance to Cd, especially considering that phytotoxicity was mainly found in high and drastic Cd concentrations (≥ 27 mg kg^{-1}), in which root integrity was barely affected. At lower Cd concentrations, *P. trichocarpa* displayed similar tolerance mechanisms and translocation patterns found in plants with hyperaccumulator phenotypes; in which metal partitioning appears to play a major role in Cd tolerance. Decrease in translocation at high metal concentrations was achieved partly by down-regulating the expression of *Pt-HMA4* in roots. Zn promoted Cd uptake and shoot accumulation without compromising plant growth. Such results suggest that *P. trichocarpa* has the potential to survive, stabilise and extract Cd from soils in areas contaminated with both Cd and Zn and be a valid candidate for phytoremediation, especially in a short rotation coppice system. However, it is still necessary to better comprehend the interactions between Cd, Zn and other toxic metals in this species, as well as consider its interactions with surrounding soil microbiota (e. g. mycorrhizal symbiosis).

Appendix A. Supplementary data

Supplementary data can be found at: http://… and consist of the following tables. **Table S1**. Root morphologic parameters, leaf transpiration (E) and stomatal conductance (gs) of *Populus trichocarpa* exposed to different Cd and Zn concentrations for five weeks. **Table S2**. Spearman correlation (r_s) matrix between 14 different variables from *Populus trichocarpa* grown under different Cd concentrations. Variables were considered monotonic correlated for $p < 0.05$. **Table S3**. Spearman correlation (rs) matrix between 14 different variables from *Populus trichocarpa* grown under different Zn concentrations. Variables were considered monotonic correlated for $p < 0.05$. **Table S4**. Cadmium concentration, total uptake, translocation factor (*Tf*: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* ‘Trichobel’ grown for five weeks under different Cd doses. **Table S5**. Zinc concentration, total uptake, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* ‘Trichobel’ grown for five weeks under different Zn doses.

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FIGURE CAPTIONS

Fig. 1 - Phytotoxic effects of Cd and Zn in *Populus trichocarpa* at different soil concentrations, after five weeks of exposure.

Fig. 2 - Root scans of *Populus trichocarpa* exposed to different Cd and Zn concentrations during five weeks. Images were used for length, area, volume and diameter analyses.

Fig. 3 - Toxicity ranks of *P. trichocarpa* exposed to different Cd and Zn concentrations. Symptomatic leaf tissue area (SLTA) was assessed visually and scored from 0 to 5 (each score represent 20% of leaf area). Significant differences are represented by different letters by Tukey test ($p < 0.05$) in Cd treatments and Dunn's test ($p < 0.05$) in Zn treatments.

Fig. 4 - Cd and Zn concentrations (mg kg^{-1}) in leaves, stems and roots of *Populus trichocarpa* grown for five weeks in sandy substrate at different Cd or Zn doses. Error bars indicate standard error of the mean (n = 4). Different letters correspond to significant differences between doses applied (Cd: Tukey test, $p < 0.05$; Zn: Dunn's test, $p < 0.05$). To better visualise the complete data, x axis was set in log scale and breaks were added to both axes. Dotted lines indicate gaps between axis breaks.

Fig. 5 - Transcript levels of the PtHMA4 gene in roots and leaves of *P. trichocarpa* after growing for eight weeks under Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) stress, and without any metal addition (Control). The mRNA levels were quantified by real-time qPCR and normalised in relation to Ubiquitin (UBQ) expression. Different letters represent significant differences among treatments, determined by Tukey test after ANOVA ($p = 0.0167$). There were no differences among treatments in leaf tissues.

Fig. 6 - Concentrations of Cd and Zn in leaves, stems and roots of *Populus trichocarpa* exposed to different metal combinations: 27 mg kg^{-1} Cd, 90 or 270 mg kg^{-1} Zn. Different letters correspond to significant differences among treatments for the same plant tissue, Tukey test ($p < 0.05$) in the top figure and Dunn's test ($p < 0.05$) in the bottom figure.

APPENDIX A. SUPPLEMENTARY INFORMATION

Table S1. Root morphologic parameters, leaf transpiration (E) and stomatal conductance (gs) of *Populus trichocarpa* exposed to different Cd and Zn concentrations for five weeks.

Metal	**Length**	**Projected area**	**Average diameter**	**Root volume**	**E**	**gs**
Cd (mg kg^{-1})	-- cm --	-- cm^2 --	-- mm --	-- cm^3 --	mmol m^{-2} s^{-1}	mol m^{-2} s^{-1}
Control	1963 a	100.8 a	0.52 a	4.08 a	2.65 a	0.08 a
1	2228 a	108.0 a	0.49 a	4.15 a	2.81 a	0.08 a
3	2080 a	100.1 a	0.48 a	3.83 a	2.60 a	0.08 a
9	1980 a	95.3 a	0.49 a	3.65 ab	2.67 a	0.08 a
27	2028 a	101.4 a	0.50 a	4.02 a	2.50 a	0.07 a
81	2002 a	86.8 a	0.43 ab	2.97 ab	2.40 a	0.06 a
243	233 b	9.3 b	0.37 b	0.37 b	0.48 b	0.01 b
Zn (mg kg^{-1})						
Control	2106 a	100.8 a	0.52 a	4.43 a	2.89 a	0.08 a
30	2046 a	92.9 a	0.49 ab	3.87 a	2.81 a	0.08 a
90	1966 a	93.5 a	0.49 ab	3.81 a	2.76 a	0.08 a
270	1833 ab	63.4 ab	0.47 abc	2.88 ab	2.47 a	0.06 a
810	763 bc	22.6 bc	0.35 cd	0.85 bc	1.94 a	0.05 a
2430	333 c	13.1 c	0.41 bcd	0.40 c	x	x
7290	363 c	10.5 c	0.33 d	0.34 c	x	x

Significant differences among treatments (for each metal) are represented by different letters.

Cd treatments: Tukey test: $p < 0.05$, n = 4;

Zn treatments: Dunn test, $p < 0.05$, n = 3.

x's represent dead leaves and measurements were not recorded.

Table S2. Spearman correlation (r_s) matrix between 14 different variables from *Populus trichocarpa* grown under different Cd concentrations. Variables were considered monotonic correlated for $p < 0.05$.

Variables	Cd applied	Cd leaf	Cd root	Cd stem	DW leaf	DW root	DW stem	n. of leaves	Shoot growth	Root diam.	E	gs	symptoms	pH
Cd applied	1													
Cd leaf	0.94	1												
Cd root	0.98	0.93	1											
Cd stem	0.96	0.98	0.96	1										
DW leaf	-0.72	-0.73	-0.68	-0.71	1									
DW root	-0.60	-0.68	-0.58	-0.65	0.83	1								
DW stem	-0.64	-0.69	-0.61	-0.67	0.82	0.76	1							
n. of leaves	-0.61	-0.52	-0.58	-0.51	0.60	ns*	0.45	1						
Shoot growth	-0.71	-0.63	-0.68	-0.64	0.76	0.51	0.67	0.88	1					
Root diam.	-0.54	-0.53	-0.53	-0.51	0.66	0.73	0.52	0.46	0.57	1				
E	-0.48	ns	-0.47	-0.40	0.61	ns	0.46	0.77	0.84	0.47	1			
gs	-0.61	-0.47	-0.61	-0.51	0.50	ns	0.41	0.73	0.76	0.41	0.81	1		
symptoms	0.77	0.66	0.76	0.72	-0.39	ns	-0.48	-0.50	-0.46	ns	ns	-0.52	1	
pH	ns	-0.43	ns	-0.39	0.40	0.53	ns	ns	ns	ns	ns	ns	ns	1

Cd applied: Cd solutions applied in the substrate (0; 1; 3; 9; 27; 81; 243 mg kg^{-1});
Cd leaf, stem, root: Cd concentration in plant tissues;
DW: dry weight;
n. of leaves: number of expanded leaves at harvest;
Shoot growth: difference (in cm) of shoot height before and after Cd treatment;
Root diam.: mean root diameter;
E: leaf transpiration;
gs: stomatal conductance;
symptoms: toxicity symptoms in leaves at harvest;
pH: substrate pH after harvest.

Table S3. Spearman correlation (r_s) matrix between 14 different variables from *Populus trichocarpa* grown under different Zn concentrations. Variables were considered monotonic correlated for $p < 0.05$.

Spearman correlations	Zn applied	Zn leaf	Zn root	Zn stem	DW leaf	DW root	DW stem	n. of leaves	Shoot growth	Root diam.	E	gs	symptoms	pH
Zn applied	1													
Zn leaf	0.97	1												
Zn root	0.97	0.94	1											
Zn stem	0.98	0.98	0.96	1										
DW leaf	-0.91	-0.87	-0.89	-0.89	1									
DW root	-0.89	-0.89	-0.92	-0.90	0.87	1								
DW stem	-0.88	-0.82	-0.86	-0.84	0.91	0.82	1							
n. of leaves	-0.89	-0.84	-0.86	-0.87	0.83	0.77	0.83	1						
Shoot growth	-0.95	-0.90	-0.95	-0.92	0.89	0.83	0.89	0.91	1					
Root diam.	-0.84	-0.86	-0.81	-0.84	0.81	0.83	0.68	0.74	0.77	1				
E	ns	ns	ns	ns	ns	ns	ns	0.60	ns	ns	1			
gs	ns	ns	ns	ns	ns	ns	ns	0.53	ns	ns	0.91	1		
symptoms	0.83	0.81	0.85	0.82	-0.78	-0.85	-0.86	-0.83	-0.81	-0.69	ns	ns	1	
pH	-0.91	-0.90	-0.89	-0.92	0.84	0.88	0.77	0.82	0.87	0.81	ns	ns	-0.76	1

[a] Zn applied: Zn solutions applied in the substrate (0; 30; 90; 270; 810; 2430; 7290 mg kg^{-1});
Zn leaf, stem, root: Zn concentration in plant tissues;
DW: dry weight;
n. of leaves: number of expanded leaves at harvest;
Shoot growth: difference (in cm) of shoot height before and after Zn treatment;
Root diam.: mean root diameter;
E: leaf transpiration;
gs: stomatal conductance;
symptoms: toxicity symptoms in leaves at harvest;
pH: substrate pH after harvest.

Table S4**.** Cadmium concentration, total uptake, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* ‘Trichobel’ grown for five weeks under different Cd doses.

Cd ($mg\ kg^{-1}$)	Cd concentration ($mg\ kg^{-1}$)			Cd uptake ($\mu g\ plant^{-1}$)	T*f*	BCF	
	Leaves	Stems	Roots			Leaf	Root
Control	0.5 ± 0.1 aA	1.4 ± 0.3 aA	2.5 ± 0.4 aB	3.2 ± 0.3	20	---	---
1	2.6 ± 0.1 bA	3.4 ± 0.4 bB	14.4 ± 2.7 bC	14.8 ± 3.1	18	2.6	14.4
3	6.2 ± 0.3 cA	8.30 ± 0.8 cA	35.1 ± 4.4 cB	31.0 ± 5.2	18	2.1	11.7
9	42.9 ± 4.4 dA	41.1 ± 7.1 dA	105 ± 18 dA	119 ± 11	41	4.8	11.7
27	42.0 ± 4.3 dA	42.6 ± 7.3 dA	163 ± 29 dB	167 ± 22	26	1.6	6.0
81	53.9 ± 2.0 dA	67.4 ± 9.7 dA	487 ± 64 eB	267 ± 52	11	0.7	6.0
243	681 ± 31 eA	434 ± 98 eA	6,537 ± 816 fB	629 ± 157	6	2.8	47.6

Different lowercase letters denote significant difference between treatments by Tukey test ($p < 0.05$);
Different uppercase letters denote significant differences between plant organs in the same treatment by Tukey test ($p < 0.01$).
Tf = (leaf concentration / root concentration) × 100.
BCF = (plant concentration / soil concentration).

Table S5. Zinc concentration, total uptake, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* 'Trichobel' grown for five weeks under different Zn doses.

Zn ($mg\ kg^{-1}$)	Zn concentration ($g\ kg^{-1}$)			Zn uptake ($mg\ plant^{-1}$)	*Tf*	BCF	
	Leaves	Stems	Roots			Leaf	Root
Control	0.09 ± 0.01 aB	0.04 ± 0.01 aA	0.2 ± 0.01 aC	0.3 ± 0.01	33	---	---
30	0.1 ± 0.01 aA	0.3 ± 0.1 bAB	0.4 ± 0.1 aC	0.5 ± 0.1	21	3.1	14.8
90	0.2 ± 0.02 bA	0.5 ± 0.01 bB	0.6 ± 0.1 aB	0.9 ± 0.1	40	2.6	6.5
270	0.7 ± 0.1 cA	0.8 ± 0.1 bcA	2.5 ± 0.9 bA	2.0 ± 0.2	26	2.4	9.3
810	1.5 ± 0.1 cdA	1.9 ± 0.2 cdAB	5.6 ± 1.3 bcB	2.5 ± 0.5	27	1.9	6.9
2,430	2.2 ± 0.4 dA	4.8 ± 1.1 dA	10.1 ± 1.5 cdB	3.5 ± 1.1	22	0.9	4.2
7,290	12.8 ± 3.5 eA	24.3 ± 4.2 eA	21.5 ± 5.3 dA	17.9 ± 2.2	59	1.7	2.9

Different lowercase letters denote significant difference between treatments by Dunn test ($p < 0.05$);
Different uppercase letters denote significant differences between plant organs in the same treatment by Dunn test ($p < 0.01$).
Tf = (leaf concentration / root concentration) × 100.
BCF = (plant concentration / soil concentration).