



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

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1 **Original Article**

2

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

4 Vinicius H. **DE OLIVEIRA** and Mark **TIBBETT***

5

6 *Centre for Agri-Environmental Research & Soil Research Centre, School of Agriculture*

7 *Policy and Development, University of Reading, Berkshire, RG6 6AR, United Kingdom*

8

9 Running title: Tolerance, toxicity and transport of Cd and Zn in poplar

10

11 *For correspondence. E-mail: m.tibbett@reading.ac.uk*

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16

17 **Abstract**

18 Metal inputs to terrestrial ecosystems are of great concern due their toxicity to biota,
19 especially for elements with no biological function such as cadmium. Fast-growing trees such
20 as poplars may have potential in phytoremediation schemes. We assessed accumulation, metal
21 partitioning, gene expression (*Pt-HMA4*) and overall tolerance to, and interaction between,
22 cadmium (Cd) and zinc (Zn) in *Populus trichocarpa* ‘Trichobel’. We predicted that Zn would
23 have an antagonistic effect in Cd accumulation and anticipated some level of tolerance to these
24 metals. Poplars were grown in sandy substrate under different metal applications, ranging from
25 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
26 Cd with 90 or 270 mg kg⁻¹ Zn. Growth parameters and metal contents in shoots and roots
27 were determined. Transcriptional levels of the *Pt-HMA4* gene were assessed in roots and
28 leaves. *P. trichocarpa* showed a surprisingly high tolerance to Cd, with root biomass being
29 affected only at the highest doses applied. Metals accumulated mainly in roots (up to 6,537 mg
30 kg⁻¹ Cd and 21,500 mg kg⁻¹ Zn), root-to-shoot translocation peaked at the 9 mg kg⁻¹ dose for
31 Cd (41%) and 90 mg kg⁻¹ for Zn (40%). At high Cd/Zn applications, expression of *Pt-HMA4*
32 in roots decreased significantly. Contrary to the initial presumption, Zn addition increased Cd
33 uptake, reaching hyperaccumulator-like concentrations in shoots (≥ 100 mg kg⁻¹ Cd).
34 Differential root-to-shoot partitioning has a major role in Cd tolerance in *P. trichocarpa*; partly
35 by down-regulating the *Pt-HMA4* gene in roots. Zn addition promoted high Cd uptake without
36 any detriment to plant growth. *P. trichocarpa* was tolerant to extreme Cd concentrations,
37 offering a great potential to be used in phytoremediation techniques for stabilization/extraction
38 of Cd from soils contaminated by both Cd and Zn.

39

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

42

43

44 1. Introduction

45 Cadmium (Cd) is one of the most hazardous metals in the environment, ranked seventh
46 in toxicity by the Agency for Toxic Substance and Disease Registry (ATSDR, 2017), it lacks
47 any known biological function, being toxic to humans and other organisms at relatively low
48 concentrations (Alloway 2013) and has a high mobility in soils (Lei *et al.*, 2010). Cd is
49 frequently found in zinc (Zn) bearing minerals (Alloway 2013) and, due to their similar
50 geochemical characteristics Zn is often associated with Cd in soils (Kabata-Pendias and
51 Pendias 2001), although not as toxic, high concentrations of Zn can be extremely harmful to
52 biota. Plant exposure to Cd often leads to phytotoxicity depending on the concentration, plant
53 genotype, soil characteristics and exposure time (Das *et al.*, 1997; Benavides *et al.*, 2005)
54 mainly due to the fact that Cd has a chemical similarity to other essential elements, such as Ca,
55 Fe and particularly Zn (Clemens, 2006; Verbruggen *et al.*, 2009). Growth impairment, biomass
56 decrease, foliar necrosis and chlorosis are typical effects from Cd toxicity in plants (He *et al.*,
57 2017; Tran and Popova, 2013; Pál *et al.*, 2006). Similar to Cd, Zn toxicity effects in plants
58 include growth inhibition, leaf chlorosis and necrosis, oxidative stress, inhibition of protein
59 functions and impairment of photosynthesis (Todeschini *et al.*, 2011; Hasan *et al.*, 2017). Cd²⁺
60 and Zn²⁺ are long known for being competing ions in the soil matrix due to their chemical
61 similarities and same uptake pathways in plants (Clemens, 2006; Kirkham, 2006) in which Zn
62 is often responsible for decreasing Cd uptake and even considered as a soil amendment to
63 reduce Cd concentration in edible crops (Green *et al.*, 2003; Garg and Kaur, 2013). However,
64 it has been reported recently that Zn not always impact Cd accumulation (Green *et al.*, 2017).

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

69 Watson, 2003; Guerra *et al.*, 2011). Poplars (*Populus* sp.) are trees widely considered for
70 phytoextraction of several metals, such as Cd, Zn, Pb and Cu (Castiglione *et al.*, 2009; Zacchini
71 *et al.*, 2009; Guerra *et al.*, 2011; Dai *et al.*, 2013; Luo *et al.*, 2016), mainly due to their biomass
72 production, deep root systems (Bhargava *et al.*, 2012), tolerance to high metal concentrations
73 and fast growth (Robinson *et al.*, 2009). *Populus* species can also rapidly invade disturbed sites,
74 reproduce asexually (Sebastiani *et al.*, 2004; Hamberg *et al.*, 2011) and are not a source of food
75 for farm animals, reducing the risk of heavy metals entering the human food chain (Shim *et al.*,
76 2013).

77 Metal tolerance and partitioning in plants are important features to be considered in
78 phytoextraction (Luo *et al.*, 2016), in which root-to-shoot translocation of Cd is regarded as a
79 major factor in determining its toxicity thresholds in poplar (Durand *et al.*, 2011). Several
80 transmembrane proteins are involved in cation efflux from the cytoplasm, from which HMA4
81 (Heavy Metal ATPase 4), a common metal transporter from the P-type ATPase family, is
82 known to play a role in the xylem-loading of metals (Hanikenne *et al.*, 2008; Luo *et al.*, 2016),
83 affecting transport and accumulation in poplar (Adams *et al.*, 2011). The *HMA4* gene is
84 considered to be key in Zn and Cd hyperaccumulation and also tolerance, which was previously
85 verified in *Arabidopsis thaliana* (Mills *et al.*, 2005), *Noccaea caerulescens* (Lochlainn *et al.*,
86 2011) and transgenic *Nicotiana tabacum* plants (Grispen *et al.*, 2011).

87 *Populus trichocarpa* (black cottonwood) is considered a model tree species (Bradshaw
88 *et al.*, 2000), with its genome already fully sequenced (Tuskan *et al.*, 2006). However, little is
89 known about heavy metal accumulation, toxicity and translocation in *P. trichocarpa*, most
90 studies being mainly focused on other species from the *Populus* genus. The objectives of this
91 work were to investigate (1) the effects of different concentrations of Cd and Zn on *P.*
92 *trichocarpa*, (2) the accumulation and distribution of Cd and Zn within the plant and their
93 effects on the expression of the metal transporter *Pt-HMA4*, and (3) the interactive effects

94 between Cd and Zn in terms of phytotoxicity and metal distribution. We predicted that Zn could
95 prevent Cd uptake, consequently alleviating toxicity effects and that tolerance is associated
96 with different metal translocation patterns, influenced by the expression of *Pt-HMA4*.

97

98 2. Materials and Methods

99 2.1 *Plant material and pre-growth*

100 Cuttings (15 cm, two nodes) of *Populus trichocarpa* ‘Trichobel’ clones were rooted in
101 sand for four weeks, and fertilised three times with 10 mL of a modified Long Ashton’s solution
102 (macronutrients: (NH₄)₂SO₄ (4 mM), K₂SO₄ (2 mM), CaCl₂·2H₂O (3 mM), MgSO₄·7H₂O (1.5
103 mM), NaNO₃ (8 mM), FeEDTA (0.1 mM); micronutrients: H₃BO₃ (2.86 mg l⁻¹), MnCl₂·4H₂O
104 (1.81 mg l⁻¹), CuSO₄·5H₂O (0.08 mg l⁻¹), NaMoO₄·2H₂O (0.025 mg l⁻¹), ZnSO₄·7H₂O (0.22
105 mg l⁻¹)), according to Kariman *et al.*, (2014) and 1 mL of a solution with KH₂PO₄ (1 mM).
106 This clone is an intraspecific hybrid of *Populus trichocarpa* Torrey & A. Gray ex Hook
107 (Burgess *et al.*, 2005).

108 All rooted cuttings were transplanted to plastic pots (without holes in the bottom) filled
109 with 1 kg of substrate: 50 g vermiculite, 50 g peat moss and 900 g of sand (pH 6.9); one cutting
110 per pot. Water holding capacity was maintained at 70% (300 mL of distilled water). The
111 experiment was carried out in the glasshouse of the University of Reading, between December
112 2015 and February 2016. The temperature average recorded in the glasshouse during this
113 period was 24.5°C (± 2.4), and artificial light was provided (18h/day). Poplar cuttings were
114 obtained from AF Hill & Son, Redditch, UK and were kept refrigerated at 4°C until the
115 experiment.

116

117 2.2 *Treatments and Experimental Design*

118 The experiment was designed in randomized blocks, cuttings with similar sizes were
119 assigned to one of the flour blocks. After one week, the final fertilisation was applied and all
120 cuttings had their expanded leaves counted and stems measured from the node sprouting to
121 the apex; a sample from the substrate was also taken for further analysis. All pots were spiked
122 with either Cd or Zn solutions on the following day. Cd was added via CdCl₂ stock solutions
123 to make up six different concentrations in the pot substrate: 1, 3, 9, 27, 81 and 243 mg kg⁻¹
124 Cd; Zn was added via ZnSO₄ stock solutions, making up six different concentrations in the
125 substrate: 30, 90, 270, 810, 2430 and 7290 mg kg⁻¹ Zn. Two further treatments included both
126 Cd and Zn: 27 mg kg⁻¹ Cd + 90 mg kg⁻¹ Zn (Cd₂₇ + Zn₉₀); and 27 mg kg⁻¹ Cd + 270 mg kg⁻¹
127 Zn (Cd₂₇ + Zn₂₇₀). Control had water only instead of the metal solutions, and all pots
128 contained only one poplar cutting. Metals were added in a single dose and each treatment had
129 four replicates arranged in blocks.

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

136

137 2.3 Harvest and Phytotoxicity assessment

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

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

146 Plants were then harvested and separated into roots, stems and leaves (initial cuttings were
147 not included in any analyses). Roots were washed thoroughly with tap water and immersed in
148 a 0.05 mM CaCl₂ solution for 30 minutes to remove any surface adhering metals (Marmiroli *et*
149 *al.*, 2013), roots were rinsed with deionized water and scanned using the software WinRhizo®,
150 to determine the root length, diameter, root tips, surface area and volume. All plant parts were
151 dried separately in an oven at 70°C for seven days, then dry weight (DW) was determined. Soil
152 was air dried, sieved (2 mm) and soil pH was determined in a water-soil suspension (2.5:1)
153 shook for 15 min at 120 rpm (Rowell, 1994).

154

155 2.4 Acid Digestion and Metal Determination

156 Dried samples were ground and 50 mg of plant material was digested for 8 hours in 5 mL
157 of 70% HNO₃ (≥69% TraceSELECT® for trace analysis) in closed glass vessels in heating
158 blocks at 110°C (Huang *et al.*, 2004). All digestions were performed in duplicates, and for
159 quality control, a blank and a plant certified reference material (IAEA-359 cabbage leaves)
160 were included. Digested extracts were then diluted in a solution of 2% HNO₃ + 5 ppb Rh, and
161 filtered. The concentrations of Cd and Zn were determined by inductively coupled plasma mass
162 spectrometry (Thermo Scientific™ iCAP™ Q ICP-MS), using rhodium as an internal standard.

163

164 2.5 Bioconcentration Factor, Translocation Factor and Tolerance Index

165 The bioconcentration factor (BCF), the translocation factor (*Tf*), and tolerance index (TI)
166 are used as indices to assess the plant's capacity to accumulate, translocate (from roots to
167 shoots) and tolerate heavy metals (Rafati *et al.*, 2011). BCF is the ratio between the metal

168 concentrations within the plant tissue and in the soil or substrate; Tf is the ratio between the
169 metal concentrations in leaves and roots; and TI is the ratio between a parameter assess in
170 heavy metal treated plants and the control (Saraswat and Rai, 2009; Zacchini *et al.*, 2009; Rafati
171 *et al.*, 2011); see equations below, in which [M]: metal concentration; T: treated plants; C:
172 control plants.

173

$$174 \quad BCF = \frac{[M]_{plant}}{[M]_{soil}} \quad (1)$$

175

$$176 \quad Tf = \frac{[M]_{leaf}}{[M]_{root}} \times 100 \quad (2)$$

177

$$178 \quad TI \% = \frac{T}{C} \times 100 \quad (3)$$

179

180 2.6 *Pt-HMA4* expression in roots and leaves

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

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

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

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

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

213

214 2.7 Statistical Analyses

215 Statistical analyses were performed for all parameters assessed using R software. Metal
216 treatments were considered as categorical factors and therefore ANOVA was performed for
217 each parameter assessed ($p < 0.05$). When significant differences were detected, a Tukey test
218 ($p < 0.05$) was carried out to discriminate differences between treatments. Pearson correlation

219 was also performed. Data was transformed when necessary (determined by Shapiro-Wilk
220 normality test and Levene's test, $p < 0.05$) to attain normal distribution and homoscedasticity,
221 in order to meet ANOVA and Pearson correlation assumptions (Zar, 2010). Transformation
222 was carried out mainly by two equations: $\log(x)$ or x^2 ; root dry weight data from Zn treatments
223 were transformed by $\sqrt[3]{x}$ after a BoxCox plot. Data that could not be transformed to attain
224 normality (i.e. a few root morphology parameters), Kruskal-Wallis followed by a Dunn's test
225 ($p < 0.05$) were performed. A non-parametric correlation test (Spearman) was done for 14
226 different variables to verify possible monotonic relationships and only significant r_s values (p
227 < 0.05) were reported.

228

229 3. Results

230 3.1 Growth, biomass production and transpiration rate

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

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



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

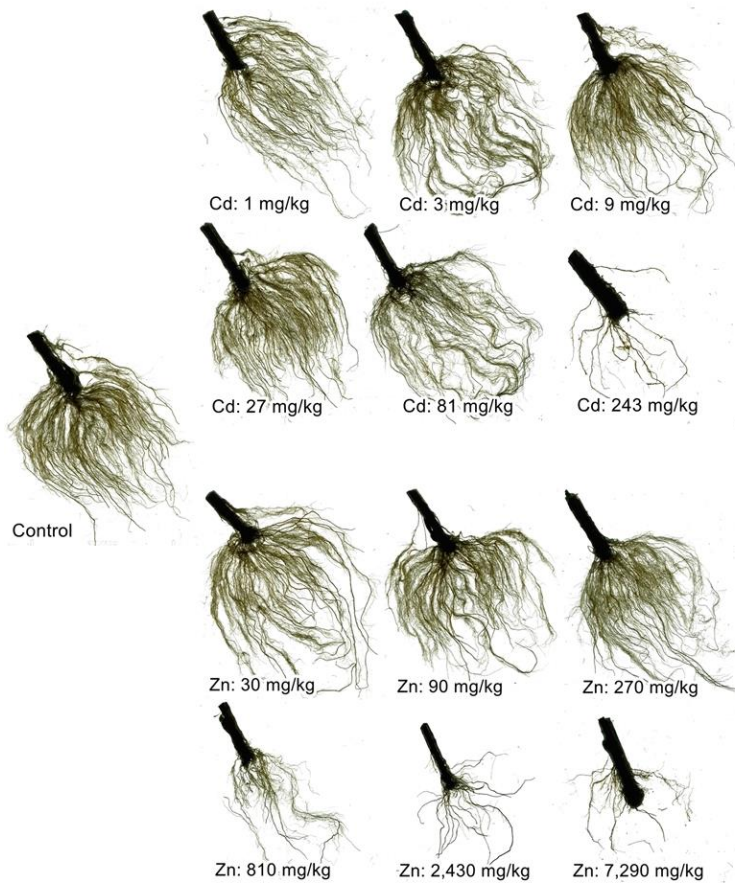


Fig. 2 - Root scans of *Populus trichocarpa* exposed to different Cd and Zn concentrations during five weeks. Images were used for length, area, volume and diameter analyses.

242

243

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.

244 Foliar symptoms of phytotoxicity were more evident in Cd treatments than in Zn

245 treatments, when compared to control at lower concentrations, 30 to 270 mg kg⁻¹ Zn (Fig. 3).

246 All treatments displayed marginal necrosis in the leaves assessed (older leaves), including the

247 control, but chlorosis and discoloration were present only in Cd-treated plants. Although

248 necrosis and chlorosis were both considered for the toxicity scoring, chlorosis were

249 predominantly in Cd treatments. At the highest Zn concentrations (2430 and 7290 mg kg⁻¹) all

250 leaves were scored as a 5, due to extensive foliar necrosis (Fig. 2).

251

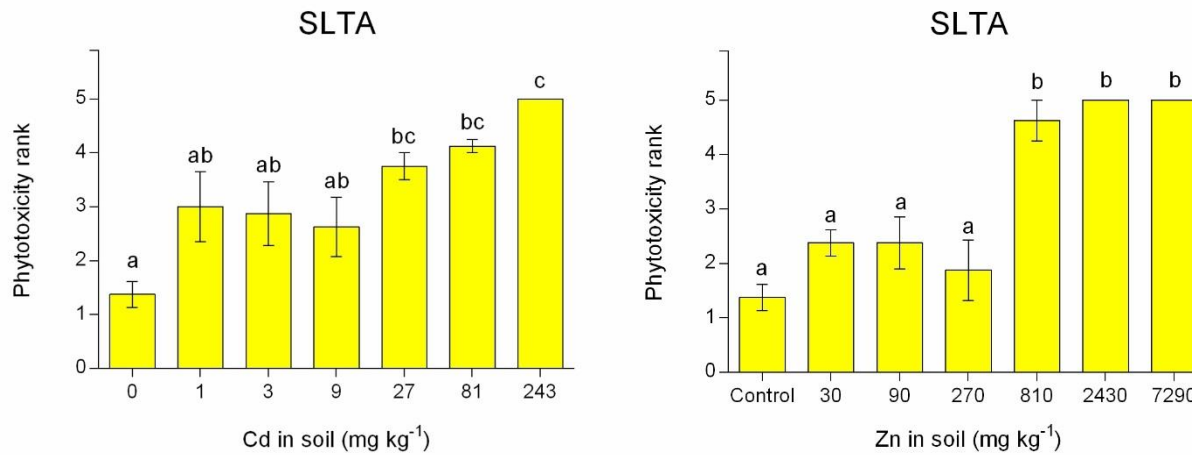


Fig. 3 - Toxicity ranks of *P. trichocarpa* exposed to different Cd and Zn concentrations. Symptomatic leaf tissue area (SLTA) was assessed visually and scored from 0 to 5 (each score represent 20% of leaf area). Significant differences are represented by different letters by Tukey test ($p < 0.05$)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

252

253 Root scanning allowed the determination of root total length, area, volume and diameter
254 for *P. trichocarpa* grown in different Cd and Zn concentrations. Results for root morphology
255 parameters, leaf transpiration and stomatal conductance can be found in Table S1. Roots under
256 Cd treatments displayed a similar response as the other parameters assessed, with evident
257 toxicity effects only at the highest concentration of 243 mg kg⁻¹. In the case of Zn, length, area
258 and volume reduction of roots was caused mainly at 810 mg kg⁻¹ or higher concentrations. As
259 for the analyses of stomatal conductance (gs) and transpiration rate (E), there were no
260 significant differences among Zn treatments (Control – 810 mg kg⁻¹) or among Cd treatments,
261 except for the highest concentration of 243 mg kg⁻¹, in which there was a reduction in the
262 transpiration rate (E) in comparison to the control, from 2.65 to 0.48 mmol m⁻² s⁻¹, and in
263 stomatal conductance (gs), from 0.084 to 0.008 mol m⁻² s⁻¹ (Tukey test, $p = 0.0009$ and $p =$
264 0.0004, respectively).

265

266 3.2 Cadmium and zinc uptake, accumulation and translocation

267 Cd uptake in poplar roots increased almost exponentially and was at least 10 times the
268 concentration applied in some treatments (1 to 9 mg kg⁻¹ Cd) (Fig. 4). In leaves, an increasing
269 uptake is observed only until the concentration of 9 mg kg⁻¹ Cd, after which there is a plateau
270 and Cd concentration is maintained around 50 mg kg⁻¹ (Fig. 4). However, in the treatment with
271 243 mg kg⁻¹, Cd accumulation surpasses the plateau concentration in more than 10 times (from
272 an average of 45 to 681 mg kg⁻¹). The bioconcentration factor (BCF) shows a decrease in Cd
273 accumulation for both roots and leaves as concentrations in soil increases (Table 2), except at
274 the highest concentration which had a BCF of 47.6 in poplar roots (tissue concentration of
275 6,537 mg kg⁻¹ Cd), suggesting a loss of regulation in Cd uptake and excessive metal
276 accumulation (Fig. 4). Overall the concentration of 9 mg kg⁻¹ Cd appears to be the threshold in
277 Cd translocation from roots to shoots ($Tf = 41\%$, the highest in this study), after which the ratio
278 between root and leaf concentration was reduced almost by half ($Tf = 26\%$ at 27 mg kg⁻¹ Cd).
279 At the applied dose of 81 mg kg⁻¹ Cd, root biomass was not affected despite tissue
280 concentrations reaching nearly 500 mg kg⁻¹ Cd (Table 1 and Fig. 4). Cadmium concentration,
281 translocation factor (Tf : roots-to-leaves) and bioconcentration factor (BCF) can be found in
282 Table S4.

283 Unlike with Cd, Zn content in roots did not differ significantly at lower soil concentrations
284 (≤ 90 mg kg⁻¹), increasing only after 270 mg kg⁻¹ Zn (Fig. 4). Zn content in leaves was a direct
285 result of the concentration applied, although only a slight increase was observed between
286 treatments of 810 and 2430 mg kg⁻¹ Zn.

287

288

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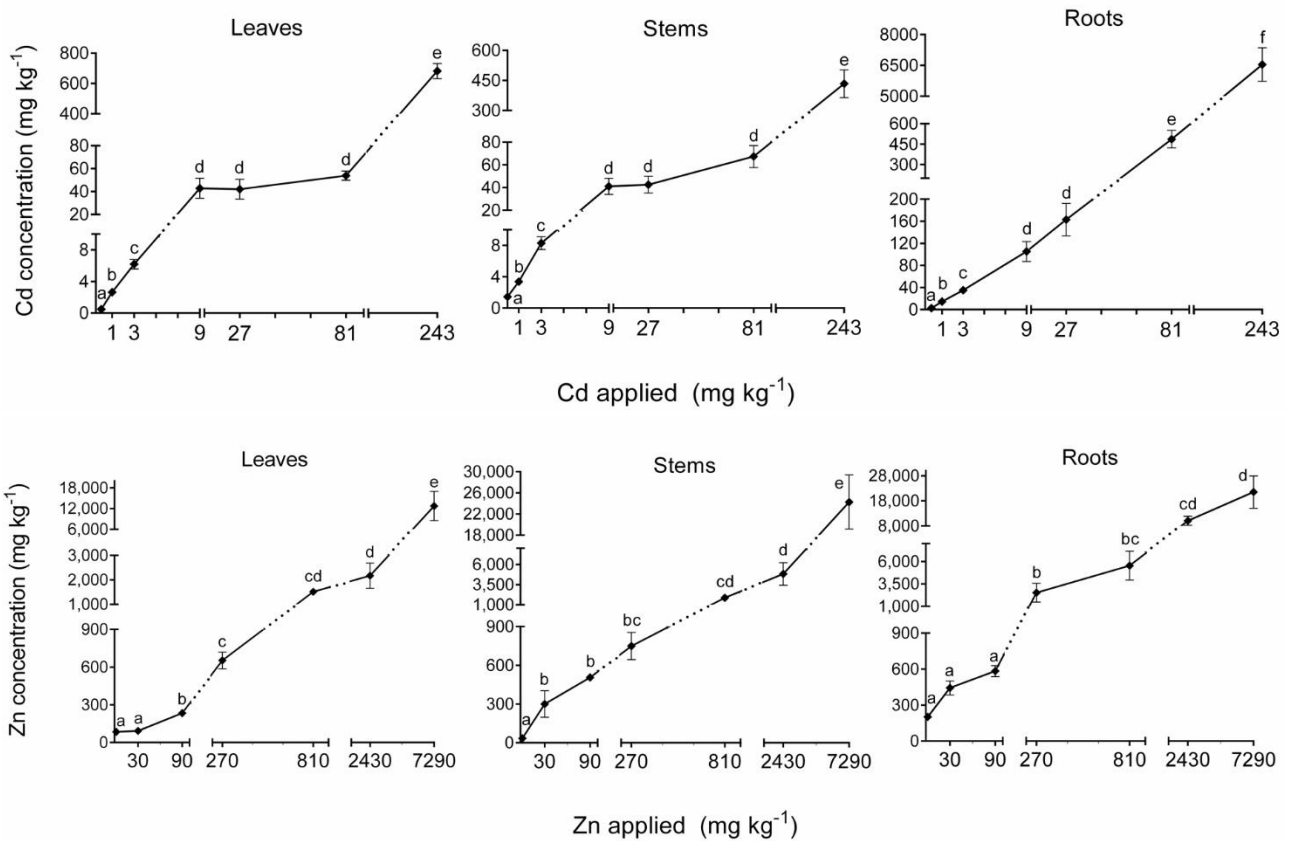


Fig. 4 - Cd and Zn concentrations (mg kg^{-1}) in leaves, stems and roots of *Populus trichocarpa* grown for five weeks in sandy substrate at different Cd or Zn doses. Error bars indicate standard error of the mean ($n = 4$). Different letters correspond to significant differences between doses applied (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.

290

291 Zn accumulation in roots varied across all treatments, and the highest BCF was found at
 292 30 mg kg^{-1} , and lowest at 7290 mg kg^{-1} (Table 2), however at the latter, translocation of Zn
 293 from roots to leaves was the highest found in this study ($Tf = 59\%$). Considering the tolerance
 294 indexes, 90 mg kg^{-1} Zn was the threshold for toxicity in both poplar roots and shoots (Table 1).
 295 Interestingly, this treatment showed a translocation factor of 40%, nearly the same factor found
 296 at the Cd threshold concentration of 9 mg kg^{-1} . Zinc concentration, translocation factor (Tf:
 297 roots-to-leaves) and bioconcentration factor (BCF) can be found in Table S5.

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

299 Stomatal conductance (gs) and transpiration rates (E) had a lower correlation to almost all other
 300 parameters assessed (especially root parameters), however both variables were highly
 301 correlated ($r_s > 0.70$) to the number of leaves (NL) and shoot growth (SG) (Table S2). Overall
 302 Zn treatments had a similar correlation among all the parameters assessed to Cd treatments
 303 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) | <i>Tf</i> | 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) | <i>Tf</i> | 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.

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307 3.3 Expression of *Pt-HMA4* under Cd and Zn stress

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

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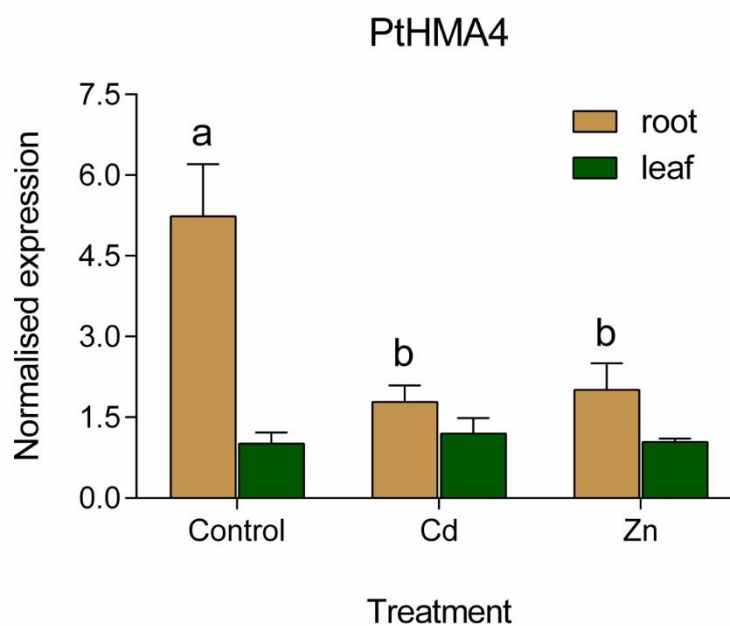


Fig. 5 - Transcript levels of the *PtHMA4* gene in roots and leaves of *P. trichocarpa* after growing for eight weeks under Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) stress, and without any metal addition (Control). The mRNA levels were quantified by real-time qPCR and normalised in relation to Ubiquitin (*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.

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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 Cd₂₇ + Zn₉₀, almost three times higher than the concentration found when Cd was added singly (Cd₂₇), 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 (Zn₉₀ and Zn₂₇₀) (Fig. 6).

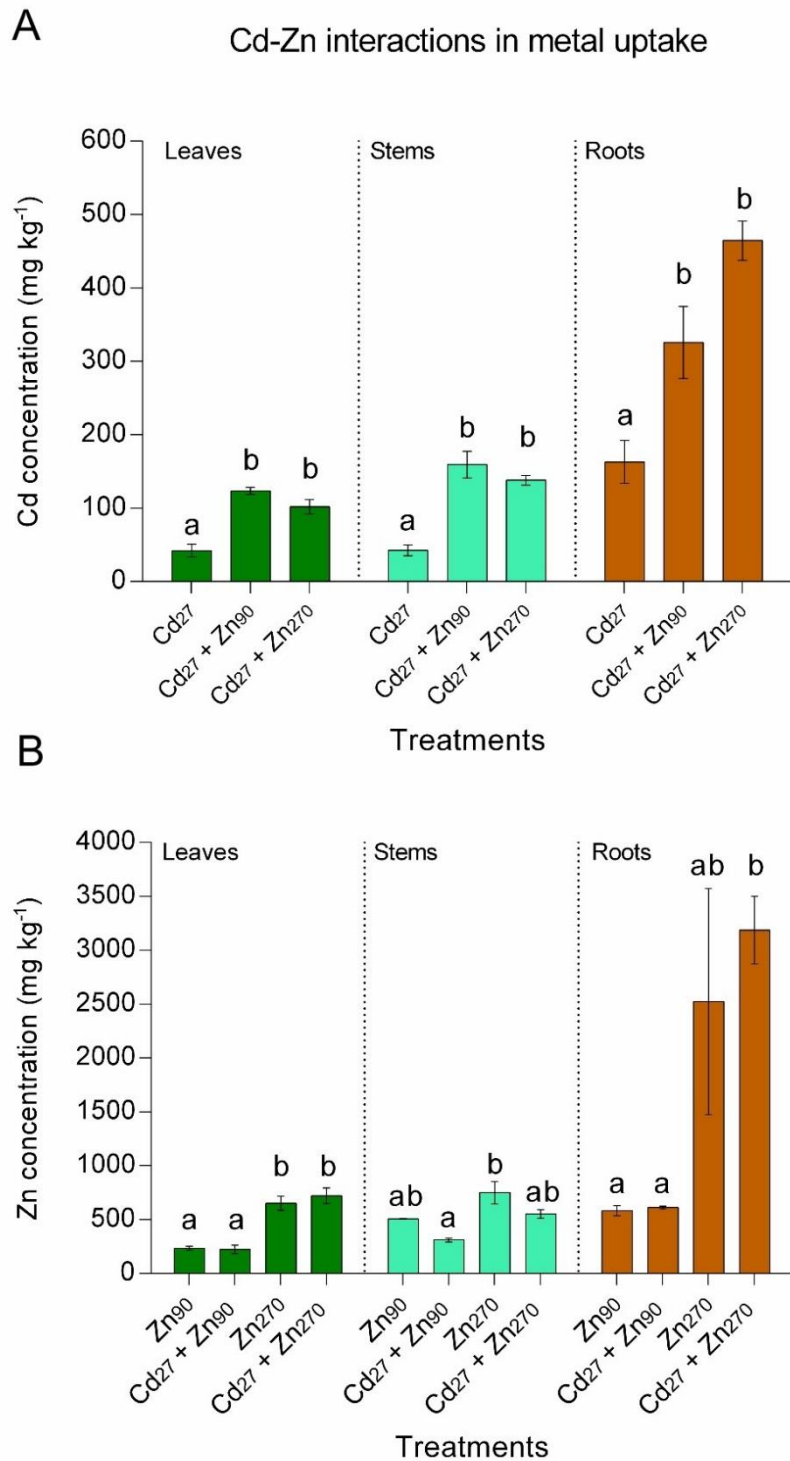


Fig. 6 - Concentrations of Cd (A) and Zn (B) in leaves, stems and roots of *Populus trichocarpa* exposed to different metal combinations: 27 mg kg⁻¹ Cd, 90 or 270 mg kg⁻¹ Zn. Different letters correspond to significant differences among treatments for the same plant tissue, Tukey test, $p < 0.05$ (A)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

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335 4. Discussion

336 4.1 Cadmium accumulation, distribution and toxicity

337 Exposure to Cd often leads to oxidative stress and phytotoxicity (Benavides *et al.*, 2005)
338 as a result of Cd replacing other essential elements (e.g. Ca, Fe, Mg and Zn) in enzymes, which
339 usually lose their function (Clemens, 2006; Verbruggen *et al.*, 2009; Kupper and Andresen,
340 2016). Growth impairment is a typical effect from Cd toxicity (Pal *et al.*, 2006), biomass
341 decrease in roots and shoots are commonly reported (Tran and Popova, 2013), as well as foliar
342 chlorosis and necrosis (Das *et al.*, 1997). In the current experiment, despite *P. trichocarpa*
343 showing symptoms of toxicity in leaves under Cd exposure, particularly at soil concentrations
344 higher than 27 mg kg⁻¹ Cd, loss of biomass was not evident in most of the treatments. Only at
345 the highest concentration did all roots, stems and leaves present obvious toxic effects,
346 indicating a remarkable tolerance to Cd in comparison to other published studies (Table 3).
347 According to Audet and Charest (2008), plants from the Brassicaceae family, known for their
348 high tolerance to metals, tend to maintain a constant biomass allocation to roots despite
349 exposure to higher metal concentrations in soils, similar to that observed for poplars exposed
350 to Cd in the present study, suggesting that in both cases metal partitioning plays a larger role
351 in tolerance than does biomass plasticity.

352 Tolerance index (TI) is a good measure to compare different studies regarding metal
353 toxicity. In this work, the tolerance index ranged from 107 to 75% in leaves across all Cd
354 treatments, excluding the highest Cd concentration, which displayed a conspicuous toxicity.
355 These values are within the bounds reported for poplars exposed to Cd concentrations lower
356 than 30 mg kg⁻¹: TI of 90 to 78% in *P. x canescens* (Dai *et al.*, 2013) and 91% in *P. nigra*
357 (Gaudet *et al.*, 2011). The most important mechanism for Cd tolerance in plants is the metal
358 chelation and compartmentalization into the vacuoles (Sharma *et al.*, 2016), especially via the
359 phytochelatin (PC) pathway (Clemens, 2006). Expression of genes encoding metallothioneins

360 (metal chelation) and heat shock proteins (proper protein folding) due to Cd exposure were
361 also associated with stress tolerance mechanisms in poplars (Hassinen *et al.*, 2009; Hasan *et*
362 *al.*, 2017).

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

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

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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.

| <i>Populus</i> species | Growth substrate | Metal concentration | Phytotoxicity | LOAEC | Reference |
|------------------------|------------------|--------------------------|--------------------------------|---------------------|-----------|
| <i>P. alba</i> | 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 |
| <i>P. canescens</i> | 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 |
| <i>P. deltoides</i> | soil | 8.14 Cd | photosynthesis | 8.14 Cd | 16 |
| | soil + waste | 10,300 Zn; 5.5 Cd | x | x | 17 |
| <i>P. euramericana</i> | 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.*

| <i>Populus</i> species | Growth medium | Metal concentration | Parameter affected | LOAEC | Reference |
|------------------------|----------------|-------------------------------|----------------------------|---------|---------------|
| <i>P. nigra</i> | 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 |
| <i>P. pyramidalis</i> | soil | 0 – 100 Cd | leaf biomass | 25 Cd | 26 |
| <i>P. tremula</i> | 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 |
| <i>P. trichocarpa</i> | 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 |
| <i>Populus</i> 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] Cicitelli *et al.*, 2010; [4] Cicitelli *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.

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387 At 9 mg kg⁻¹ the high translocation of Cd to aboveground parts (Tf: 41%) is considered

388 to be a common mechanism of hyperaccumulators, in which the metal is detoxified by

389 chelation, vacuole storage and rapidly translocation to shoots via the xylem (Tran and Popova,

390 2013). However, at 81 kg kg⁻¹, there is a much higher Cd uptake in roots, which is a reflection391 of the non-specific mechanisms by which Cd enters the plant system (Lux *et al.*, 2011), thus in

392 order to avoid toxicity in the photosynthetic apparatus, there is a limited transport of Cd to the

393 shoots (Tf: 11%). Restricting root-to-shoot translocation is a strategy typical of woody species

394 that may contribute to metal tolerance (Arduini *et al.*, 1996) since the first important barrier

395 against Cd toxicity is the immobilization in cell walls in roots (Sanita di Toppi and Gabbrielli,
396 1999). Lower translocation of Cd to shoots can be due to different mechanisms, such as down-
397 regulation of transporter proteins (i. e. heavy metal ATPases and ABC transporters) responsible
398 for Cd loading in the xylem or increasing production of metal chelators (Lux *et al.*, 2011).
399 Lignification of cortical cells, sclerenchyma walls and vascular tissues can also be triggered by
400 Cd (Luković *et al.*, 2012; Kupper and Andresen, 2016; Tylova *et al.*, 2017), which may
401 contribute to the thickening of the Casparian bands in the root apex (Schreiber *et al.*, 1999;
402 White, 2001) where high influx of Cd²⁺ occurs (He *et al.*, 2011).

403

404 4.2 Zn accumulation, distribution and toxicity

405 Phytotoxic effects of Zn in plants is characterised by growth inhibition, leaf chlorosis
406 and necrosis, oxidative stress, impairment of photosynthesis, degradation of mitochondria and
407 chloroplasts (Todeschini *et al.*, 2011) and, in general, Zn concentration in leaves above 300 mg
408 kg⁻¹ induces visible toxicity symptoms (Marschner, 1995). Although there were no differences
409 from control in terms of foliar symptoms at lower Zn doses applied (≤ 270 mg kg⁻¹ Zn), *P.*
410 *trichocarpa* had significantly less leaf and stem biomasses even at the lowest dose of 30 mg
411 kg⁻¹, considered to be a sub-lethal concentration (< 65 mg kg⁻¹) (Romeo *et al.*, 2014). It should
412 be noted that in our experiment the metal solutions were applied in a single pulse, in which a
413 rapid uptake could have occurred in these plants immediately after contamination and may
414 have impaired plant growth due to salinity or osmotic stress (Polle *et al.*, 2013). Recent studies
415 have classified poplars as being sensitive to moderately sensitive to salinity stress (Mirck and
416 Zalesny, 2015). Moreover, high cation additions (≥ 270 mg kg⁻¹ Zn; or 243 mg kg⁻¹ Cd)
417 significantly decreased the substrate pH, especially at extreme Zn concentrations (2430 and
418 7290 mg kg⁻¹), thus it is evident that this acidification could have led to an acute toxicity by
419 enhancing Zn²⁺ availability in the rhizosphere (Alloway, 2008).

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

431 Although Zn doses applied were 10 times higher than Cd, Zn translocation response
432 (based on Tf values) was somewhat analogous to the patterns seen in Cd-treated poplars. This
433 suggests that *P. trichocarpa* adopts similar strategies for dealing with Cd and Zn toxicity by
434 drastically decreasing metal translocation after a certain concentration threshold, in this case at
435 270 mg kg⁻¹ Zn. Reducing Zn translocation as a protective effect was also seen in *P. alba*
436 (Romeo *et al.*, 2014) and *P. nigra* (Dos Santos Utmazian and Wenzel, 2007).

437

438 4.3 *Pt-HMA4* is down-regulated in roots under Cd and Zn stress

439 The significant decrease in root-to-shoot translocation of Cd and Zn observed at the doses
440 applied of 81 mg kg⁻¹ Cd and 270 mg kg⁻¹ Zn, led us to investigate if the ATPase HMA4, which
441 plays a pivotal role in metal detoxification and long distance transport in plants (Luo *et al.*,
442 2016; Sarwar *et al.*, 2017), could help explain such findings. *Pt-HMA4* was expressed highly
443 in roots, similar to what has been observed for other members of the HMA family in poplar,
444 specifically around xylem vessels (Migeon *et al.*, 2010). In *A. halleri*, exposure to Zn clearly

445 showed an abundance of HMA4 transcripts in the root xylem adjacent to the pericycle layer,
446 which emphasises HMA4 involvement in xylem loading and justifies its high expression in
447 root tissues (Hanikenne *et al.*, 2008).

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

461

462 4.4 Cd and Zn interactions in poplar

463 Decrease in Cd uptake in plants due to elevated Zn supply has been commonly shown and
464 is often associated with competitive interactions during root uptake, in which Cd is believed to
465 enter the plant via transport processes inherent to Zn (Marschner, 1995; Hart *et al.*, 2002; Garg
466 and Kaur, 2013). The opposite can also be observed, for instance in wheat, a decrease in Zn
467 translocation was attributed to competition with high Cd concentrations in soil (Green *et al.*,
468 2010). We predicted similar outcomes, in which Zn would be preferentially taken up by the
469 roots, therefore reducing Cd accumulation in the plant. However Zn had the opposite effect in

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

472 A pH decline in the substrate due to high cationic concentration (Zn^{2+}) may have played
473 an important part in increasing Cd uptake, which is known for the inverse relationship with soil
474 pH (Chuan *et al.*, 1996; Smolders and Mertens, 2013). But substrate pH was unaffected by the
475 addition of 90 mg kg^{-1} Zn compared to when Cd was added singly (pH of 6.1 in both cases),
476 yet it still lead to a significant increase in Cd concentrations in all plant parts: for instance Cd
477 concentration in leaves increased from 42 mg kg^{-1} under single metal treatment to 123 mg kg^{-1}
478 ¹ under the combined treatment. Similar effect was observed in *Nocceae caerulescens*, in which
479 combined treatments of Zn ($500\text{ }\mu\text{M}$) and Cd ($200\text{ }\mu\text{M}$) in hydroponic cultures resulted in
480 increasing Cd^{2+} influx into root tissues and higher accumulation in shoots (Papoyan *et al.*,
481 2007), and this response has been associated with hyperaccumulator phenotypes (Lasat *et al.*,
482 1998; Papoyan and Kochian, 2004). Moreover, the hyperaccumulator *Brassica juncea* had an
483 increase in Cd uptake after Zn addition, leading also to a higher tolerance in comparison to
484 plants exposed to Cd and Zn separately (Kutrowska *et al.*, 2017). In field conditions, positive
485 correlation between Zn and Cd accumulation in shoots was also reported in Cacao trees
486 (Arévalo-Gardini *et al.* 2017). Such response might be related to an up-regulation of genes
487 encoding some metal transporters in roots triggered by the exposure to Zn^{2+} , through which
488 Cd^{2+} could have been actively transported. For instance, in *Salix caprea* the combined
489 treatment of Cd and Zn induced the expression of transporters ZIP6 and HMA1 (Konlechner
490 *et al.*, 2013). Another reason for higher Cd uptake can be attributed to the direct competition
491 between Zn and Cd for the soil adsorption sites (Lu and Xu 2009), for these elements have
492 similar atomic characteristics and are both affected by electrostatic interactions (Moreira and
493 Alleoni, 2010). Considering that the concentrations of Zn added were at least three times higher
494 than Cd, it is likely that Zn caused a displacement of Cd into the solution, increasing its

495 availability for plant uptake.

496 Metal accumulation in *P. trichocarpa* varied depending on external metal contents and
497 also the plant's own regulatory system, which in some cases presented responses analogous to
498 hyperaccumulator plants. Foliar concentration of 123 mg kg⁻¹ Cd is not high compared to well
499 established Cd-hyperaccumulators such as *N. caerulescens*, that can accumulate more than
500 3000 mg kg⁻¹ DW (Papoyan *et al.*, 2007). However Cd is naturally in plants at levels lower
501 than 1 mg kg⁻¹ (Reeves, 2006) and, according to Baker *et al.* (2000) and He *et al.* (2017),
502 concentrations higher than 100 mg kg⁻¹ Cd are exceptional and can be the threshold for
503 recognizing a hyperaccumulator of Cd (0.01% of dry weight).

504 Zn addition lead to higher Cd accumulation in leaves and stems, but this did not result in
505 higher toxicity, suggesting that Zn also had a protective effect. According to Cherif *et al.*
506 (2011), Zn addition can restore and enhance the functional activities of antioxidant enzymes
507 such as superoxide dismutase, catalase and glutathione reductase that are suppressed by Cd
508 toxicity. Concentration around 65 mg L⁻¹ Zn improved the photoprotective and antioxidant
509 responses (α -Tocopherol and reduced glutathione) in two poplar clones in hydroponics
510 (Fernandez-Martinez *et al.*, 2014). Overall, Zn can protect cells from damaging reactions
511 caused by reactive oxygen species (ROS) and compete with Cd for binding sites in enzymes (-
512 SH groups) and membrane proteins (Cakmak, 2000; Cherif *et al.*, 2011).

513

514 5. Conclusions

515 Cadmium and zinc toxicity affected growth and metal allocation in *Populus trichocarpa*
516 'Trichobel', in which Cd transport appears to be strongly regulated to some extent (\leq 81 mg
517 kg⁻¹). Although shoot concentrations were not as high as found in extreme hyperaccumulator
518 plants, this variety of poplar has an exceptional tolerance to Cd, especially considering that
519 phytotoxicity was mainly found in high and drastic Cd concentrations (\geq 27 mg kg⁻¹), in which

520 root integrity was barely affected. At lower Cd concentrations, *P. trichocarpa* displayed similar
521 tolerance mechanisms and translocation patterns found in plants with hyperaccumulator
522 phenotypes; in which metal partitioning appears to play a major role in Cd tolerance. Decrease
523 in translocation at high metal concentrations was achieved partly by down-regulating the
524 expression of *Pt-HMA4* in roots. Zn promoted Cd uptake and shoot accumulation without
525 compromising plant growth. Such results suggest that *P. trichocarpa* has the potential to
526 survive, stabilise and extract Cd from soils in areas contaminated with both Cd and Zn and be
527 a valid candidate for phytoremediation, especially in a short rotation coppice system. However,
528 it is still necessary to better comprehend the interactions between Cd, Zn and other toxic metals
529 in this species, as well as consider its interactions with surrounding soil microbiota (e. g.
530 mycorrhizal symbiosis).

531

532 **Appendix A. Supplementary data**

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

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References

Adams JP, Adeli A, Hsu CY. et al. 2011. Poplar maintains zinc homeostasis with heavy metal genes *HMA4* and *PCS1*. *Journal of Experimental Botany*. **62**: 3737-3752.

Alloway BJ. 2008. *Zn in Soils and Crop Nutrition*, 2nd edn. Brussels, Belgium and Paris: IZA and IFA.

Alloway BJ. 2013. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer.

Arduini I, Godbold DL, Onnis A. 1996. Cadmium and copper uptake and distribution in Mediterranean tree seedlings, *Physiologia Plantarum* **97**: 111–117. doi: 10.1111/j.1399-3054.1996.tb00486.x.

Arévalo-Gardini E, Arévalo-Hernández CO, Baligar VC, He ZL. Heavy metal accumulation in leaves and beans of cacao (*Theobroma cacao* L.) in major cacao growing regions of Peru, *Science of the Total Environment* **605-606**: 792-800. doi: 10.1016/j.scitotenv.2017.06.122.

ATSDR, Agency for Toxic Substances and Disease Registry. 2017. *CERCLA Priority List of Hazardous Substances*. <https://www.atsdr.cdc.gov/spl/index.html> . 24 Oct. 2017.

Audet P, Charest C. 2008. Allocation plasticity and plant–metal partitioning: Meta-analytical perspectives in phytoremediation, *Environmental Pollution*. **156**: 290–296. doi: 10.1016/j.envpol.2008.02.010.

Baker AJM, McGrath SP, Reeves RD, Smith JAC. 2000. Metal hyperaccumulator plants: A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry N, Banuelos G, Vangrosveld J, eds. *Phytoremediation of Contaminated Soil And Water*. London: LewisPublisher, 85–107.

Benavides MP, Gallego SM, Tomaro ML. 2005. Cadmium toxicity in plants, *Brazilian Journal of Plant Physiology*. **17**: 21–34.

Bhargava A, Carmona FF, Bhargava M, Srivastava S. 2012. Approaches for enhanced phytoextraction of heavy metals, *Journal of Environmental Management*. **105**: 103–120.

Bradshaw H, Ceulemans R, Davis J, Stettler R. 2000. Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree, *Journal of Plant Growth Regulation*. **19**: 306–313. <https://doi.org/10.1007/s003440000030>

590 **Brunner I, Luster J, Gunthardt-Goerg S, Frey B. 2008.** Heavy metal accumulation and
591 phytostabilisation potential of tree fine roots in a contaminated soil, *Environmental Pollution*.
592 **152:** 559-568.
593

594 **Burgess PJ, Incoll LD, Corry DT, Beaton A, Hart BJ. 2005.** Poplar (*Populus spp*) growth
595 and crop yields in a silvoarable experiment at three lowland sites in England, *Agroforestry*
596 *Systems*. **63:** 157-169. doi.org/10.1007/s10457-004-7169-9.
597

598 **Cakmak I. 2000.** Possible roles of zinc in protecting plant cells from damage by reactive
599 oxygen species, *New Phytologist*. **146:** 185–205. doi: 10.1046/j.1469-8137.2000.00630.x.
600

601 **Castiglione S, Franchin C, Fossati T, Lingua G, Torrigiani P, Biondi S. 2007.** High zinc
602 concentrations reduce rooting capacity and alter metallothionein gene expression in white
603 poplar (*Populus alba* L. cv. Villafranca), *Chemosphere*, **67:** 1117–1126. doi:
604 <http://dx.doi.org/10.1016/j.chemosphere.2006.11.039>.
605

606 **Castiglione S, Todeschini V, Franchin C, et al. 2009.** Clonal differences in survival capacity,
607 copper and zinc accumulation, and correlation with leaf polyamine levels in poplar: a large-
608 scale field trial on heavily polluted soil, *Environmental Pollution*. **157:** 2108–2117.
609

610 **Cherif J, Mediouni C, Ammar WB, Jemal F. 2011.** Interactions of zinc and cadmium toxicity
611 in their effects on growth and in antioxidative systems in tomato plants (*Solanum*
612 *lycopersicum*), *Journal of Environmental Sciences*. **23:** 837–844. doi: 10.1016/S1001-
613 0742(10)60415-9.
614

615 **Chuan MC, Shu GY, Liu JC. 1996.** Solubility of heavy metals in a contaminated soil: Effects
616 of redox potential and pH, *Water, Air and Soil Pollution*. **90:** 543–556. doi:
617 10.1007/BF00282668.
618

619 **Ciadamidaro L, Madejon P, Madejon E. 2014.** Soil chemical and biochemical properties
620 under *Populus alba* growing: Three years study in trace element contaminated soils, *Applied*
621 *Soil Ecology*. **73:** 26-33.
622

623 **Cicatelli A, Lingua G, Todeschini V, Biondi S, Torrigiani P, Castiglione S. 2010.**
624 Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy
625 metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and
626 polyamine biosynthetic gene expression, *Annals of Botany*. **106:** 791–802.
627

628 **Cicatelli A, Lingua G, Todeschini V, Biondi S, Torrigiani P, Castiglione S. 2012.**
629 Arbuscular mycorrhizal fungi modulate the leaf transcriptome of a *Populus alba* L. clone
630 grown on a zinc and copper-contaminated soil, *Environmental and Experimental Botany*. **75:**
631 25–35.
632

633 **Claus J, Bohmann A, Chavarria-Krauser A. 2013.** Zinc uptake and radial transport in roots
634 of *Arabidopsis thaliana*: a modelling approach to understand accumulation. *Annals of Botany*.
635 **112:** 369-380.
636

637 **Clemens S. 2006.** Toxic metal accumulation, responses to exposure and mechanisms of
638 tolerance in plants, *Biochimie*. **88:** 1707–1719.
639

640 **Dai HP, Shan CJ, Jia GL. et al. 2013.** Responses to cadmium tolerance, accumulation and
641 translocation in *Populus × canescens*, *Water, Air and Soil Pollution*. **224**: 1-9.
642

643 **Das P, Samantaray S, Rout GR. 1997.** Studies on cadmium toxicity in plants: a review,
644 *Environal Pollution*. **98**: 29–36.
645

646 **Di Baccio D, Kopriva S, Sebastiani L, Rennenberg H. 2005.** Does glutathione metabolism
647 have a role in the defence of poplar against zinc excess?, *New Phytologist*. **167**: 73-80.
648

649 **Di Baccio D, Minnocci A, Sebastiani L. 2010.** Leaf structural modifications in *Populus x*
650 *euramericana* subjected to Zn excess, *Biologia Plantarum*. **54**: 502-508.
651

652 **Di Baccio D, Tognetti R, Minnocci A, Sebastiani L. 2009.** Responses of the *Populus x*
653 *euramericana* clone I-214 to excess zinc: Carbon assimilation, structural modifications, metal
654 distribution and cellular localization, *Environmental and Experimental Botany*. **67**: 153-163.
655

656 **Di Lonardo S, Capuana M, Arnetoli M, Gabbrielli R, Gonnelli C. 2011.** Exploring the
657 metal phytoremediation potential of three *Populus alba* L. clones using an in vitro screening,
658 *Environmental Science and Pollution Research*. **18**: 82–90.
659

660 **Dos Santos Utmazian MN, Wenzel WW. 2007.** Cadmium and zinc accumulation in willow
661 and poplar species grown on polluted soils, *Journal of Plant Nutrition and Soil Science*. **170**:
662 265–272.
663

664 **Dos Santos Utmazian MN, Wieshammer G, Vega R, Wenzel WW. 2007.** Hydroponic
665 screening for metal resistance and accumulation of cadmium and zinc in twenty clones of
666 willows and poplars, *Environmental Pollution*. **148**, 155–165. doi:
667 <http://dx.doi.org/10.1016/j.envpol.2006.10.045>.
668

669 **Durand TC, Baillif P, Alberic P, Carpin S, Label P, Hausman JF, Morabito D. 2011.**
670 Cadmium and zinc are differentially distributed in *Populus tremula* x *P. alba* exposed to metal
671 excess, *Plant Biosystems*. **145**: 397–405.
672

673 **Durand TC, Hausman JF, Carpin S, Alberic P, Baillif P, Label P, Morabito D. 2010a.**
674 Zinc and cadmium effects on growth and ion distribution in *Populus tremula* x *P. alba*,
675 *Biologia Plantarum*. **54**: 191-194.
676

677 **Durand TC, Sergeant K, Planchon S. et al. 2010b.** Acute metal stress in *Populus tremula* x
678 *P. alba* (717-1B4 genotype): Leaf and cambial proteome changes induced by cadmium²⁺,
679 *Proteomics*. **10**: 349-368.
680

681 Fernandez-Martinez J, Zacchini M, Fernandez-Marin B, Garcia-Plazaola JI, Fleck I. 2014. Gas-
682 exchange, photo- and antioxidant protection, and metal accumulation in I-214 and Eridano
683 *Populus* sp. clones subjected to elevated zinc concentrations, *Environmental and Experimental*
684 *Botany*. **107**: 144-153.
685

686 **Franchin C, Fossati T, Pasquini E, Lingua G, Castiglione S, Torrigiani P, Biondi S. 2007.**
687 High concentrations of zinc and copper induce differential polyamine responses in
688 micropropagated white poplar (*Populus alba*), *Physiologia Plantarum*. **130**: 77–90.
689

690 **Garg N, Kaur H. 2013.** Impact of cadmium-zinc interactions on metal uptake, translocation
691 and yield in pigeonpea genotypes colonized by arbuscular mycorrhizal fungi, *Journal of Plant*
692 *Nutrition*. **36**: 67–90.
693

694 **Gaudet M, Pietrini F, Beritognolo I. et al. 2011.** Intraspecific variation of physiological and
695 molecular response to cadmium stress in *Populus nigra* L., *Tree Physiology*. **31**: 1309–1318.
696

697 **Green CE, Chaney RL, Bouwkamp J. 2003.** Interactions between cadmium uptake and
698 phytotoxic levels of zinc in hard red spring wheat, *Journal of Plant Nutrition*. **26**: 417–430.
699 doi: 10.1081/PLN-120017144.
700

701 **Green CE, Chaney RL, Bouwkamp J. 2017.** Increased zinc supply does not inhibit cadmium
702 accumulation by rice (*Oryza sativa* L.), *Journal of Plant Nutrition*. **40**: 869-877. doi:
703 10.1080/01904167.2016.1262407
704

705 **Green ID, Diaz A, Tibbett M. 2010.** Factors affecting the concentration in seven-spotted
706 ladybirds (*Coccinella septempunctata* L.) of Cd and Zn transferred through the food chain,
707 *Environmental Pollution*. **158**: 135-141.
708

709 **Green ID, Jeffries C, Diaz A, Tibbett M. 2006.** Contrasting behaviour of cadmium and zinc
710 in a soil-lant-arthropod system, *Chemosphere*. **61**: 1115-1121.
711

712 **Green ID, Tibbett M. 2008.** Differential uptake, partitioning and transfer of Cd and Zn in the
713 soil-pea plant-aphid system, *Environmental Science & Technology*. **42**: 450–455. doi:
714 10.1021/es071992c.
715

716 **Grispen VMJ, Hakvoort HWJ, Blik T, Verkleij JAC, Schat H. 2011.** Combined
717 expression of the *Arabidopsis* metallothionein MT2b and the heavy metal transporting ATPase
718 HMA4 enhances cadmium tolerance and the root to shoot translocation of cadmium and zinc
719 in tobacco. *Environmental and Experimental Botany*. **72**: 71-76.
720

721 **Guerra F, Gainza F, Perez R, Zamudio F. 2011.** Phytoremediation of heavy metals using
722 poplars (*Populus* Spp): A glimpse of the plant responses to copper, cadmium and zinc stress,
723 in: Golubev IA, ed. *Handbook of Phytoremediation*. New York: NOVA, 387–413.
724

725 **Hamberg L, Malmivaara-Lamsa M, Lofstrom I, Vartiamaki H, Valkonen S, Hantula J.**
726 **2011.** Sprouting of *Populus tremula* L. in spruce regeneration areas following alternative
727 treatments, *European Journal of Forest Research*. **130**: 99–106.
728

729 **Hamon RE, Holm PE, Lorenz SE, McGrath SP, Christensen TH. 1999.** Metal uptake by
730 plants from sludge-amended soils: caution is required in the plateau interpretation, *Plant and*
731 *Soil*. **216**: 53-64.
732

733 **Hanikenne M, Talke IN, Haydon MJ. et al. 2008.** Evolution of metal hyperaccumulation
734 required *cis*-regulatory changes and triplication of *HMA4*, *Nature*. **453**: 391-395.
735

736 **Hart J, Welch RM, Norvell WA, Kochian LV. 2002.** Transport interactions between
737 cadmium and zinc in roots of bread and durum wheat seedlings, *Physiologia Plantarum*. **116**:
738 73-78.
739

740 **Hasan K, Cheng Y, Kanwar MK, Chu XY, Ahammed GJ, Qi ZY. 2017.** Responses of plant
741 proteins to heavy metal stress - A review, *Frontiers in Plant Science*. **8**: 1492.
742

743 **Hassinen V, Vallinkoski VM, Issakainen S, Tervahauta A, Karenlampi S, Servomaa K.**
744 **2009.** Correlation of foliar *MT2b* expression with Cd and Zn concentrations in hybrid aspen
745 (*Populus tremula x tremuloides*) grown in contaminated soil. *Environmental Pollution*. **157**:
746 922-930.
747

748 **He J, Qin J, Long L. et al. 2011.** Net cadmium flux and accumulation reveal tissue-specific
749 oxidative stress and detoxification in *Populus × canescens*, *Physiologia Plantarum*. **143**: 50–
750 63.
751

752 **He S, Yang X, He Z, Baligar VC. 2017.** Morphological and physiological responses of plants
753 to cadmium toxicity: a review, *Pedosphere*. **27**: 421–438. doi: 10.1016/S1002-0160(17)60339-
754 4.
755

756 **Hu Y, Nan Z, Jin C, Wang N, Luo H. 2014.** Phytoextraction potential of poplar (*Populus*
757 *alba* L. var. *pyramidalis* Bunge) from calcareous agricultural soils contaminated by cadmium,
758 *International Journal of Phytoremediation*. **16**: 482–495. doi:
759 10.1080/15226514.2013.798616.
760

761 **Huang L, Bell RW, Dell B, Woodward J. 2004.** Rapid nitric acid digestion of plant material
762 with an open-vessel microwave system, *Communications in Soil Science and Plant Analysis*.
763 **35**: 427–440. doi: 10.1081/CSS-120029723.
764

765 **Iori V, Gaudet M, Fabbrini, F. et al. 2016.** Physiology and genetic architecture of traits
766 associated with cadmium tolerance and accumulation in *Populus nigra* L., *Trees*. **30**: 125-139.
767

768 **Jaakola L, Pirtilla M, Halonen M, Hohtola A. 2001.** Isolation of high quality RNA from
769 Bilberry (*Vaccinium myrtillus* L.) fruit. *Molecular Biotechnology*. **19**: 201-203.
770

771 **Jun R, Ling T. 2012.** Increase of Cd accumulation in five poplar (*Populus* L.) with different
772 supply levels of Cd, *International Journal of Phytoremediation*. **14**: 101–113. doi:
773 10.1080/15226514.2010.525564.
774

775 **Kabata-Pendias A, Pendias H. 2001.** *Trace Elements In Soils And Plants*, Florida: CRC press.
776

777 **Kariman K, Barker SJ, Jost R, Finnegan PM, Tibbett M. 2014.** A novel plant-fungus
778 symbiosis benefits the host without forming mycorrhizal structures, *New Phytologist*. **201**:
779 1413–1422.
780

781 **Kariman K, Barker SJ, Jost R, Finnegan PM, Tibbett M. 2016.** Sensitivity of jarrah
782 (*Eucalyptus marginata*) to phosphate, phosphite, and arsenate pulses as influenced by fungal
783 symbiotic associations, *Mycorrhiza*. **26**: 401–415. doi: 10.1007/s00572-015-0674-z.
784

785 **Kieffer P, Planchon S, Oufir M. et al. 2009.** Combining proteomics and metabolite analyses
786 to unravel cadmium stress-response in poplar leaves, *Journal of Proteome Research*. **8**: 400-
787 417.
788

789 **Kirkham MB. 2006.** Cadmium in plants on polluted soils: effects of soil factors,

790 hyperaccumulation, and amendments, *Geoderma*. **137**: 19–32.

791

792 **Konlechner C, Turktas M, Langer I. et al. 2013.** Expression of zinc and cadmium responsive
793 genes in leaves of willow (*Salix caprea* L.) genotypes with different accumulation
794 characteristics. *Environmental Pollution*. **178**: 121-127.

795

796 **Kupper H, Andresen E. 2016.** Mechanisms of metal toxicity in plants, *Metallomics*. **8**: 269-
797 285.

798

799 **Kutrowska A, Malecka A, Piechalak A et al. 2017.** Effects of binary metal combinations on
800 zinc, copper, cadmium and lead uptake and distribution in *Brassica juncea*, *Journal of Trace*
801 *Elements in Medicine and Biology*. **44**: 32–39. doi: 10.1016/j.jtemb.2017.05.007.

802

803 **Langer I, Krpata D, Fitz WJ, Wenzel WW, Schweiger PF. 2009.** Zinc accumulation
804 potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone
805 in a dose–response study, *Environmental Pollution*. **157**: 2871–2877. doi:
806 <http://dx.doi.org/10.1016/j.envpol.2009.04.003>.

807

808 **Lasat MM, Baker AJM, Kochian LV. 1998.** Altered Zn compartmentation in the root
809 symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn
810 hyperaccumulation in *Thlaspi caerulescens*, *Plant Physiology*. **118**: 875-883.

811

812 **Laureysens I, Blust R, De Temmerman L, Lemmens C, Ceulemans R. 2004.** Clonal
813 variation in heavy metal accumulation and biomass production in a poplar coppice culture: I.
814 Seasonal variation in leaf, wood and bark concentrations, *Environmental Pollution*. **131**: 485-
815 494.

816

817 **Lei M, Zhang Y, Khan S, Qin P, Liao B. 2010.** Pollution, fractionation, and mobility of Pb,
818 Cd, Cu, and Zn in garden and paddy soils from a Pb/Zn mining area, *Environmental Monitoring*
819 *and Assessment*. **168**: 215–222.

820

821 **Lingua G, Franchin C, Todeschini V. et al. 2008.** Arbuscular mycorrhizal fungi differentially
822 affect the response to high zinc concentrations of two registered poplar clones, *Environmental*
823 *Pollution*. **153**: 137–147.

824

825 **Lochlainn SO, Bowen HC, Fray RG. et al. 2011.** Tandem quadruplication of HMA4 in the
826 Zinc (Zn) and Cadmium (Cd) hyperaccumulator *Noccaea caerulescens*. *PLOS One*. **6**: e17814.
827 doi: 10.1371/journal.pone.0017814.

828

829 **Lu SG, Xu QF. 2009.** Competitive adsorption of Cd, Cu, Pb and Zn by different soils of
830 Eastern China, *Environmental Geology*. **57**: 685–693. doi: 10.1007/s00254-008-1347-4.

831

832 **Luković J, Merkulov L, Pajević S. et al. 2012.** Quantitative assessment of effects of cadmium
833 on the histological structure of poplar and willow leaves', *Water, Air and Soil Pollution*. **223**:
834 2979–2993. doi: 10.1007/s11270-012-1081-0.

835

836 **Luo ZB, He J, Polle A, Rennenberg H. 2016.** Heavy metal accumulation and signal
837 transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation
838 efficiency, *Biotechnology Advances*. **34**: 1131-1148.

839

840 **Lux A, Martinka M, Vaculik M, White PJ. 2011.** Root responses to cadmium in the
841 rhizosphere: a review, *Journal of Experimental Botany*. **62**: 21–37. doi: 10.1093/jxb/erq281.
842

843 **Marmioli M, Imperiale D, Maestri E, Marmioli N. 2013.** The response of *Populus* spp. to
844 cadmium stress: Chemical, morphological and proteomics study, *Chemosphere*. **93**: 1333–
845 1344.
846

847 **Marmioli N, Marmioli M, Maestri E. 2006.** Phytoremediation and phytotechnologies: a
848 review for the present and the future. In: Twardowska I, Allen HE, Häggblom MM, Stefaniak
849 S, eds. *Soil and Water Pollution Monitoring, Protection and Remediation, NATO Science*
850 *Series vol 69*. Dordrecht: Springer, 403–416.
851

852 **Marschner H. 1995.** Mineral Nutrition of Higher Plants, 1st edn. London: Academic Press.
853

854 **Migeon A, Blaudez D, Wilkins O. et al. 2010.** Genome-wide analysis of plant metal
855 transporters with an emphasis on poplar. *Cellular and Molecular Life Sciences*. **67**: 3763–3784.
856

857 **Mills RF, Francini A, Ferreira da Rocha PSC. et al. 2005.** The plant P_{1B}-type ATPase
858 AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied
859 at elevated levels. *FEBS Letters*. **579**: 783–791.
860

861 **Mirck J, Zalesny RS. 2015.** Mini-review of knowledge gaps in salt tolerance of plants applied
862 to willows and poplars, *International Journal of Phytoremediation*. **17**: 640–650. doi:
863 :10.1080/15226514.2014.950414.
864

865 **Moreira CS, Alleoni LRF. 2010.** Adsorption of Cd, Cu, Ni and Zn in tropical soils under
866 competitive and non-competitive systems, *Scientia Agricola*. **67**: 301–307. doi:
867 10.1590/S0103-90162010000300008.
868

869 **Obata H, Umebayashi M. 1997.** Effects of cadmium on mineral nutrient concentrations in
870 plants differing in tolerance for cadmium, *Journal of Plant Nutrition*. **20**: 97–105.
871

872 **Pál M, Horvath E, Janda T, Paldi E, Szalai G. 2006.** Physiological changes and defense
873 mechanisms induced by cadmium stress in maize, *Journal of Plant Nutrition and Soil Science*.
874 **169**: 239–246.
875

876 **Pajevic S, Borisec M, Nikolic N, Krstic B, Pilipovic A, Orlovic S. 2009.** Phytoremediation
877 capacity of poplar (*Populus* spp.) and willow (*Salix* spp.) clones in relation to photosynthesis,
878 *Archives of Biological Sciences*. **61**: 239–247.
879

880 **Papoyan A, Kochian LV. 2004.** Identification of *Thlaspi caerulescens* genes that may be
881 involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy
882 metal transporting ATPase, *Plant Physiology*. **136**: 3814–3823. doi: pp.104.044503.
883

884 **Papoyan A, Piñeros M, Kochian LV. 2007.** Plant Cd²⁺ and Zn²⁺ status effects on root and
885 shoot heavy metal accumulation in *Thlaspi caerulescens*, *New Phytologist*. **175**: 51–58. doi:
886 10.1111/j.1469-8137.2007.02073.x.
887

888 **Pilon-Smits E. 2005.** Phytoremediation, *Annu. Rev. Plant Biol.* **56**, 15–39.
889

- 890 **Polle A, Klein T, Kettner C. 2013.** Impact of cadmium on young plants of *Populus euphratica*
891 and *P. × canescens*, two poplar species that differ in stress tolerance, *New Forest*. **44**: 13–22.
892
- 893 **Pulford ID, Watson C. 2003.** Phytoremediation of heavy metal-contaminated land by trees -
894 A review, *Environment International*. **29**: 529–540.
895
- 896 **Rafati M, Khorasani N, Moattar F, Shirvany A, Moraghebi F, Hosseinzadeh S. 2011.**
897 Phytoremediation potential of *Populus alba* and *Morus alba* for cadmium, chromium and
898 nickel absorption from polluted soil, *International Journal of Environmental Research*. **5**: 961–
899 970.
900
- 901 **Reeves RD. 2006.** Hyperaccumulation of trace elements by plants. In: Morel JL, Echevarria
902 G, Goncharova N, eds. *Phytoremediation of Metal-contaminated Soils*. Netherlands: Springer,
903 25–52.
904
- 905 **Robinson BH, Banuelos G, Conesa HM, Evangelou MWH, Schulin R. 2009.** The
906 phytomanagement of trace elements in soil, *Critical Reviews in Plant Sciences*. **28**: 240–266.
907
- 908 **Romeo S, Francini A, Ariani A, Sebastiani L. 2014.** Phytoremediation of Zn: Identify the
909 diverging resistance, uptake and biomass production behaviours of poplar clones under high
910 zinc stress, *Water, Air and Soil Pollution*. **225**: 1-12.
911
- 912 **Rowell DL. 1994.** *Soil Science: Methods and Applications*. Essex: Longman Group UK Ltd.
913
- 914 **Sanita di Toppi L, Gabbriellini R. 1999.** Response to cadmium in higher plants, *Environmental*
915 *and Experimental Botany*. **41**: 105–130.
916
- 917 **Saraswat S, Rai JPN. 2009.** Chemistry and ecology phytoextraction potential of six plant
918 species grown in multimetal contaminated soil, *Chemistry and Ecology*. **25**: 1–11. doi:
919 10.1080/02757540802657185.
920
- 921 **Sarwar N, Imran M, Shaheen MR. et al. 2017.** Phytoremediation strategies for soils
922 contaminated with heavy metals: Modifications and future perspectives. *Chemosphere*. **171**:
923 710-721.
924
- 925 **Schreiber L, Hartmann K, Skrabs M, Zeier J. 1999.** Apoplastic barriers in roots: chemical
926 composition of endodermal and hypodermal cell walls, *Journal of Experimental Botany*. **50**:
927 1267–1280. doi: 10.1093/jxb/50.337.1267.
928
- 929 **Sebastiani L, Scebba F, Tognetti R. 2004.** Heavy metal accumulation and growth responses
930 in poplar clones Eridano (*Populus deltoides* × *maximowiczii*) and I-214 (*P. × euramericana*)
931 exposed to industrial waste, *Environmental and Experimental Botany*. **52**: 79–88. doi:
932 <http://dx.doi.org/10.1016/j.envexpbot.2004.01.003>.
933
- 934 **Sergeant K, Kieffer P, Dommes J, Hausman JF, Renaut J. 2014.** Proteomic changes in
935 leaves of poplar exposed to cadmium and low-temperature, *Environmental and Experimental*
936 *Botany*. **106**: 112-123.
937
- 938 **Sharma SS, Dietz KJ, Mimura T. 2016.** Vacuolar compartmentalization as indispensable
939 component of heavy metal detoxification in plants. *Plant, Cell and Environment*. **39**: 1112-

940 1126.
941
942 **Shim D, Kim S, Choi, Y. et al. 2013.** Transgenic poplar trees expressing yeast cadmium factor
943 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil,
944 *Chemosphere*. **90**: 1478–1486.
945
946 **Smolders E, Mertens J. 2013.** Cadmium. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn.
947 Netherlands: Springer, 283–311.
948
949 **Takahashi R, Bashir K, Ishimaru Y, Nishizawa NK, Nakanishi H. 2012.** The role of heavy-
950 metal ATPases, HMAs, in zinc and cadmium transport in rice. *Plant Signaling and Behaviour*.
951 **7**: 1605-1607.
952
953 **Todeschini V, Lingua G, D'Agostino G, Carniato F, Roccotiello E, Berta G. 2011.** Effects
954 of high zinc concentration on poplar leaves: A morphological and biochemical study,
955 *Environmental and Experimental Botany*. **71**: 50–56. doi:
956 <http://dx.doi.org/10.1016/j.envexpbot.2010.10.018>.
957
958 **Tran TA, Popova LP. 2013.** Functions and toxicity of cadmium in plants: Recent advances
959 and future prospects, *Turkish Journal of Botany*. **37**: 1–13. doi: 10.3906/bot-1112-16.
960
961 **Tuskan GA, DiFazio SP, Jansson S. et al. 2006.** The genome of black cottonwood, *Populus*
962 *trichocarpa* (Torr. & Gray). *Science*. **313**: 1596–1604.
963
964 **Tylova E, Peckova E, Blascheova Z, Soukoup A. 2017.** Casparian bands and suberin lamellae
965 in exodermis of lateral roots: an important trait of root systems response to abiotic stress
966 factors. *Annals of Botany*. **120**: 71-85.
967
968 **Verbruggen N, Hermans C, Schat H. 2009.** Mechanisms to cope with arsenic or cadmium
969 excess in plants, *Current Opinion in Plant Biology*. **12**: 364–372. doi:
970 <http://dx.doi.org/10.1016/j.pbi.2009.05.001>.
971
972 **White PJ. 2001.** The pathways of calcium movement to the xylem, *Journal of Experimental*
973 *Botany*. **52**: 891–899. doi: 10.1093/jexbot/52.358.891.
974
975 **Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012.** Primer-
976 BLAST: A tool to design target-specific primers for polymerase chain reaction. *BCM*
977 *Bioinformatics*. **13**: 134. doi: 10.1186/1471-2105-13-134.
978
979 **Zacchini M, Pietrini F, Mugnozza GS, Iori V, Pietrosanti L, Massacci A. 2009.** Metal
980 tolerance, accumulation and translocation in poplar and willow clones treated with cadmium
981 in hydroponics, *Water, Air and Soil Pollution*. **197**: 23–34. doi: 10.1007/s11270-008-9788-7.
982
983 **Zar JH. 2010.** *Biostatistical analysis*, 5th edn. New Jersey: Prentice Hall.
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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.

1021 **Fig. 6** - Concentrations of Cd and Zn in leaves, stems and roots of *Populus trichocarpa* exposed
1022 to different metal combinations: 27 mg kg⁻¹ Cd, 90 or 270 mg kg⁻¹ Zn. Different letters
1023 correspond to significant differences among treatments for the same plant tissue, Tukey test (p
1024 < 0.05) in the top figure and Dunn's test (p < 0.05) in the bottom figure.

1025 **APPENDIX A. SUPPLEMENTARY INFORMATION**

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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 |
|---------------------------------------|---------------|-----------------------|-------------------------|-----------------------|--------------------------------------|-------------------------------------|
| <i>Cd (mg kg⁻¹)</i> | -- 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 |
| <i>Zn (mg kg⁻¹)</i> | | | | | | |
| 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.

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

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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 $p < 0.05$.

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

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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 (µg plant ⁻¹) | Tf | 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 ($p < 0.01$).

$Tf = (\text{leaf concentration} / \text{root concentration}) \times 100$.

$BCF = (\text{plant concentration} / \text{soil concentration})$.

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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).

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