Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in Populus trichocarpa

Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in Populus trichocarpa

Journal Pre-proof

Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in *Populus trichocarpa*

Vinicius Henrique De Oliveira, Ihsan Ullah, Jim M. Dunwell, Mark Tibbett

PII: S0098-8472(19)31522-9

DOI: https://doi.org/10.1016/j.envexpbot.2019.103925

Reference: EEB 103925

To appear in: *Environmental and Experimental Botany*

Received Date: 26 August 2019

Revised Date: 10 October 2019

Accepted Date: 23 October 2019

Please cite this article as: De Oliveira VH, Ullah I, Dunwell JM, Tibbett M, Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in *Populus trichocarpa*, *Environmental and Experimental Botany* (2019), doi: https://doi.org/10.1016/j.envexpbot.2019.103925

Type: Original Paper

Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in *Populus trichocarpa*

Authors: Vinicius Henrique **DE OLIVEIRA**[1,2], Ihsan **ULLAH**[1], Jim M. **Dunwell**[1], Mark **TIBBETT** [1,2 *]

[1] School of Agriculture, Policy and Development, University of Reading, Earley Gate, Reading, RG6 6AR, UK.

[2] Centre for Agri-Environmental Research & Soil Research Centre, University of Reading, RG6 6AR, UK

Running title: Mycorrhizal influence on Cd/Zn partitioning in poplar tree

*Corresponding author: M. Tibbett: m.tibbett@reading.ac.uk / +44 (0)118 378 6026

Highlights:

- Under excess, *P. trichocarpa* restricts Cd transport to shoots but not Zn
- Expression patterns of *HMA4* and *ZIP1* suggest they transport both Cd and Zn
- Arbuscular mycorrhizal fungus modulates Cd/Zn accumulation and distribution in poplar

- Symbiosis up-regulates metallothionein *PtMT2b* in roots regardless of contamination
- *PtMT2b* greatly increases Cd tolerance in transgenic yeast under Cd stress

Abstract

We investigated how arbuscular mycorrhizal symbiosis can alter trace element uptake, distribution and toxicity in plants by examining some of the molecular mechanisms behind *Populus trichocarpa* tolerance to Cd and Zn, and the effects of AMF in metal homeostasis. Plants were grown under Cd and Zn contamination, with and without *Rhizophagus irregularis* inoculation. We determined organ metal concentrations, the expression of genes involved in trace element homeostasis, and the function of metallothionein *PtMT2b* by heterologous expression in yeast. *P. trichocarpa* was highly tolerant to both elements, with AMF increasing Zn accumulation. AMF altered the partitioning of Cd, but maintained the same patterns for Zn, indicating that despite being geochemically similar and carried mostly by the same transporters, the nutrient metal (Zn) is handled differently from the non-essential metal (Cd). High Zn and Cd down-regulated *PtHMA4* (roots), and up-regulated *PtZIP1* (leaves), suggesting their involvement in transporting both metals in poplar. *PtMT2b* was highly up-regulated in mycorrhizal roots and enhanced Cd tolerance in transformed yeast. *R. irregularis* reduced Cd transfer to poplar shoots, but did not affect Zn partitioning. The gene expression patterns observed offer a glimpse into the mechanisms behind trace element uptake/translocation dynamic in poplars, influenced by AMF symbiosis.

Keywords: arbuscular mycorrhizal fungi, heavy metal transporters, heterologous expression, metallothionein, phytoremediation

1. Introduction

Soil contamination by trace elements (TEs) – also known as heavy metals – is an increasing threat to environmental safety and human health (Ali *et al.*, 2013). Cadmium (Cd) is an extremely toxic metal even at low concentrations (Alloway, 2013) and has high mobility in soils, which can lead to groundwater contamination (Lei *et al.*, 2010). Cd is geochemically similar to zinc (Zn) and is often found in Zn bearing minerals (Alloway, 2013); therefore, Zn ores can be responsible for releasing both Cd and Zn into the environment (He *et al.*, 2015) and despite being an essential element, high concentrations of Zn in soils can be harmful to plants and other organisms in the food chain (Green and Tibbett 2008; Green *et al.*, 2010; Nagajyoti *et al.*, 2010; Ali *et al.*, 2013).

Plants have a series of transporters involved in metal uptake and homeostasis that regulates metal movement into the symplast and subsequent loading into vascular tissues (Palmer and Guerinot, 2009). Gene families encoding metal transporters in plants are very diverse and this variation is responsible for the high and low affinity systems necessary to withstand different metal availability in soils (Guerra *et al.*, 2011). Transport of metals into the symplast can be carried out by members of numerous transporter families, such as the heavy metal (Cpx-type) ATPases, the cation diffusion facilitators (CDF), the zrt-, irt-like proteins (ZIP), metal tolerance proteins (MTPs) and the natural resistance-associated macrophage proteins (NRAMP) (Yang *et al.*, 2005; Colangelo and Guerinot, 2006; Sheoran *et al.*, 2011; Ricachenevsky *et al.*, 2013). Since Cd and Zn are very similar, it is generally believed that Cd^{2+} uptake by plants happens by a carrier for Zn^{2+}, or even other divalent cations, such as Cu^{2+} or Fe^{2+}, or by Ca^{2+} and Mg^{2+} transporters/channels (Clemens, 2006; Guerra *et al.*, 2011).

Most metal ions in plants require constant chelation after being taken up by the cell. Chelators bind these ions and contribute to metal detoxification by buffering metal concentrations in the cytosol (Clemens, 2001). One of the main groups of characterised chelators in plant cells are the metallothioneins (MTs) (Clemens, 2006). These low-molecular weight proteins are rich in cysteine, which bind metals in metal-thiolate clusters (Cobbett and Goldsbrough, 2002), and they are considered to be responsible for the homeostasis of essential TEs (Kotrba *et al.*, 2009). In order to understand TE sequestration, Kohler *et al.*, (2004) characterized six MT genes (*PtdMTs*) in the hybrid *P. trichocarpa x deltoides* and verified through heterologous expression of *PtdMT* cDNAs in Cd-sensitive yeasts, that these genes could confer Cd tolerance. However, data about MT production in poplars are still very limited (Guerra *et al.*, 2011). Expression of genes

that encode TE transporters and MTs in plants can be regulated by environmental conditions, metal concentration in soil, pathogen infection and symbiotic interactions (Kohler *et al.*, 2004), such as with mycorrhizal fungi (Hildebrandt *et al.*, 2007).

Almost all terrestrial plants form mycorrhizal associations, especially with arbuscular mycorrhizal fungi (AMF) (Smith and Read, 2008), in which an interplay between direct plant uptake and the mycorrhizal pathway uptake influences the overall plant nutritional status and metal homeostasis. The fact that AMF fungi can increase Zn uptake under deficient conditions is almost a basic tenet when it comes to AM symbiosis (Smith *et al.*, 2010), in some cases, the mycorrhizal fungi pathway can account for almost 25% of the plant's Zn supply (Watts-Williams *et al.*, 2015). Although both pathways for nutrient acquisition in soils are well established (e.g. absorption via plant roots and root hairs and/or fungal hyphae and external mycelium) (Smith *et al.*, 2011), recent works, both physiological and molecular, have been elucidating a more integrated system between these pathways, mainly on the expression of P-transporter genes (Christophersen *et al.*, 2009; Kariman *et al.*, 2016).

The influence of AMF under high Zn concentrations sometimes leads to a decrease in Zn plant uptake (Cavagnaro *et al*., 2010), which can be due to a weaker contribution of the mycorrhizal pathway (Watts-Williams *et al.*, 2015), or by altering the uptake machinery in the host plant. Under Zn stress, mycorrhizal fungi was shown to down-regulate plant Zn transporters in order to promote homeostatic balance (Burleigh and Bechman, 2002), or to up-regulate the expression MT genes as a detoxification strategy (Cicatelli *et al.*, 2010). Similarly, symbiosis with AMF was also shown to alter plant gene expression patterns in host plants exposed to Cd (Rivera-Becerril *et al.*, 2005; Kumar *et al.*, 2015).

In general, AMF symbiosis will often improve plant tolerance to TE toxicity (Andrade *et al.*, 2008; Miransari, 2010; Kariman *et al*., 2018), mostly by cell wall and glomalin binding or by cytosolic chelation (Saraswat and Rai, 2011), however the molecular mechanisms by which they confer tolerance to TEs are highly variable and have not been fully clarified (Cicatelli *et al.*, 2014). For instance, despite Cd and Zn sharing similar uptake pathways in plants (Clemens, 2006) and most of the same membrane metal transporters (Martinka *et al.*, 2014), AMF can have divergent effects on the expression of transporter and tolerance genes in the same plant, depending if it is under Cd or Zn stress (Blaudez *et al.*, 2003). Cadmium and Zn may be geochemically similar, but by being inherently different in terms of essentiality for plant or fungal

development, it becomes apparent that the AMF influence on plant gene expression will not be the same in terms of toxicity avoidance. Nonetheless, studies that help elucidate these divergent molecular responses are lacking.

Phytoremediation is the use of plants and associated microbiota for environmental decontamination (Pilon-Smits, 2005; Gomes *et al.*, 2016), in which phytoextraction (uptake and translocation of metals to aboveground parts) and phytostabilisation (immobilisation of contaminants in roots reducing their availability in soils) are the most common processes for remediation of inorganic contaminants such as TEs (Ali *et al.*, 2013). Trees from the *Populus* genus (poplars) are increasingly being considered for remediation of several metals, such as Cd, Zn and Cu (Guerra *et al.*, 2011; Luo *et al.*, 2016; Redovnikovic *et al.*, 2017), due to their high biomass production, deep root systems, rapid growth and tolerance to elevated metal concentrations (Robinson *et al.*, 2009; Bhargava *et al.*, 2012; De Oliveira and Tibbett, 2018). Poplars can promptly invade disturbed sites, reproduce asexually (Hamberg *et al.*, 2011) and are not a source of food for farm animals (Sebastiani *et al.*, 2004; Shim *et al.*, 2013), reducing the risk of TEs entering the ecosystem/human food chain. Inoculation of poplar trees with AMF can significantly increase their biomass and tolerance (Cicatelli *et al.*, 2010; Ciadamidaro *et al.*, 2017), enhance Cd accumulation (Chen *et al.*, 2016) and phytostabilisation of TEs such as Cu and Zn (Cicatelli *et al.*, 2014). However, metal uptake in plants under AMF symbiosis varies greatly depending on species, cultivars and symbiont partners, factors that certainly affect their overall phytoremediation potential (Bissonnette *et al.*, 2010; Sun *et al.*, 2018).

The genome of *P. trichocarpa* has been completely sequenced (Tuskan *et al.*, 2006) and offers great opportunities for identifying candidate genes for TE uptake in the presence or the absence of AMF (Göhre and Paszkowski, 2006). Assessing the effects of AMF on the patterns of gene expression in host plants is also relevant for elucidating the extent of the mycorrhizal influence, since these fungi are known for promoting systemic effects on their symbiont's gene expression and transcriptional responses (Liu *et al.*, 2007; Kariman *et al.*, 2016).

Therefore, the objectives of this work were to investigate changes caused by mycorrhizal symbiosis (*Rhizophagus irregularis*) to the uptake and transport of Cd and Zn in *P. trichocarpa* under sub-lethal Cd and Zn stress. Our *a priori* hypotheses were that: i) mycorrhizal symbiosis enhances Cd and Zn uptake and increases plant tolerance to toxicity; ii) poplar genes for metal uptake are down-regulated under metal exposure as

an avoidance strategy, while genes associated with metal chelation are up-regulated as a detoxifying mechanism; and iii) AMF alters Cd and Zn partitioning and distribution in plant organs, and modulates the expression of transporter/chelation genes in poplar. Our results on the effect of AMF on the expression of *PtMT2b* in poplar roots, and its association with Cd immobilisation, led us to subsequently investigate its function in providing Cd tolerance by expressing this gene in a yeast system.

2. Materials and Methods

2.1 Growth substrate preparation, plant material and AMF inoculation.

Growth substrate was made up from a mixture of TerraGreen® clay (American Granules Plain, OIL-DRI, UK) and sand (1:5 w/w) (Sibelco, UK) and autoclaved twice (121^{o}C for 15 min). Plastic pots (1 kg, 13 cm diameter) were prepared with 900 g of the substrate and 100 g of the mycorrhizal inoculum (sand mix containing colonised root fragments, hyphae and fungal spores). *R. irregularis* inoculum was obtained from the University of Reading mycorrhizal collection, which is cultured using *Plantago lanceolata* as the host plant. Non-mycorrhizal treatments received 100 g of autoclaved inoculum. The substrate surface in all pots was covered with a thin layer (0.5 cm) of plastic pellets, to avoid possible cross contamination among treatments.

Poplar cuttings (*Populus trichocarpa* cv Trichobel) were obtained from AF Hill and Son, Redditch, UK and were kept refrigerated at 4^{o}C until the experiment. One cutting (15 cm, two nodes) was planted in the centre of each pot to grow for five weeks in a growth chamber (23^{o}C; light per day, 16 h; photosynthetic photon flux, 100 µmol m^{-2} s^{-1} - Philips MCFE 40W/840) and all plants were fertilised weekly for the first three weeks with 10 mL of a modified Long Ashton's solution (macronutrients: $(NH_4)_2SO_4$ (4 mM), K_2SO_4 (2 mM), $CaCl_2 \cdot 2H_2O$ (3 mM), $MgSO_4 \cdot 7H_2O$ (1.5 mM), $NaNO_3$ (8 mM), FeEDTA (0.1 mM); micronutrients: H_3BO_3 (2.86 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (1.81 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.08 mg L^{-1}), $NaMoO_4 \cdot 2H_2O$ (0.025 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.22 mg L^{-1})), according to Kariman *et al.*, (2014). Water holding capacity was maintained at 70% (300 mL of distilled water).

2.2 Contamination and experimental design

After five weeks of growth, pots were divided randomly into six different treatments: (1) Non-mycorrhizal control (Control NM); (2) mycorrhizal control (Control + M); (3) non-mycorrhizal under Cd amendment (Cd NM); (4) mycorrhizal under Cd

contamination (Cd + M); (5) non-mycorrhizal under Zn contamination (Zn NM); and (6) mycorrhizal under Zn contamination (Zn + M). For the Cd treatments, pots were spiked with a stock solution of $CdCl_2$ to reach a final concentration of 81 mg kg^{-1} Cd; to avoid osmotic shock the application was split into three consecutive days (27 mg Cd day^{-1}). For the Zn treatments, a solution of $ZnSO_4$ was used to reach a final concentration of 300 mg kg^{-1} Zn; application was also split into three consecutive days (100 mg Zn day^{-1}). These concentrations of Cd and Zn were based on a previous study, in which they caused significant yet sub-lethal toxic effects (De Oliveira and Tibbett 2018). Each treatment had six replicates and they were set up in a completely randomised design. Non-contaminated controls received deionised water instead of metal solutions.

2.3 Transpiration rate, harvest and pH

After four weeks of exposure to contamination, the two youngest expanded leaves from each plant were assessed for stomatal conductance (gs, in mol m^{-2} s^{-1}) and transpiration rate (mmol m^{-2} s^{-1}) using a portable infrared gas analyser (LCi Portable Photosynthesis System). Eight weeks after contamination, plants were harvested and split into leaves, stems and roots (original cutting was discarded). The 9^{th} leaf of each plant (counting from the bottom of the stem) was sampled and immediately frozen in liquid nitrogen for RNA extraction. Roots were washed thoroughly with tap water and random sections of 2 cm from the root tips were sampled both for determination of mycorrhizal colonisation and for gene expression analyses, the latter were immediately frozen in liquid nitrogen. The remaining roots were immersed in a 0.05 M $CaCl_2$ solution for 30 minutes to remove any surface adhering metals (Marmiroli *et al.*, 2013).

All plant parts were dried in an oven at 70^{o}C for seven days before dry weight (DW) was determined. Soil was air dried, sieved (2 mm) and soil pH was determined in a water-soil suspension (2.5:1) shaken for 15 min at 120 rpm (Rowell, 1994).

2.4 Mycorrhizal colonisation

Root sub-samples were cleared in KOH solution (10% w/v) at room temperature for 10 days, then stained in a 5% (v/v) black ink vinegar solution (Vierheilig *et al.*, 1998) for 1 hour before being washed and transferred to a solution of lactoglycerol (Walker, 2005). Colonisation scoring was done by the line intercept method, in which the presence of either hyphae, arbuscule or vesicle was considered as evidence of mycorrhizae (Giovanetti and Mosse, 1980).

2.5 Acid digestion and determination of metal content

Leaf, stem and root dried samples were ground and digested (50 mg) for 8 hours in 5 mL of 70% HNO_3 (≥69% TraceSELECT®) in closed glass vessels on heating blocks at 110ºC (Huang *et al.*, 2004). Every digestion run was performed in duplicate, with a blank and a plant certified material (IAEA-359 cabbage leaves) included for quality control. Extracts were diluted in a solution of 2% HNO_3 + 5 ppb Rh (rhodium), and filtered. Cd and Zn concentrations were determined by inductively coupled plasma mass spectrometry (Thermo Scientific™ iCAP™ Q ICP-MS), using Rh as an internal standard. Total metal contents – expressed in µg (Cd) or mg (Zn) per plant – were calculated by multiplying the determined metal concentration (mg kg^{-1}) by their respective biomass weight, for each organ separately (roots, stems and leaves) and then for the entire plant.

The translocation factor (T*f* %) is an index used to assess the plant's capacity to translocate TEs from roots to aboveground parts (Rafati *et al.*, 2011), and is the ratio between the metal concentrations in leaves and roots (Saraswat and Rai, 2009; Zacchini *et al.*, 2009). In the present work, total metal contents (µg or mg per organ) were used instead of the traditional metal concentrations (mg kg^{-1}), as it takes into account the biomass of each organ, and provides a more accurate representation of the proportion of metals that can be translocated and stored in leaves and roots:

$$Tf = \frac{Total\ leaf\ content}{Total\ root\ content} \times 100$$

2.6 RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 100 g of fresh weight material (leaves or roots) macerated in liquid nitrogen via TissueLyser II (Qiagen®). Extraction was performed by a modified version of the CTAB method (Jaakola *et al.*, 2001): macerated samples were incubated with CTAB buffer (hexadecyltrimethylammonium bromide) for 25 min at 65ºC (instead of 10 min), LiCl addition was 1/3 of total extract volume (instead of 1/4) and after overnight precipitation at 4ºC, extract was centrifuged for 60 min (instead of 20 min). After centrifugation, the supernatant was discarded and RNA pellets were purified with the RNeasy Plant Mini kit (Qiagen, UK), including a DNAse treatment (Qiagen, UK) for 20 min. Three replicates of each experimental treatment with the highest RNA concentration and quality were selected for cDNA

synthesis (SensiFAST cDNA synthesis kit, Bioline - UK) and following the manufacturer's instructions. Both DNA and RNAs were quantified using a NanoDrop 2000 Spectrophotometer.

2.7 Primer design and gene expression analyses by qPCR

Specific primers for all the selected *P. trichocarpa* genes were designed with the Primer-BLAST tool (Ye *et al.*, 2012) (Table S1). In some cases, homologues from *P. tremula* x *P. alba* or *P. trichocarpa* x *P. deltoides* genes were used based on their identity to the *P. trichocarpa* genome (≥ 93% identity). Eight genes were selected, four associated with metal transport (*MTP1*, *HMA4*, *ZIP1* and *NRAMP3*), three with metal chelation (*MT2a*, *MT2b* and *PCS1*) and one reference gene that encodes ubiquitin (*UBQ*), with stable expression throughout organs and experimental treatments (ANOVA, $p > 0.76$).

The qPCR was performed in duplicate for each sample, in roots and leaves using PowerUp™ SYBRGreen™ (Applied Biosystems). Parameters for the qPCR reactions were as follows: 1 cycle (2 min) at 50ºC followed by 2 min at 95ºC, then 40 cycles of 95ºC (3 sec) and 60ºC (30 sec). Primer specificity was verified by electrophoresis and confirmed by melt curve analyses. The qPCR run and analyses were performed using StepOne™ Real-Time PCR System (Applied Biosystems). Results were analysed by the standard curve method, and gene expression was normalised using *UBQ* as the house keeping gene.

2.8 Expression of PtMT2b in Saccharomyces cerevisiae

PtMT2b expression in roots was up-regulated and highly correlated to mycorrhizal colonisation, which appears to be involved in the restriction of Cd transport from roots to shoots (see Results section; Fig**.** 4, 5). Therefore we hypothesised that this gene is highly effective in conferring Cd tolerance. This was tested by overexpressing *PtMT2b* in yeast under different Cd concentrations.

The wild-type *S. cerevisiae* strain DY1457 (WT) was used for transformation. The cDNA synthesised previously was used as template to amplify the open reading frame (ORF) of *PtMT2b* using a primer set containing *attB* overhang (annealing temperature: 58ºC), with sequences (5' – 3'):

F: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTTGCTGTGGAGGAAA;

R: GGGGACCACTTTGTACAAGAAAGCTGGGTCTCA TTTGCAGGAGCATGGAT.

The gene was introduced into a Gateway® donor vector pDONR221 (with kanamycin resistance gene – Fig. S1) using Gateway® BP Clonase® II enzyme mix. Chemically competent *E. coli* cells (TOP10) were transformed with the entry clone and grown overnight in LB agar + Kanamycin medium at 37ºC. Plasmids were isolated from transformed *E. coli* and introduced into destination vector pDR195 (Fig. S2) using the

Gateway® LR Clonase® II enzyme mix. *E. coli* cells were transformed with the expression vector and grown in LB agar + Ampicillin, same parameters as before. WT yeast was transformed with the expression vector containing *PtMT2b*, and an empty vector (as control). The transformants were selected on synthetic complete (SC) drop-out medium without uracil [1 g/L drop out medium Y1501 Sigma® + 6.7 g L^{-1} yeast nitrogen base Invitrogen™] + 2% dextrose (v/v). Plasmids were restricted (entry vector: *Sac*I and *Ssp*I; expression vector: *Sac*I and *Hind*III) and sequenced at every stage to confirm ORF integrity and direction.

Yeast cells were grown overnight at 30ºC (250 rpm) in SC liquid media (5 mL). OD_{600} was recorded using SpectraMax i3x (Molecular Devices) microplate reader. Cultures were diluted in sterile water to reach OD_{600} of 0.1, and then used for serial dilutions (1:10 v/v). All dilutions of transformed yeast ('*PtMT2b*') and empty vector control yeast ('WT') were spotted (5 µL) in SC agar plates (2% bacteriological agar w/v) at 0; 10; 20; and 50 µM Cd (via $CdCl_2$), then grown at 30ºC for 72 hours in the dark (three replicates).

2.9 Statistical analyses

All statistical analyses were performed using R software. Dry weight (DW), leaf transpiration (E), and stomatal conductance (gs) were analysed by two-way ANOVA and further Tukey tests ($p < 0.05$). One-Way ANOVA and Tukey test ($p < 0.05$) were used to determine the differences in colonisation percentage of mycorrhizal roots, and the differences in metal concentrations between leaves, stems and root organs. The effects of both AMF and contamination (Cd or Zn) on plant metal concentrations (leaves, stems and roots, in mg kg^{-1}) had an inverse Gaussian distribution and therefore were analysed using generalised linear models (GLM; $p < 0.05$) – instead of two-way ANOVA – followed by Tukey contrast analyses. The overall extraction (µg of Cd or mg of Zn per plant) in mycorrhizal and non-mycorrhizal poplars was compared by t-test ($p < 0.05$).

Gene expression data was analysed by two-way ANOVA, followed by a Tukey test when significance was detected ($p < 0.05$), however two variables (*MTP1*-root and *NRAMP3*-root) were transformed to attain the ANOVA's normality and homoscedasticity assumptions, using log(x). To compare the overall gene expression between leaves and roots, a simple t-test was performed. A pair-wise Spearman correlation ($p < 0.05$) was also carried out among the gene expression values in roots and leaves, as well as other

parameters assessed such as metal concentrations and colonisation scores (untransformed data).

3 Results

3.1 Biomass, transpiration rate and AMF colonisation.

Shoot biomass (dry weight) ranged from 5.6 to 7.0 g, while root biomass (dry weight) ranged from 0.4 to 0.5 g (Table 1), with no significant differences among treatments (ANOVA, $p < 0.05$). Although biomass was virtually the same in all treatments, two-way ANOVA detected an overall higher shoot biomass (~ 0.7 g) in mycorrhizal poplars than in non-inoculated plants regardless of metal additions (F value: 4.25; $p = 0.048$). Similarly, transpiration rates (E) were in general 15% higher in mycorrhizal poplars than in non-mycorrhizal (two-way ANOVA, F value: 13.1; $p = 0.001$).

No colonisation was detected in non-inoculated poplars. In inoculated treatments, percentage of colonisation did not differ from the non-contaminated control, but plants exposed to Cd (81 mg kg^{-1}) had significantly higher colonisation than plants under Zn treatment (300 mg kg^{-1}). Overall there was no apparent visual toxicity symptoms in comparison to control plants, regardless of metal addition or AMF inoculation.

3.2 Translocation and accumulation of Cd and Zn in mycorrhizal poplars

Cd accumulation in poplar shoots (leaves and stems) was generally the same in both mycorrhizal and non-mycorrhizal plants when growing in non-contaminated soil (Control vs Control + M), except for roots, in which Cd concentration doubled from ~ 1.3 to ~ 2.6 mg kg^{-1} (Table 2), where translocation (T*f*) decreased sharply from 524% to 233% in mycorrhizal poplars. Under Cd exposure the opposite effect was observed; in this case, root concentrations were similar, but in leaves Cd accumulation decreased by at least 40% in mycorrhizal poplars, in which an interactive effect was also detected between metal addition and inoculation (leaves, $p = 0.022$; stems, $p = 0.015$). Under Cd treatment, root-to-shoot translocation was much lower than found in non-contaminated soil (T*f* %, Table 2), where roots were the main sink for Cd storage.

Unlike with Cd, AMF did not affect Zn accumulation, partitioning or translocation in poplars growing in the non-contaminated soil (Table 3), with roots accumulating at

least three times more Zn than leaves in this case. In poplars growing under 300 mg kg^{-1} Zn, concentrations were at least 10 times higher in roots and stems, and 50 times higher in leaves than in control treatments (Table 3). Zinc partitioning also shifted under contamination, where both leaves and roots acted equally as the main sinks for accumulation. However, the overall Zn concentrations were not increased by AMF (Table 3), and no interactive effects were detected by GLM analyses ($p > 0.2$ for all plant organs).

Considering the total amount of metals extracted from the contaminated soil (µg per plant), Cd contents were similar between non- and mycorrhizal poplars, both with the following order: roots > stems > leaves (Fig. 1). Yet, inoculation with *R. irregularis* clearly affected Cd partitioning, which increased Cd percentage content in roots from 64 to 78%. Under Zn contamination, mycorrhizal poplars extracted overall 38% more Zn (in mg per plant) than their non-mycorrhizal counterparts, although metal allocation followed the same pattern of: leaves > stems > roots (Fig. 1), with only 8% of Zn being sequestered in roots for both cases. It should be noted, however, that Zn concentrations in poplar tissues (mg kg^{-1}) were not significantly affected by mycorrhization (Table 3), only the total metal content (mg per plant/organ), which takes into account the overall plant biomass produced (Fig. 1).

3.3 *Effects of Cd and AMF on gene expression*

Gene expression varied greatly depending on the treatment applied (Cd or inoculation) and the organ assessed (roots or leaves). The membrane metal transporter *PtHMA4* was down-regulated in poplar roots subjected to Cd contamination, however in leaves, AMF lead to a slightly lower expression regardless of metal addition (Fig. 2A). *PtMTP1* expression was not significantly different across treatments in both leaf and root organs (Fig. 2B), but its overall expression was two times higher in leaves than in roots (t-test, $p < 0.001$). Similar results were found for transporter *PtNRAMP3* (Fig. 2C), which had lower expression in roots (t-test, $p < 0.001$) and was overall unaffected by either metal or mycorrhizal treatments.

Metallothionein gene *PtMT2a* was mostly expressed in leaves (t-test, $p = 0.0012$), and was up-regulated due to Cd stress only in non-inoculated plants (Fig. 2E), where there was an interaction effect between mycorrhizal and metal treatments ($p = 0.0031$). The opposite was observed for *PtMT2b*, this gene was highly expressed in the root system (t-

test, $p < 0.001$) and up-regulated considerably by AMF symbiosis, around four times higher than in non-inoculated plants (Fig. 2F). *PtPCS1* expression was similar across all treatments (Fig. 2D) and organs assessed (t-test, p = 0.209). The expression of the zinc-Iron transporter *PtZIP1* was twofold higher with Cd exposure, but was not affected by AMF (Fig. 2G).

3.4 *Effects of Zn and AMF on gene expression*

In poplars exposed to excess Zn, gene expression patterns were similar to Cd treatments, in most cases. *PtHMA4* was also down-regulated in roots due to Zn exposure (Fig. 3A), which also had higher expression than in leaves (t-test, $p = 0.006$), although no effects were found in leaves. *PtMTP1* was not differentially expressed (Fig. 3B) with higher expression observed in leaves than in roots (t-test, $p < 0.001$). *PtNRAMP3* was mostly expressed in leaves (t-test, $p < 0.001$), but overall was not affected by either AMF or Zn treatments (Fig. 3C).

As with Cd, expression of the *PtZIP1* transporter was only affected by metal treatment, however Zn exposure quadrupled its expression in poplar leaves (Fig. 3G) while only a twofold increase was observed under Cd (Fig. 2G). In roots, *PtMT2a* expression was down-regulated under both high Zn and AMF symbiosis (Fig. 3E), while in leaves up-regulation was caused by AMF alone; overall this gene was mostly expressed in leaves (t-test, $p = 0014$). The other metallothionein gene assessed, *PtMT2b*, was more highly expressed in roots than in leaves (t-test, $p < 0.001$), with up-regulation occurring in control treatments under AMF symbiosis, but only slightly higher after Zn addition (Fig. 3F). Similar to Cd treatments, no changes were verified in *PtPCS1* expression between treatments (Fig. 3D) or organs (t-test, $p = 0.139$).

Expression levels of metal transporter genes were more correlated to metal treatments (Cd or Zn) than were genes involved in metal chelation (Table S2). Relationships among the genes assessed were mainly negative, with the only positive correlations observed between *PtHMA4* and *PtMTP1* in both roots and leaves (Fig. 4). *PtMT2b* expression in either leaves or roots had no correlations with the other genes; however, the level of *PtMT2b* transcripts in roots was highly correlated ($r_s = 0.76$) to the percentage of *R. irregularis* colonisation (Fig. 4).

3.5 *Functional expression of PtMT2b in yeast*

Yeasts carrying the metallothionein gene *MT2b* from *P. trichocarpa* were grown in Cd contaminated media. At lower concentrations of Cd, both WT and transformed yeast presented similar growth (Fig. 5); however, at 20 µM Cd there was a clear distinction in growth between the strains. At the highest treatment applied (50 µM Cd), only the transformed strain was able to withstand the Cd toxicity and grew even at 1/1000 dilution, demonstrating the role of *PtMT2b* in increasing Cd tolerance.

4 Discussion

4.1 Mycorrhizal effects in Cd and Zn tolerance

Populus trichocarpa cv. Trichobel showed a very high tolerance to Cd and Zn stress (Table 1). The distinct tolerance of this poplar clone has been demonstrated before under a range of Cd and Zn concentrations (De Oliveira and Tibbett, 2018). Such attributes may account for the small change in biomass production found between mycorrhizal and non-mycorrhizal plants growing under metal stress. Although it has been reported that symbiosis with *R. irregularis* can increase biomass production in poplars under Cd stress (Ciadamidaro *et al.*, 2017), in general, biomass increment as a response to AMF inoculation is highly variable, depending on the plant hosts and ecotypes, fungal partners, metal concentrations, soil attributes etc. (Gaurg and Bhandari, 2014; Coninx *et al.*, 2017).

All inoculated poplars presented on average 40% of root colonisation, including metal contaminated treatments (Table 1). This is not surprising considering AMF are commonly found in roots of plants growing in soils contaminated by TEs (Bedini *et al.*, 2010; Javaid, 2011). Comparable colonisation rates by *R. irregularis* were also found in *P. deltoides* under Cd contamination (Chen *et al.*, 2016).

4.2 Decreased Cd and increased Zn translocation to shoots occurred under symbiosis

Despite not showing any toxicity symptoms nor biomass reduction, *P. trichocarpa* exposed accumulated considerable amounts of Cd (Table 2) and Zn (Table 3), mainly in roots for Cd which are usually reported as the main sink for Cd in poplars (Zacchini *et al.*, 2009; Di Lonardo *et al.*, 2011; De Oliveira and Tibbett, 2018).

Mycorrhizal symbiosis decreased Cd concentration in leaves and stems by around 40%, but did not affect root concentrations (Table 2). A similar response was reported previously for some individuals of *P. deltoides* colonised by *R. irregularis* (Chen *et al.*, 2016), while in *P. nigra*, Cd concentrations were not affected by mycorrhization (Mrnka *et al.*, 2012). Nonetheless, the question of whether *R. irregularis* enhances or decreases

metal accumulation cannot be viewed on a whole plant basis, and will vary depending on metal concentration and soil characteristics (Audet and Charest, 2007). Different plant partners can lead to different outcomes for Cd partitioning. For example, in *Nicotiana tabacum* and the macrophyte *Phragmites australis, R. irregularis* significantly increased Cd concentration in shoots (Janouskova *et al.*, 2006; Huang *et al.*, 2018).

Overall Cd extraction (µg per plant) was similar for both non- and inoculated treatments (Fig. 1), however, mycorrhizal roots accumulated 78% of the total Cd (14% higher than the control), suggesting that *R. irregularis* can promote Cd phytostabilisation by limiting Cd transport to aboveground organs. Mycorrhizal fungi have several defence mechanisms against TE toxicity which may have contributed to Cd immobilisation in roots, mainly cell wall binding, chelation in cytoplasm and metal transport into intracellular compartments (Coninx *et al.*, 2017), or spores (Gonzalez-Guerrero *et al.*, 2008). Furthermore, *R. irregularis* forms vesicles: thick-walled ovoid structures abundant in lipids that can act as storage units (Smith and Read, 2008) and are believed to be a sink for TE storage within mycorrhizal roots (Göhre and Paszkowski, 2006; Nayuki *et al.*, 2014).

Regardless of inoculation, *P. trichocarpa* accumulated high contents of Zn under 300 mg kg^{-1} Zn amendment (Table 3), and despite concentrations in leaves and roots being similar, the overall Zn accumulated (in mg per plant) had a very different distribution among organs (Fig. 1), with at least 60% accumulated in leaves against only 8% in roots. Our findings confirm the initial hypothesis that AMF symbiosis increases Zn accumulation in *P. trichocarpa* and are in line with reports on other *Salicaceae* species (Laureysens *et al.*, 2004; Lingua *et al.*, 2008; Castiglione *et al.*, 2009; Cicatelli *et al.*, 2010; Todeschini *et al.*, 2011).

Although the role of AMF in increasing Zn uptake is well established, especially under Zn deficiency, their effect in plants under high Zn concentrations vary (Toler et al. 2005; Ferrol *et al.*, 2016). For instance, in Zn-contaminated soil, inoculation of two poplar hybrids resulted in higher Zn concentration in leaves of one clone, but not the other (Phanthavongsa *et al.*, 2017). In *P. alba* under 950 mg kg^{-1} Zn, inoculation with *Funneliformis mossae* increased Zn accumulation in both roots and leaves, while symbiosis with *R. irregularis* had no effects (Cicatelli *et al.*, 2010).

In the present work, mycorrhizal poplars had a mean concentration of 1,227 mg kg^{-1} Zn in leaves (Table 3), a concentration considered to be highly toxic for foliar tissues (> 300 mg kg^{-1} Zn) (Marschner, 1995), but in this case did not impair plant growth. It has

been suggested that for host plants with high accumulation capacity and TE translocation towards shoots – such as in the present work – AMF would increase this phenomenon and enhance phytoextraction (Affholder *et al.*, 2014).

4.4 AMF and gene expression under metal stress

Seven poplar genes involved in metal transport and chelation processes were assessed under AMF colonisation and Cd/Zn stress.

PtHMA4: HMA4 transporters can selectively transport essential metals as well as TEs, especially Zn^{2+} and Cd^{2+} (Hussain *et al.*, 2004). Both metals were responsible for a sharp down-regulation in *PtHMA4* expression in poplar roots, regardless of inoculation (Fig. 3A, 4A). *HMA4* is highly expressed in the root pericycle and is involved in xylem loading of Zn and Cd (Verret *et al.*, 2004; Hanikenne *et al.*, 2008; Migeon *et al.*, 2010) playing an important role in long distance transport in plants (Luo *et al.*, 2016; Sarwar *et al.*, 2017). Thus, down-regulating its expression is probably one of the mechanisms by which *P. trichocarpa* avoids metal toxicity in aboveground organs, and is a common response in non-hyperaccumulator plants (Hammond *et al.* 2006). Similar results were verified in *P. nigra* exposed to high Zn (Adams *et al.*, 2011), however this gene has many splice variants, which were not assessed in the present study (Li *et al.*, 2015).

PtMTP1: MTPs are involved in metal efflux from the cytoplasm, either to extracellular spaces or into organelles, e.g. vacuoles and Golgi apparatus (Peiter *et al.*, 2007; Ricachenevsky *et al.*, 2013), in which MTP1 usually acts on the transport of Zn, Cd, Fe and Mn and tends to have similar roles and localisation across different species (Kramer *et al.*, 2005; Hammond *et al.*, 2006; Ricachenevsky *et al.*, 2013). Contrary to our hypothesis, *PtMTP1* expression was not different in *P. trichocarpa* regardless of Cd/Zn addition or AMF inoculation (Fig. 3B, 4B). Blaudez *et al.* (2003) have shown that in *P. deltoides* MTP1 expression was influenced by Zn, but not Cd. Nonetheless, it is important to consider that qPCR analyses was performed after eight weeks of metal exposure, at which time the transcript levels may have returned to their original baseline (control).

PtNRAMP3: NRAMPs are membrane metal transporters usually located in tonoplasts, from which NRAMP3 is involved in the metal trafficking between the vacuole and the cytoplasm (Iori *et al.* 2016; Sharma *et al.*, 2016). Although there are not many studies of *NRAMP3* in poplars, there is agreement that this gene is usually affected by metal exposure in *P. trichocarpa* itself (Le Thi, 2015), or its homologues in *A. thaliana* and *Nocceae caerulescens* (Oomen *et al.*, 2009). The fact that no treatments affected the

expression of this gene in the present study (Fig. 3C, 4C) suggests that its transcriptional regulation happened early on during Cd and Zn exposure, since gene expression during Cd stress varies depending on time of exposure (Rome *et al.*, 2016).

PtPCS1: Phytochelatins (PCs) are proteins involved in Cd and Zn chelation and sequestration in plant cells, with their syntheses catalysed by the enzyme phytochelatin synthase (PCS) (Cobbett and Goldsbrough, 2002). Although a variation due to metal exposure was hypothesised, *PtPCS1* expression was not significantly different across treatments. Similar results were observed in tomato plants inoculated with AMF, in which neither symbiosis nor TE exposure affected the expression of this gene (Ouziad *et al.*, 2005). In a poplar hybrid exposed to Cd, up-regulation of a *PCS* gene occurred after 12 hours, only to decrease to control levels after 240 hours (Lin *et al.*, 2016), suggesting that this gene is also regulated during early Cd/Zn exposure.

PtZIP1: Members of the ZIP family are able to transport several cations, such as Zn and Cd into the cytosol (Pottier *et al.*, 2015; Iori *et al.*, 2016). Expression of *PtZIP1* was around three times greater in leaves of poplars exposed to high Zn than in non-polluted soil, due to the high influx of Zn to those organs (Fig. 3G). Cd also up-regulated *PtZIP1* expression, but to a lesser extent, highlighting the role of this gene in Cd transport in poplars (Fig. 2G). In the present study, the level of *ZIP1* transcripts was too low to be detected in roots via qPCR (data not shown), similar to results from Shi *et al.* 2016, in willow (*Salix integra*)

4.5 AMF effects on metallothioneins: gene expression and functional expression of PtMT2b *in yeast.*

Metallothioneins are small proteins rich in cysteine (Cys) residues capable of binding a range of transition metal ions, such as Zn and Cd, which are mainly bound by members of the MT2 subfamily (Hassinen *et al.*, 2011). Thus, tolerance and homeostasis are considered to be their main functions (Cobbett and Goldsbrough, 2002).

PtMT2a: Expression of MTs is generally responsive to TE exposure (Chen *et al.*, 2014), but in this study, only *MT2a* was affected by metals (Fig. 3E, 4E), with higher expression found in leaves of non-mycorrhizal poplars exposed to Cd, the treatment with the highest foliar Cd concentration. *MT2a* expression in willow leaves (*S. caprea*) was also induced by Cd exposure (Konlechner *et al.*, 2013), while in *P. trichocarpa x deltoides* both Cd and Zn affected its expression in leaves (Kohler *et al.*, 2004).

PtMT2b: Despite not being influenced much by Cd and Zn, *MT2b* expression in roots was significantly increased by AMF symbiosis, which helps explain the high percentage of Cd found in colonised roots (78% of total Cd). Up-regulation of *MT2b* solely by AMF symbiosis is in accordance with other studies involving *R. irregularis* inoculation of poplars (Cicatelli *et al.*, 2012; Pallara *et al.*, 2013), highlighting AMF ability in protecting plants against stress by activating detoxifying defences in plants (Miransari, 2017). The up-regulation of MTs in mycorrhizal roots regardless of TE exposure could be related to their secondary role in ROS (reactive oxygen species) scavenging (Wong *et al.*, 2004; Ruttkay-Nedecky *et al.*, 2013), which occurs through the same Cys residues responsible for metal binding (Hassinen *et al.*, 2011). During the symbiosis establishment, fungal hyphae trigger an intracellular burst of ROS in the host plant, and even accumulation of H_2O_2 (Kapoor and Singh, 2017), thus it is possible that MT2b up-regulation in roots is a result of the colonisation itself, and an indirect mechanism of alleviating TE stress. This explanation is supported by results from Hrynkiewicz *et al.*, (2012) and Haq *et al.*, (2003).

Spearman analyses showed an interesting positive correlation (76%) between *PtMT2b* expression in roots and the colonisation rates (Fig. 4). Therefore, immobilisation of Cd in roots is probably a combined effect from both fungal binding and MT2b chelation as a detoxifying strategy. Indeed we demonstrate here that *PtMT2b* is able to successfully enhance Cd tolerance when expressed in yeast (50 µM Cd). To the best of our knowledge, the function of *PtMT2b* in Cd tolerance has not been previously tested in *S. cerevisiae*, the closest being the work from Kohler *et al.*, (2004), with a poplar hybrid, although most studies are still from herbaceous species (Guo *et al.*, 2008; Zhang *et al.*, 2014).

5 Conclusions

Mycorrhizal symbiosis with *R. irregularis* clearly altered the partitioning of Cd in *P. trichocarpa*, but maintained the same patterns for Zn, indicating that despite these metals being geochemically similar and carried mostly by the same transporters, the nutrient metal (Zn) is handled differently from the non-essential metal (Cd). Increase in Zn accumulation and decrease in Cd root-to-shoot transport suggest that this association enhances the potential for Zn phytoextraction and Cd phytostabilisation. Overall, the results from this work advance the knowledge on the effects of AMF in poplars under Cd and Zn stress, not only in terms of tolerance and remediation applications but also on the transcriptional level, contributing to unravel the mechanisms behind AMF symbiosis in

woody species, and highlighting potential candidate genes for future investigations and biotechnological applications, such as the possibility of *PtMT2b* to be used in transgenic plants or microorganisms for Cd remediation.

6. Supplementary data

Table S1. List of primers.

Table S2. Spearman correlations between parameters assessed in *P. trichocarpa*

Table S3. Total Cd and Zn accumulation (per organ) in *P. trichocarpa*

Fig. S1. Donor vector.

Fig. S2. Expression vector.

Declaration of contributions

VHDO; MT, and IU have made substantial contributions to conception and design, or acquisition of data, analysis and interpretation of data;

VHDO; MT and JMD have been involved in drafting the manuscript or revising it critically for important intellectual content.

Final approval of the version was given by all authors.

Declarations of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author: m.tibbett@reading.ac.uk.

8. Funding

This research was supported by CAPES (Coordination for the Improvement of Higher Education Personnel - Brazil) [project: 13462-13-0].

7. Acknowledgements

We thank Dr John Hammond and Dr Jeanette Hartley-Whitaker for their valuable inputs in this work.

9. References

Adams JP, Adeli A, Hsu CY, Harkess RL, Page GP, dePamphilis CW, Schultz EB, Yuceer C. 2011. Poplar maintains zinc homeostasis with heavy metal genes *HMA4* and *PCS1*. Journal of Experimental Botany **62**, 3737-3752.

Affholder MC, Pricop AD, Laffont-Schwob I, Coulomb B, Rabier J, Borla A, Demelas C, Prudent P. 2014. As, Pb, Sb, and Zn transfer from soil to root of wild rosemary: do native symbionts matter? Plant and Soil **382**, 219-236.

Ali H, Khan E, Sajad MA. 2013. Phytoremediation of heavy metals – Concepts and applications. Chemosphere **91**, 869-881.

Alloway BJ. 2013**.** *Heavy Metals in Soils*. Netherlands, Springer.

Andrade SAL, Silveira APD, Jorge RA, Abreu MF. 2008. Cadmium accumulation in sunflower plants influenced by arbuscular mycorrhiza. International Journal of Phytoremediation **10**, 1-13.

Audet P, Charest C. 2007**.** Dynamics of arbuscular mycorrhizal symbiosis in heavy metal phytoremediation: Meta-analytical and conceptual perspectives. Environmental Pollution **147,** 609-614.

Bedini S, Turrini A, Rigo C, Argese E, Giovannetti M. 2010**.** Molecular characterization and glomalin production of arbuscular mycorrhizal fungi colonizing a heavy metal polluted ash disposal island, downtown Venice. Soil Biology & Biochemistry **42**, 758-765.

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

Bissonnette L, St-Arnaud M, Labrecque M. 2010**.** Phytoextraction of heavy metals by two Salicaceae clones in symbiosis with arbuscular mycorrhizal fungi during the second year of a field trial. Plant and Soil **332**, 55-67.

Blaudez D, Kohler A, Martin F, Sanders D, Chalot M. 2003**.** Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. The Plant Cell **15**, 2911-2928.

Burleigh SH, Bechmann IE. 2002**.** Plant nutrient transporter regulation in arbuscular mycorrhizas. Plant and Soil **244**, 247-251.

Castiglione S, Todeschini V, Franchin C, Torrigiani P, Gastaldi D, Cicatelli A, Rinaudo C, Berta G, Biondi S, Lingua G. 2009**.** Clonal differences in survival capacity, copper and zinc accumulation, and correlation with leaf polyamine levels

in poplar: a large-scale field trial on heavily polluted soil. Environmental Pollution **157**, 2108–2117.

Cavagnaro TR, Dickson S, Smith FA. 2010. Arbuscular mycorrhizas modify plant responses to soil zinc addition. Plant and Soil **329**, 307-313.

Chen L, Ma L, Bai Q, Zhu X, Zhang J, Wei Q, Li D, Gao C, Li J, Zhang Z et al. 2014**.** Heavy metal-induced metallothionein expression is regulated by specific protein phosphatase 2A complexes. The Journal of Biological Chemistry **298**, 22413-22426.

Chen L, Zhang D, Yang W, Liu Y, Zhang L, Gao S. 2016**.** Sex-specific responses of *Populus deltoides* to *Glomus intraradices* colonization and Cd pollution. Chemosphere **155**, 196-206.

Ciadamidaro L, Girardclos O, Bert V, Zappelini C, Yung L, Foulon J, Papin A, Roy S, Blaudez D, Chalot M. 2017**.** Poplar biomass production at phytomanagement sites is significantly enhanced by mycorrhizal inoculation. Environmental and Experimental Botany **139**, 48-56.

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

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

Cicatelli A, Todeschini V, Lingua G, Biondi S, Torrigiani P, Castiglione S. 2014**.** Epigenetic control of heavy metal stress response in mycorrhizal versus non-mycorrhizal poplar plants. Environmental Science and Pollution Research **21**, 1723-1737.

Clemens S. 2001**.** Molecular mechanisms of plant metal tolerance and homeostasis Planta **212**, 475-486.

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

Cobbett C, Goldsbrough P. 2002**.** Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annual Review in Plant Biology **53**, 159-

182.

Colangelo EP, Guerinot ML. 2006**.** Put the metal to the petal: metal uptake and transport throughout plants. Current Opinion in Plant Biology **9**, 322-330.

Coninx L, Martinova V, Rineau F. 2017**.** Mycorrhiza-Assisted Phytoremediation. In: Cuypers A, Vangronsveld J, eds. Advances in Botanical Research: Phytoremediation vol. *83*. Elsevier, 127-188.

Christophersen HM, Smith FA, Smith SE. 2009. Arbuscular mycorrhizal colonization reduces arsenate uptake in barley via downregulation of transporters in the direct epidermal phosphate uptake pathway. New Phytologist **184**, 962-974.

De Oliveira VH, Tibbett M. 2018**.** Tolerance, toxicity and transport of Cd and Zn in *Populus trichocarpa*. Environmental and Experimental Botany **155**, 281-292.

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

Ferrol N, Tamayo E, Vargas P. 2016**.** The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. Journal of Experimental Botany **67**, 6253-6265.

Garg N, Bhandari P. 2014**.** Cadmium toxicity in crop plants and its alleviation by arbuscular mycorrhizal (AM) fungi: An overview. Plant Biosystems **148**, 609-621.

Giovannetti M, Mosse B. 1980**.** An evaluation of techniques for measuring vesicular-arbuscular infection in roots. New Phytologist **84**, 489-500.

Gohre V, Paszkowski U. 2006**.** Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta **223**, 1115-1122.

Gomes MAC, Hauser-Davis RA, Souza AN, Vitoria AP. 2016**.** Metal phytoremediation: General strategies, genetically modified plants and applications in metal nanoparticle contamination. Ecotoxicology and Environmental Safety **134**, 133-147.

Gonzalez-Guerrero M, Melville LH, Ferrol N, Lott JNA, Azcon-Aguilar C, Peterson RL. 2008**.** Ultrastructural localization of heavy metals in the extraradical mycelium and spores of the arbuscular mycorrhizal fungus *Glomus intraradices*. Canadian Journal of Microbiology **54**, 103-110.

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

Green ID, Tibbett M. 2008**.** Differential uptake, partitioning and transfer of Cd and Zn in the soil−pea plant−aphid system. Environmental Science & Technology **42**, 450-455.

Guarino C, Paura B, Sciarrillo R. 2018**.** Enhancing phytoextraction of HMs at Real Scale, by combining Salicaceae trees with microbial consortia. *Frontiers in Environmental Science* **6,** 1-11.

Guerinot ML. 2000**.** The ZIP family of metal transporters. Biochimica et Biophysica Acta **1465**, 190-198.

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

Guo WJ, Meetam M, Goldsbrough PB. 2008**.** Examining the specific contributions of individual *Arabidopsis* metallothioneins to copper distribution and metal tolerance. Plant Physiology **146**, 1697-1706.

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

Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJM, Whiting SN, May ST, Broadley MR. 2006**.** A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. New Phytologist **170**, 239-260.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Kramer U. 2008**.** Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of *HMA4*. Nature **453**, 391-395.

Haq F, Mahoney M, Koropatnick J. 2003**.** Signaling events for metallothionein induction. Mutation Research **533**, 211-226.

Hassinen VH, Tervahauta AI, Schat H, Karenlampi SO. 2011**.** Plant metallothioneins – metal chelators with ROS scavenging activity? Plant Biology **13,** 225-232.

He S, He Z, Yang X, Stoffella PJ, Baligar VC. 2015**.** Soil biogeochemistry, plant physiology, and phytoremediation of cadmium-contaminated soils. In: Sparks DL, ed. Advances in Agronomy. Elsevier, 135–225.

Hildebrandt U, Regvar M, Bothe H. 2007**.** Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry **86**, 139-146.

Hrynkiewicz K, Dabrowska G, Baum G, Niedojadlo K, Leinweber P. 2012.

Interactive and single effects of ectomycorrhiza formation and *Bacillus cereus* on metallothionein MT1 expression and phytoextraction of Cd and Zn by willows. Water, Air and Soil Pollution **223**, 957-968.

Huang L, Bell RW, Dell B, Woodward J. 2004**.** Rapid nitric acid digestion of plant material with an open-vessel microwave system. Communications in Soil Science and Plant Analysis **35**, 427–440.

Huang X, Wang L, Zhu S, Ho SH, Wu J, Kalita PK, Ma F. 2018**.** Unraveling the effects of arbuscular mycorrhizal fungus on uptake, translocation, and distribution of cadmium in *Phragmites australis* (Cav.) Trin. ex Steud. Ecotoxicology and Environmental Safety **149**, 43-50.

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS. 2004**.** P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. The Plant Cell **16**, 1327-1339.

Iori V, Gaudet M, Fabbrini, F, Pietrini F, Beritognolo I, Zaina G, Mugnozza GS, Zacchini M, Massacci A, Sabatti M. 2016**.** Physiology and genetic architecture of traits associated with cadmium tolerance and accumulation in *Populus nigra* L. Trees **30**, 125-139.

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

Janouskova M, Pavlikova D, Vosatka M. 2006**.** Potential contribution of arbuscular mycorrhiza to cadmium immobilisation in soil. Chemosphere **65**, 1959-1965.

Javaid A. 2011**.** Importance of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. In: Khan MS, Zaidi A, Goel R, Musarrat J, eds. Biomanagement of Metal-Contaminated Soils, Environmental Pollution 20. Springer Science, 125-141.

Kapoor R, Singh N. 2017**.** Arbuscular mycorrhiza and Reactive Oxygen Species. In: Wu QS ed. Arbuscular Mycorrhizas and Stress Tolerance of Plants. Singapore. Springer, 225-243.

Kariman K, Barker SJ, Tibbett M. 2018. Structural plasticity in root-fungal symbiosis: diverse interactions lead to improved plant fitness. PeerJ ***6****:e6030 doi.org/10.7717/peerj.6030*

Kariman K, Barker SJ, Jost R, Finnegan PM, Tibbett M. 2016**.** Sensitivity of jarrah (*Eucalyptus marginata*) to phosphate, phosphite, and arsenate pulses as influenced by fungal symbiotic associations. Mycorrhiza **26**, 401–415.

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

Kohler A, Blaudez D, Chalot M, Martin F. 2004**.** Cloning and expression of multiple metallothioneins from hybrid poplar. New Phytologist **164,** 83-93.

Konlechner C, Turktas M, Langer I, Vaculik M, Wenzel WW, Puschenreiter M, Hauser MT. 2013**.** Expression of zinc and cadmium responsive genes in leaves of willow (*Salix caprea* L.) genotypes with different accumulation characteristics. Environmental Pollution **178**, 121-127.

Kotrba P, Najmanova J, Macek T, Ruml T, Mackova M. 2009**.** Genetically modified plants in phytoremediation of heavy metal and metalloid soil and sediment pollution. Biotechnology Advances **27**, 799-810.

Kramer U. 2005**.** MTP1 mops up excess Zn in *Arabidopsis* cells. TRENDS in Plant Science **10**, 313-315.

Kumar P, Lucini L, Rouphael Y, Cardarelli M, Kalunke RM, Colla G. 2015**.** Insight into the role of grafting and arbuscular mycorrhiza on cadmium stress tolerance in tomato. Frontiers in Plant Science **6**, 1-16.

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

Le Thi VA. 2015**.** Characterization of poplar metal transporters to improve rehabilitation of metal polluted soils. PhD thesis, Université Paris-Sud, Paris, France. English. Available at: www.theses.fr/2015PA112004.pdf

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

Li D, Xu X, Hu X, Liu Q, Wang Z, Zhang H, Wang H, Wei M, Wang H, Liu H et al. 2015**.** Genome-wide analysis of heavy metal-induced expression profiling of the HMA gene family in *Populus trichocarpa*. Frontiers in Plant Science **6**, 1-15.

Lin T, Wan X, Zhang F. 2016**.** The short-term responses of glutathione and phytochelation synthetic pathways genes to additional nitrogen under cadmium stress in poplar leaves. Russian Journal of Plant Physiology **63**, 754-762.

Lingua G, Franchin C, Todeschini V, Castiglione S, Biondi S, Burlando B,

Parravicini V, Torrigiani P, Berta G. 2008**.** Arbuscular mycorrhizal fungi differentially affect the response to high zinc concentrations of two registered poplar clones. Environmental Pollution **153**, 137–147.

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ. 2007**.** Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. The Plant Journal **50**, 529-544.

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

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

Marschner H. 1995. Mineral Nutrition of Higher Plants. London: Academic Press.

Migeon A, Blaudez D, Wilkins O, Montanini B, Campbell MM, Richaud P, Thomine S, Chalot M. 2010**.** Genome-wide analysis of plant metal transporters, with an emphasis on poplar. Cellular and Molecular Life Sciences **67**, 3763-3784.

Miransari M. 2010**.** Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biology **12**, 563-569.

Miransari M. 2017**.** Arbuscular mycorrhizal fungi and heavy metal tolerance in plants. In: Wu QS ed. Arbuscular Mycorrhizas and Stress Tolerance of Plants. Singapore. Springer, 147-161.

Mrnka L, Kuchar M, Cieslarova Z, Matejka P, Szakova J, Tlustos P, Vosatka M. 2012**.** Effects of endo- and ectomycorrhizal fungi on physiological parameters and heavy metals accumulation of two species from the family Salicaceae. Water, Air and Soil Pollution **223,** 399-410.

Nagajyoti PC, Lee KD, Sreekanth TVM. 2010. Heavy metals, occurrence and toxicity for plants: a review. Environmental Chemistry Letters **8**, 199-216.

Nayuki K, Chen B, Ohtomo R, Kuga Y. 2014**.** Cellular imaging of cadmium in resin sections of arbuscular mycorrhizas using Synchrotron micro X-ray fluorescence. Microbes and Environments **29**, 60-66.

Oomen RJFJ, Wu J, Lelievre F, Blanchet S, Richaud P, Barbier-Brygoo H, Aarts MGM, Thomine S. 2009**.** Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. New Phytologist **181**, 637-

650.

Ouziad F, Hildebrandt U, Schmelzer E, Bothe H. 2005**.** Differential gene expressions in arbuscular mycorrhizal-colonized tomato grown under heavy metal stress. Journal of Plant Physiology **162**, 634-649.

Pallara G, Todeschini V, Lingua G, Camussi A, Racchi ML. 2013**.** Transcript analysis of stress defence genes in a white poplar clone inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* and grown on a polluted soil. *Plant* Physiology and Biochemistry **63**, 131-139.

Palmer CM, Guerinot ML. 2009**.** Facing the challenges of Cu, Fe and Zn homeostasis in plants. Nature Chemical Biology **5**, 333-340.

Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D. 2007**.** A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. PNAS **104**, 8532-8537.

Phanthavongsa P, Chalot M, Papin A, Lacercat-Didier L, Roy S, Blaudez D, Bert V. 2017**.** Effect of mycorrhizal inoculation on metal accumulation by poplar leaves at phytomanaged sites. Environmental and Experimental Botany **143**, 72-81.

Pilon-Smits E. 2005**.** Phytoremediation. Annual Review of Plant Biology **56**, 15–39.

Pottier M, Torre VSG, Victor C, David LC, Chalot M, Thomine S. 2015**.** Genotypic variations in the dynamics of metal concentrations in poplar leaves: A field study with a perspective on phytoremediation. Environmental Pollution **199**, 73-82.

Rafati M, Khorasani N, Moattar F, Shirvany A, Moraghebi F, Hosseinzadeh S. 2011**.** Phytoremediation potential of *Populus alba* and *Morus alba* for cadmium, chromuim and nickel absorption from polluted soil. International Journal of Environmental Research **5**, 961–970.

Redovnikovic IR, De Marco A, Proietti C, Hanousek K, Sedak M, Bilandzic N, Jakovljevic T. 2017**.** Poplar response to cadmium and lead in soil contamination. Ecotoxicology and Environmental Safety **144**, 482-489.

Ricachenevsky FK, Menguer PK, Sperotto RA, Williams LE, Fell JP. 2013**.** Roles of plant metal tolerance proteins (MTP) in metal storage and potential use in biofortification strategies. Frontiers in Plant Science **4**, 1-16.

Rivera-Becerril F, van Tuinen D, Martin-Laurent F, Metwally A, Dietz KJ, Gianinazzi S, Gianinazzi-Pearson V. 2005. Molecular changes in Pisum sativum L. roots during arbuscular mycorrhiza buffering of cadmium stress. Mycorrhiza **16**, 51-60.

Robinson BH, Banuelos G, Conesa HM, Evangelou MWH, Schulin R. 2009**.** The phytomanagement of trace elements in soil. Critical Reviews in Plant Sciences **28**, 240–266.

Rome C, Huang XY, Danku J, Salt DE, Sebastiani L. 2016**.** Expression of specific genes involved in Cd uptake, translocation, vacuolar compartmentalisation and recycling in *Populus alba* Villafranca clone. Journal of Plant Physiology **202**, 83-91.

Rowell DL. 1994. Soil Science: Methods and Applications. Essex: Longman Group UK Ltd.

Saraswat S, Rai JPN. 2009**.** Chemistry and ecology phytoextraction potential of six plant species grown in multimetal contaminated soil. Chemistry and Ecology **25**, 1–11.

Saraswat R, Rai JPN. 2011**.** Mechanism of metal tolerance and detoxification in mycorrhizal fungi. In: Khan MS, Zaidi A, Goel R, Musarrat J, eds., Biomanagement of Metal-Contaminated Soils. Springer, 225–240.

Sarwar N, Imran M, Shaheen MR, Ishaque W, Kamran MA, Matloob A, Rehim A, Hussain S. 2017**.** Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives. Chemosphere **171**, 710-721.

Sebastiani L, Scebba F, Tognetti R. 2004**.** Heavy metal accumulation and growth responses in poplar clones Eridano (*Populus deltoides* × *maximowiczii*) and I-214 (*P.* × *euramericana*) exposed to industrial waste. Environmental and Experimental Botany **52**, 79–88.

Sharma SS, Dietz KJ, Mimura T. 2016**.** Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. Plant, Cell and Environment **39**, 1112-1126.

Sheoran V, Sheoran AS, Poonia P. 2011**.** Role of hyperaccumulators in phytoextraction of metals from contaminated mining sites: a review. Environmental Science and Technology **41**, 168-214.

Shi X, Sun H, Chen Y, Pan H, Wang S. 2016**.** Transcriptome sequencing and expression analysis of cadmium (Cd) transport and detoxification related to genes in Cd-accumulating *Salix integra*. Frontiers in Plant Science **7,** 1-13.

Shim D, Kim S, Choi YI, Son WY, Park J, Youk ES, Jeong SC, Martinoia E, Noh EW, Lee Y. 2013**.** Transgenic poplar trees expressing yeast cadmium factor 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil. Chemosphere **90**, 1478–1486.

Smith SE, Read D. 2008. Mycorrhizal Symbiosis. Academic Press, London.

Smith SE, Jakobsen I, Gronlund M, Smith FA. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant physiology **156**, 1050-1057.

Sun H, Xie Y, Zheng Y, Lin Y, Yang F. 2018**.** The enhancement by arbuscular mycorrhizal fungi of the Cd remediation ability and bioenergy quality-related factors of five switchgrass cultivars in Cd-contaminated soil. PeerJ doi.org/10.7717/peerj.4425

Todeschini V, Lingua G, D'Agostino G, Carniato F, Roccotiello E, Berta G. 2011**.** Effects of high zinc concentration on poplar leaves: A morphological and biochemical study. Environmental and Experimental Botany **71**, 50–56.

Toler HD, Morton JB, Cumming JR. 2005**.** Growth and metal accumulation of mycorrhizal sorghum exposed to elevated copper and zinc. Water, Air and Soil Pollution **164**, 155-172.

Tuskan GA, DiFazio SP, Jansson S, *et al*. 2006**.** The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science **313**, 1596–1604.

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P. 2004**.** Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Letters **576**, 306-3012.

Vierheilig H, Coughlan AP, Wyss U, Piche Y. 1998**.** Ink and vinegar, a simple staining technique of arbuscular-mycorrhizal fungi. Applied and Environmental Microbiology **64**, 5004-5007.

Walker C. 2005. A simple blue staining technique for arbuscular mycorrhizal and other root-inhabiting fungi. Inoculum **56**, 68-69.

Watts-Williams SJ, Smith FA, McLaughlin MJ, Patti AF, Cavagnaro TR. 2015**.** How important is the mycorrhizal pathway for plant Zn uptake. Plant and Soil **390**, 157-166.

Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K. 2004**.** Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase *OsRac1* in rice. Plant Physiology **135**, 1447-1456.

Yang X, Feng Y, He Z, Stoffella PJ. 2005**.** Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation. Journal of Trace Elements in Medicine and Biology **18**, 339-353.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012**.** Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BCM Bioinformatics **13**, 134. doi: 10.1186/1471-2105-13-134.

Zacchini M, Pietrini F, Mugnozza GS, Iori V, Pietrosanti L, Massacci A. 2009**.** Metal tolerance, accumulation and translocation in poplar and willow clones treated with cadmium in hydroponics. Water, Air and Soil Pollution **197**, 23–34.

Zhang M, Takano T, Liu S, Zhang X. 2014**.** Abiotic stress response in yeast and metal-binding ability of a type 2 metallothionein-like protein (PutMT2) from *Puccinellia tenuiflora*. Molecular Biology Reports **41**, 5839-5849.

10. Figure Captions

Fig. 1 Organ distribution of Cd and Zn in *P. trichocarpa* plants under 81 mg kg^{-1} Cd or 300 mg kg^{-1} Zn, with and without inoculation of *R. irregularis*. Cd NM: under Cd non-mycorrhizal; Cd + M: under Cd mycorrhizal; Zn NM: under Zn non-mycorrhizal; Zn + M: under Zn mycorrhizal. Total content extracted per plant of Cd or Zn were compared by t-test, different letters represent significant differences ($p < 0.05$; n = 6).

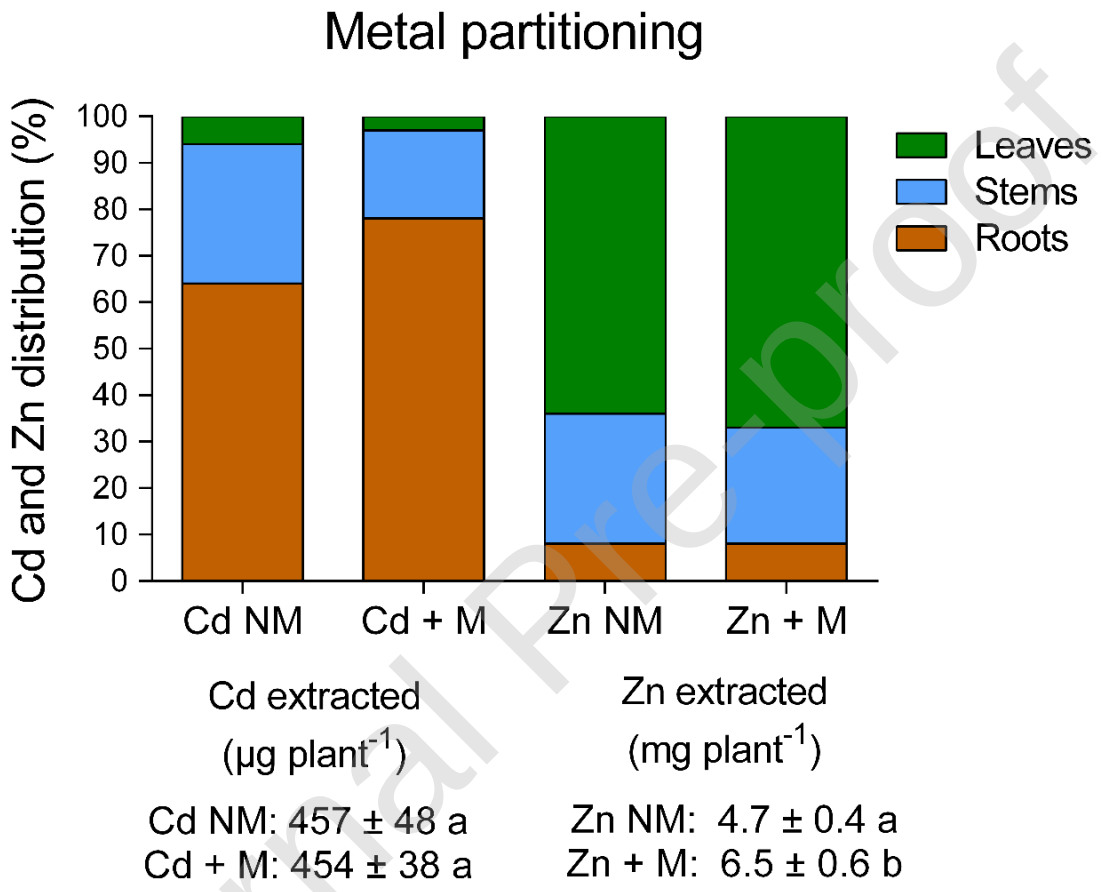


Fig. 2 Relative gene expression of *PtHMA4* (a), *PtMTP1*(b), *PtNRAMP3* (c), *PtPCS1* (d), *PtMT2a* (e), *PtMT2b* (f), and *PtZIP1*(g) in *P. trichocarpa* cv. Trichobel grown under 81 mg kg^{-1} Cd for eight weeks, with or without mycorrhizal symbiosis (*R. irregularis*). Values are means ± standard error (n = 3) of expression normalised by UBQ. Different letters represent significant differences by ANOVA, Tukey test ($p < 0.05$) for each plant organ. n.s. = not significant.

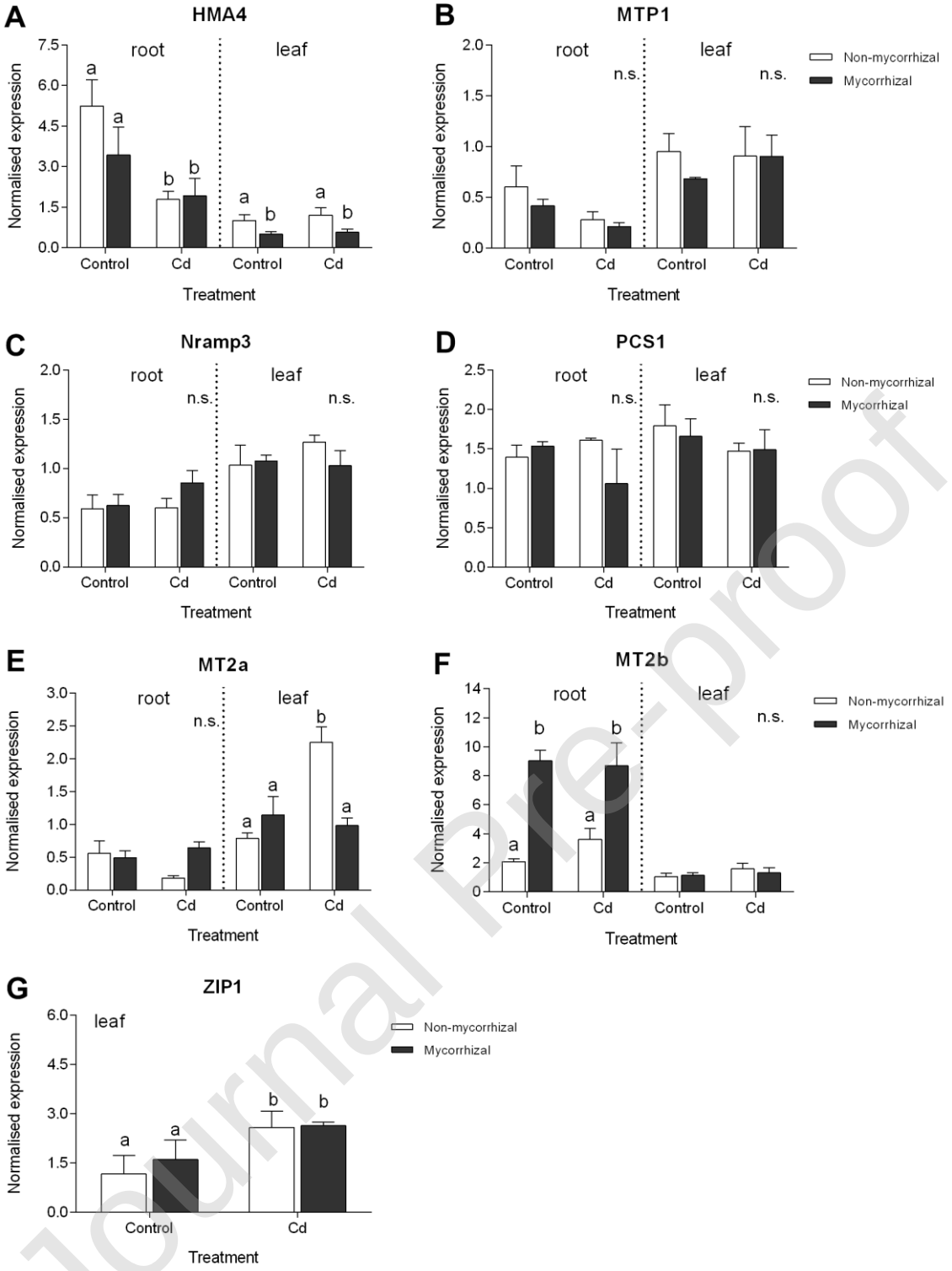


Fig. 3 Relative gene expression of *PtHMA4* (a), *PtMTP1* (b), *PtNRAMP3* (c), *PtPCS1* (d), *PtMT2a* (e), *PtMT2b* (f), and *PtZIP1* (g) in *P. trichocarpa* cv. Trichobel grown under 300 mg kg^{-1} Zn for eight weeks, with or without mycorrhizal symbiosis (*R. irregularis*). Values are means ± standard error (n = 3) of expression normalised by UBQ. Different letters represent significant differences by ANOVA + Tukey test ($p < 0.05$) for each plant organ. n.s. = not significant.

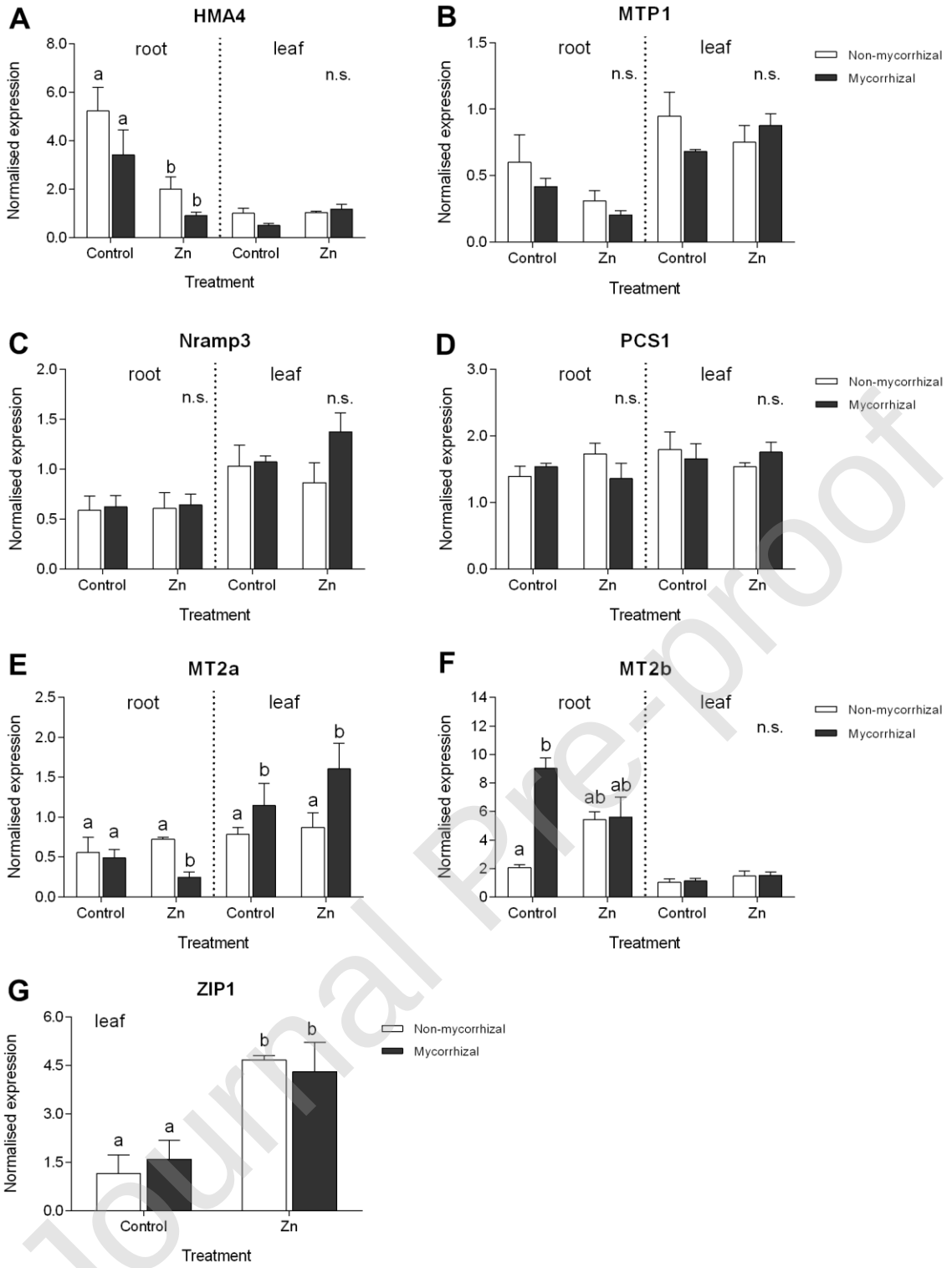


Fig. 4 Diagram representing significant correlations between the expression of different genes in roots and leaves of *Populus trichocarpa* under Cd and Zn stress, with or without mycorrhizal inoculation (*R. irregularis*). Circles: genes associated with metal chelation; Rectangles: genes involved in metal transport; Squares: metal concentration (Cd or Zn) in leaves and roots. Line: positive correlation; Dotted line: negative correlation; AMF: percentage of mycorrhizal colonisation. Pair-wise Spearman; $p < 0.05$.

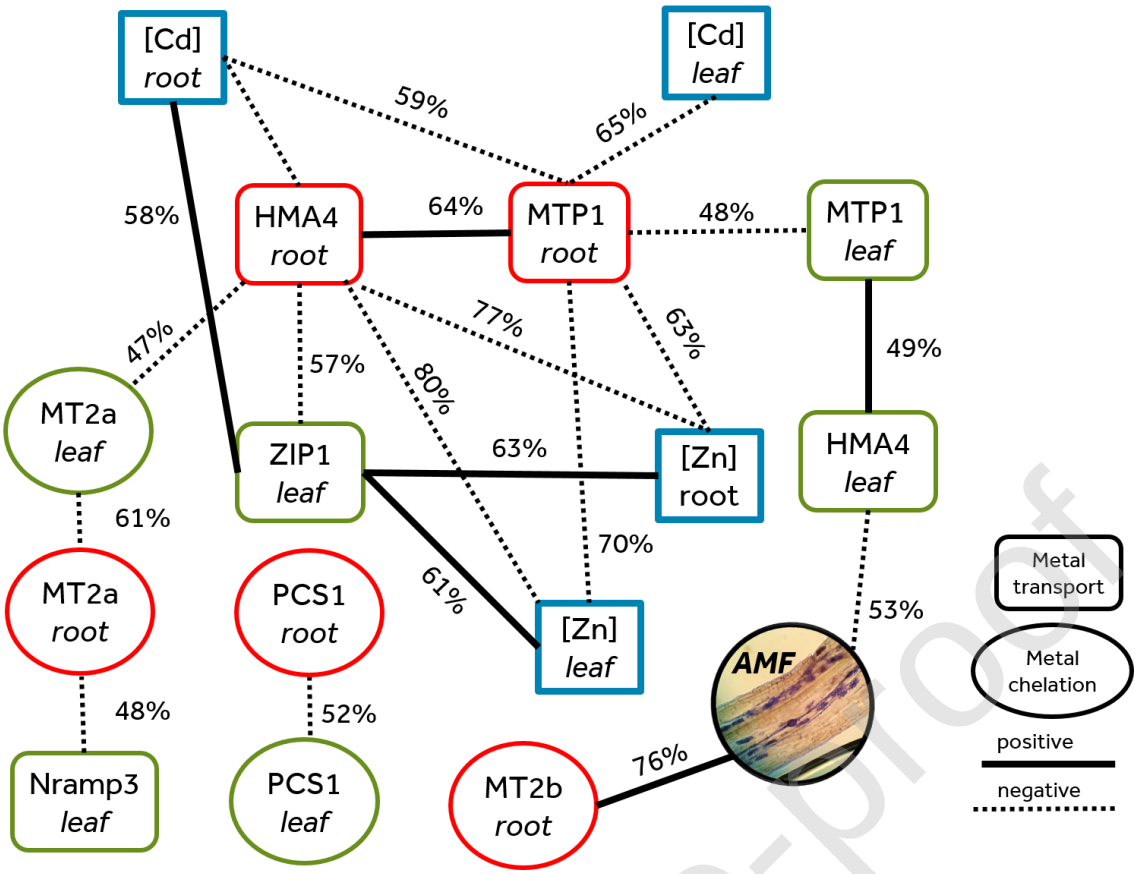
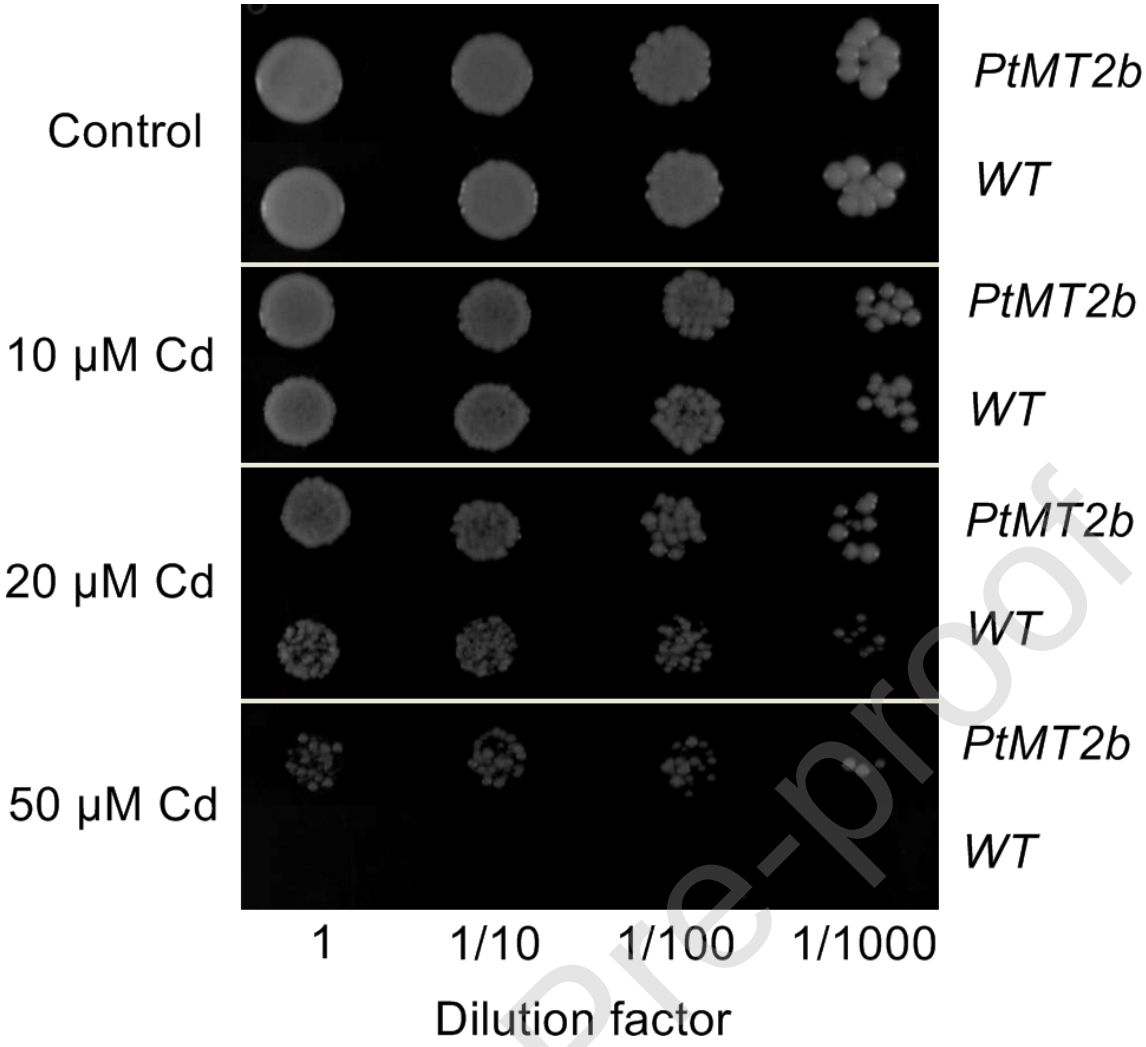


Fig. 5 Growth of *Saccharomyces cerevisiae* (DY1457) expressing the metallothionein gene *PtMT2b* under increasing Cd concentrations. The wild-type (WT) strain transformed with an empty vector was included as a control. Plates were grown in SC agar medium at 30ºC for 72 hours in the dark.



Tables 123

Table 1. Biomass (dry weight), transpiration rates (E) and root colonisation of *Populus trichocarpa* under Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) stress, with mycorrhizal inoculation (*R. irregularis*; + M) or without (autoclaved inoculum, NM).

Treatment	Biomass (g)		E	Colonisation
	Shoot	Root	mmol m^{-2} s^{-1}	%
Control NM	5.6 ± 1.9	0.5 ± 0.1	1.6 ± 0.2	-
Control + M	6.4 ± 0.9	0.4 ± 0.0	2.0 ± 0.1	46 ± 8 ab
Cd NM	6.1 ± 0.5	0.4 ± 0.1	1.7 ± 0.2	-
Cd + M	6.6 ± 0.6	0.4 ± 0.1	2.1 ± 0.2	50 ± 6 a
Zn NM	6.3 ± 0.3	0.5 ± 0.1	1.9 ± 0.3	-
Zn + M	7.0 ± 0.9	0.5 ± 0.0	1.9 ± 0.2	36 ± 9 b

Values are the means ± standard deviations, n = 6.

Significant differences among colonisation percentages are represented by different letters, by ANOVA ($p = 0.022$) followed by Tukey test.

No colonisation was detected in non-inoculated treatments.

Table 2. Cd concentration (mg kg^{-1}) and translocation factor (T*f*) in *Populus trichocarpa* under Cd stress (81 mg kg^{-1}) with (+ M) or without (NM) inoculation of *R. irregularis*.

Treatment	---------- Cd concentration ----------			T*f* (%)
	Leaf	Stem	Root	
Control NM	0.99 ± 0.3 aA	1.23 ± 0.3 aA	1.34 ± 0.3 aA	74
Control + M	0.76 ± 0.2 aA	0.96 ± 0.1 aA	2.57 ± 0.7 bB	30
Cd NM	8.47 ± 2.4 bA	48.0 ± 11 bA	725 ± 240 cB	1.2
Cd + M	5.02 ± 1.7 cA	26.2 ± 6.6 cA	871 ± 248 cB	0.6

Values are the means ± standard deviations, n = 6.

Different lowercase letters represent significant differences between treatments (columns) by GLM, followed by Tukey contrasts ($p < 0.05$).

Different uppercase letters represent significant differences between plant organs within the same treatment (rows), by ANOVA and Tukey test ($p < 0.05$).

T*f* = (leaf concentration / root concentration) × 100.

Table 3. Zn concentration (mg kg^{-1}) and translocation factor (T*f*) in *Populus trichocarpa* under Zn stress (300 mg kg^{-1}) with (+ M) or without inoculation of *R. irregularis*.

Treatment	---------- Zn concentration ----------			T*f* (%)
	Leaf	Stem	Root	
Control NM	21.0 ± 8.5 aA	40.0 ± 5.4 aAB	72.2 ± 41 aB	29
Control + M	21.4 ± 10 aA	39.4 ± 8.5 aAB	84.3 ± 57 aB	25
Zn NM	926 ± 225 bA	426 ± 68 bB	780 ± 102 bA	119
Zn + M	1227 ± 214 bA	472 ± 142 bB	1071 ± 63 bA	115

Values are the means ± standard deviations, n = 6.

Different lowercase letters represent significant differences between treatments (columns) by GLM, followed by Tukey contrasts ($p < 0.05$).

Different uppercase letters represent significant differences between plant organs within the same treatment (rows), by ANOVA and Tukey test ($p < 0.05$).

T*f* = (leaf concentration / root concentration) × 100.