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Uptake processes of Cd and Zn in mycorrhizal poplars and their potential for environmental remediation

Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Peer reviewed publications related to this thesis

De Oliveira VH, Tibbett M. 2018. Cd and Zn interactions and toxicity in ectomycorrhizal basidiomycetes in axenic culture, *PeerJ.* 6:e4478; DOI 10.7717/peerj.4478

De Oliveira VH, Tibbett M. **2018**. Tolerance, toxicity and transport of Cd and Zn in *Populus trichocarpa*, *Environmental and Experimental Botany*. 155: 281-292. DOI: https://doi.org/10.1016/j.envexpbot.2018.07.011

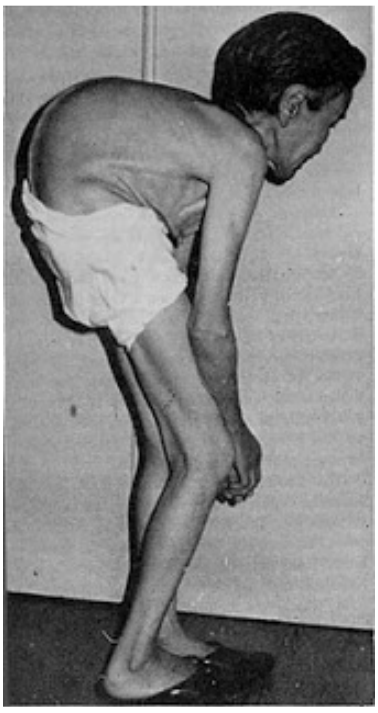
Thesis Abstract

Cadmium (Cd) is one of the most hazardous contaminants in the environment and it is often associated with zinc (Zn) in polluted soils, a nutrient that can also cause toxicity at high concentrations. Among soil remediation techniques, phytoremediation – the use of plants to immobilise and/or extract contaminants from soils - is a promising technique, considered to be less harmful to the environment. This thesis studies Cd and Zn fungi- and phytotoxicity, and the biotechnological potential of different organisms (ecto- and arbuscular mycorrhizal fungi, trees and yeast) in environmental remediation. The experiments conducted in this project aimed to investigate the potential of poplar trees (*Populus trichocarpa*) in Cd and Zn phytoremediation, and the use of mycorrhizal symbiosis (*Rhizophagus irregularis*) to enhance metal extraction and sequestration in the host plant. Another aim was to understand some of the physiological and molecular processes by which poplar trees withstand Cd and Zn toxicity, and to provide additional knowledge on the metal uptake process in mycorrhizal poplars. Transgenic yeast carrying a poplar gene (*PtMT2b*) was also studied for its potential in Cd bioremediation from contaminated solutions. Results showed that *P. trichocarpa* is highly tolerant to Cd stress, and has a considerable accumulation capacity of Cd and Zn; under both Cd and Zn exposure, poplar shoots reached hyperaccumulator levels. Mycorrhizal symbiosis increased Cd sequestration in roots, and Zn accumulation in leaves, supporting their use for Cd phytostabilisation and Zn phytoextraction. Gene expression assessment indicated mainly the involvement of *PtHMA4* and *PtZIP1* in Cd and Zn transport. Expression of *PtMT2b* was associated with mycorrhizal colonisation and its role in Cd tolerance was demonstrated in transgenic yeast assays. A mutated version of the MT2b gene (*PtMT2b* ‘Y’) promoted high Cd tolerance and accumulation in transgenic yeast showing promising results for bioremediation of Cd-contaminated wastewater. This thesis offers new opportunities for this possibly sustainable soil remediation technique; the knowledge gathered in this work may serve as basis for the genetic engineering of poplars or other organisms for heavy metal remediation or further research in refining and enhancing this technique.

Contents

Chapter 1

General introduction and literature review



Left: Famous picture of a Japanese woman suffering from the Itai-itai disease caused by Cd poisoning.

Right: Heavy metal contamination in Tar Creek (mostly Zn and Pb) in the Tri-State Mining District in (USA).

Sources:
[http://pollutionpictures.blogspot.com/2010/07/itai-itai-disease-cadmium-poisoning.html]
[https://serc.carleton.edu/NAGTWorkshops/health10/index.html]

1.1 Heavy metals in soils

The group of elements known as heavy metals (HMs) can be one of the most problematic and persistent environmental contaminants. These metals may be defined as the elements having density greater than 5 g cm^{-3} (Adriano, 2001) or a density five times greater than that of water (Naja and Volesky, 2009). Under such criteria, 53 out of the 90 naturally occurring elements are classed as HMs (Kaur and Garg 2017). Despite such definition not being an accurate characterisation of all the elements included in this group (Pourret, 2018), the term 'heavy metal' is still widely used and is as generalist as any other terms such as 'potentially toxic elements', 'toxic metals', 'trace elements', etc.

Heavy metals form the main group of inorganic contaminants and occur naturally in soils at low concentrations, due to pedogenetic processes through time (Alloway, 2013). However, anthropogenic inputs often lead to high concentrations in the environment, exceeding those considered as background concentrations (Mirsal, 2010). Sources of HMs in the environment can be natural (such as mineral weathering), via agricultural inputs, industrial processes and domestic effluents (Nagajyoti et al. 2010). However, the primary sources of metal pollution are related to mining and smelting, electroplating, burning of fossil fuels, fertilisers, pesticides, sewage, atmospheric deposition, batteries and sludge application (Garbisu and Alkorta, 2001; Gadd, 2010; Chibuike and Obiora, 2014).

More than 20% of China's arable land is polluted by HMs (Ministry of Environmental Protection of China 2013 *in:* He et al. 2015), and a total area of at least 2.88 x 10^6 ha of destroyed land has been generated due to mining activities (Ali et al. 2013). As for Europe, there are at least 160,000 sites known to be potentially polluted (Montpetit and Lachapelle 2017), and HMs are considered to be one of the main soil contaminants along with mineral oil (Jones et al. 2012). But unlike the organic contaminants, most metals will not suffer chemical or microbial degradation and will persist in the environment for a long time after their introduction (Wuana and Okieimen, 2011) posing a risk to living organisms (Long et al. 1995). For instance, the half-life of cadmium in soils is estimated to be from 13 to 1,100 years (Kabata-Pendias and Pendias, 2001). It is estimated that at least

6% of all European agricultural lands need eventual remediation due to HM contents above guidelines for food safety (Toth et al. 2016).

At the correct concentrations, several metals are essential to life and ecosystems. Nonetheless, chronic low exposures or excessive concentrations of these metals can be poisonous or lead to severe environmental and health effects in living organisms (Ho and El-Khairy, 2009). High concentrations of micronutrients such as Cu, Zn, Mn, Ni and Fe along with non-essential metals (e. g. Pb, Ag and Cd) in the environment are of great ecotoxicological concern, especially when essential elements are substituted by non-essential ones, causing toxicity symptoms or death (Naja and Volesky, 2009).

Soil contamination by HMs can lead to vegetation degradation, reduction of soil quality and affects, and consequently, the functioning of a whole ecosystem (Wong, 2003). When the metal enters the food chain, it can lead to biomagnification, meaning that a low concentration can increase and become even more toxic through different trophic levels (Janssen et al. 1993). For instance, high Cd amounts were found in the colostrum of mothers (54.5 μg L^{-1}) living in a small town in Brazil, which suggests that this region may be contaminated (Nascimento et al. 2005).

HMs are transitional elements, therefore several ions of different valence states are quite common for the same metal (Srivastava et al. 2012). However, most HMs usually form cations (e. g. Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}), which can be extremely hazardous, because once these toxic metals are present in the environment, they eventually become part of biotic and abiotic components of an ecosystem, interacting with each other and posing a risk to living organisms (Galloway et al. 1982). For instance, such cations can bind to proteins, inactivating enzymes as well as DNA replication processes (Srivastava et al. 2012).

1.2 Heavy metals in plants

Soil contamination by HMs is a critical environmental concern due to their potential adverse ecological and health effects. Such phytotoxic elements are of widespread occurrence, and their acute and chronic effects on plants grown on

such soils are often reported in the literature (Yadav, 2010 – and references therein) since plants are the major accumulators of inorganic nutrients, including metals, on which a high proportion of living organisms depend. Several elements are also essential nutrients, however high concentrations are often toxic (Ross and Kaye 1994). The physiological range for essential metals between deficiency and toxicity is extremely narrow, from which homeostatic control and adjustments are a necessity for dealing with nutrient/metal availability (Clemens, 2006).

Usually the root system is the primary form of contact between plants and the metal ions in soil solution, whereby toxic metal ions (such as HMs) enter plant cells by the same uptake processes that move essential micronutrient metal ions. According to Nieboer and Richardson (1980), class A metals (e.g. K, Ca, Mg) preferentially bind with oxygen-rich ligands, such as carboxylic groups, class B metals (e.g. Hg, Pb, Pt, Au) bind mostly with sulphur- and nitrogen-rich ligands (e.g. amino acids), and borderline metals (e.g. Cd, Cu, Zn) display intermediate preferences, with the heavier metals tending towards class B characteristics. HMs can also be absorbed directly into foliar tissues due to deposition of metal particles on leaf surfaces (Nagajyoti et al. 2010), increasing the risk of biomagnification through the food chain.

Therefore, in order to survive, plants evolved and developed efficient and specific mechanisms to tolerate HM uptake from soils (Zenk 1996). Plants have a complex system of uptake/efflux, transport/chelation and sequestration for maintaining metal homeostasis (Viehweger et al. 2014). There are several intracellular strategies by which plants tolerate or avoid HM uptake, such as stimulating efflux pumping of metals from cytosol, chelation of metals by organic acids or metallothioneins (Kotrba et al. 2009) and compartmentalization into the vacuole (Hall, 2002). At an extracellular level, mycorrhizal symbiosis (Schützendübel and Polle 2002) and exudation of organic compounds like phytochelatins (Schat et al. 2002) can increase plant metal tolerance.

Despite these tolerance mechanisms, phytotoxicity due to HM uptake is quite common. Some non-essential HMs have very similar geochemical characteristics to essential elements (macro or micronutrients) such as the case with arsenic (As) and P (Wenzel 2013) or Cd and Zn (Chaney 2010); because of this

similarity to essential elements, these metals can accumulate to potentially highly toxic concentrations in plant cells (Clemens, 2006). HMs can trigger similar toxicity responses due to other environmental stresses, such as with Cu and drought stress, Zn and cold stress or Cd mimicking pathogen contact effects (Viehweger et al. 2014). Phytotoxicity generally results in chlorosis, weak plant growth, yield depression, and may even be accompanied by reduced nutrient uptake, disorders in plant metabolism and, in leguminous plants, a reduced ability to fix molecular nitrogen (Guala et al. 2010). In higher plants, HM uptake and toxicity will depend on several factors, including plant species and ecotypes, rhizosphere microbiota, transport from surface into the root, translocation capacity from roots to shoots, soil chemical and physical characteristics, metal species, mobility and bioavailability of the element, and environment conditions (Ross and Kaye 1994; Patra et al. 2004).

1.3 Cadmium and zinc in soils and plants

1.3.1 *Cadmium*

Cadmium (Cd) is an element that lacks a known biological function. It is considered to be one of the most harmful metals in the environment, because it can affect humans and other organisms at relatively low concentrations and is highly mobile in soils (Lei et al. 2010). Without human interference, Cd content in soils varies considerably according to the parent material, which bear different amounts of this element (Bradl 2005; Khan et al. 2017), but soil background concentrations are usually around 0.5 mg kg^{-1} Cd (Kabata-Pendias and Pendias 2001). Cadmium is part of several primary minerals, mainly ZnO, ZnS (sphalerite), CdS (wurtzite/greenockite) and secondary minerals such as $ZnCO_3$, due to its affinity to Zn and S (Smolders and Mertens 2013; Kaur and Garg 2017). Cadmium compounds are also known to be isotypic to other cation compounds, such as with Zn^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} (Kabata-Pendias and Pendias 2001).

Cd occurs mostly in the form of Cd^{2+} and is usually concentrated in the topsoil, with its availability increased under lower soil pH (Kirkham 2006; Lux et al. 2011). Cadmium accumulates in soil upper horizons mostly due to their higher organic matter contents and nutrient cycling processes, atmospheric deposition

and application soil amendments and fertilisers (Alloway 2013). In a fertiliser factory in the south of Brazil, atmospheric emissions are believed to be responsible for increasing Cd contents in nearby soils from 0.5 to 3.26 mg kg^{-1} over the years (Mirlean and Roisenberg 2006).

Other anthropogenic sources of Cd include the combustion of fossil fuels, metallurgical activities, wastes from the cement industry, industrial and municipal wastes, mining, smelting and metal ore processing, which are responsible for a wide range of 5.6-38 x 10^6 kg of Cd released into the environment every year (Science Communication Unit 2013; He et al. 2015; Khan et al. 2017). Although mean Cd concentration in European topsoil is of 0.2 mg kg^{-1}, areas with high Cd can be found in Italy, Slovenia, Croatia and France, with the outlier of 14.1 mg kg^{-1} Cd reported in Greece (*terra rossa*) (Figure 1.1); as for stream sediments, even higher amounts of Cd can be found, mostly in Britain, Belgium, Czech Republic, northern and central Germany, and south-western Poland, of up to 43 mg kg^{-1} Cd (Salminen et al. 2005). In general, minimally polluted soils can contain up to 7 mg kg^{-1} Cd, while those near smelters can have concentrations as high as 578 mg kg^{-1} (He et al. 2015). Some soils around mining areas in China were reported to contain nearly 80 mg kg^{-1} Cd, with an average of 11 mg kg^{-1} Cd (n=72) (Li et al. 2014).

In agricultural soils specifically, Cd sources are mainly the application of sewage sludge and phosphate fertilisers (Alloway 2013). Some commercial phosphate fertilisers can have Cd contents from 0.67 up to 43 mg kg^{-1} (Bizarro et al. 2008) and increasing Cd concentrations in plants due to the use of such fertilisers have been reported (Nicholson et al. 1994; Gonçalves et al. 2008; Freitas et al. 2009; Gao et al. 2011). Cadmium deposition rates were estimated to be around 1,900 mg ha^{-1} every year in England and Wales, mainly via atmospheric deposition and inorganic fertilisers (Nicholson et al. 2003). Cadmium in agricultural soils in the UK ranges from less than 0.2 to 40.9 mg kg^{-1} (Chaney 2010).

Increasing Cd concentrations in agricultural soils is especially worrisome, since one of the main routes by which humans are exposed to Cd is by ingesting plants grown in areas with high contents of this metal (ATSDR 2017), with exposure through vegetable consumption accounting to around 80% of total Cd intake in humans (Khan et al. 2017). In animals it can accumulate in the liver,

kidneys and reproductive organs (Kirkham 2006); with Cd contents reaching high levels in animal meat consumed as food, such as the 0.45 and 1 mg kg^{-1} Cd in pork kidney, in the UK and Denmark, respectively (Pan et al. 2010). In humans, long-term exposure to Cd may lead to renal disfunction, lung diseases, but mainly this element is most associated with bone disorders, such as demineralisation of the bone causing osteomalacia (Mirsal 2010; ATSDR 2017). Cadmium is also associated to mutagenic effects, acting as a carcinogenic agent (Templeton and Liu 2010).

In plants, Cd phytotoxicity can cause chlorosis, stunting, lipid peroxidation, necrosis, enzyme inactivation, decrease chlorophyll production, and induce oxidative stress (Pal et al. 2006; Gallego et al. 2012). Seed germination and seedling growth are also greatly affected by Cd (Chibuike and Obiora 2014; De Oliveira et al. 2016). At the cell level, Cd can alter chloroplast structure, degrade the mitochondria, inhibit mitosis and cause chromosomal aberrations (Das et al. 1997; Gallego et al. 2012). Cadmium also competes with other elements for the same membrane transporters, thus inhibiting the uptake of other elements such as K, Fe, Mg and Ca (Rivetta et al. 1997; Shah et al. 2010). Some metalloproteins can have its native metal substituted by Cd, which can alter its functionality, such as the substitution of Mg in RuBisCo, Mn in oxygen complexes from the photosystem II and Ca in calmodulin (Viehweger et al. 2014). Normally in the environment, Cd concentrations in land plants are not high enough to induce toxicity, ranging from 0.1 to 2.4 mg kg^{-1} Cd (Nagajyoti et al. 2010).

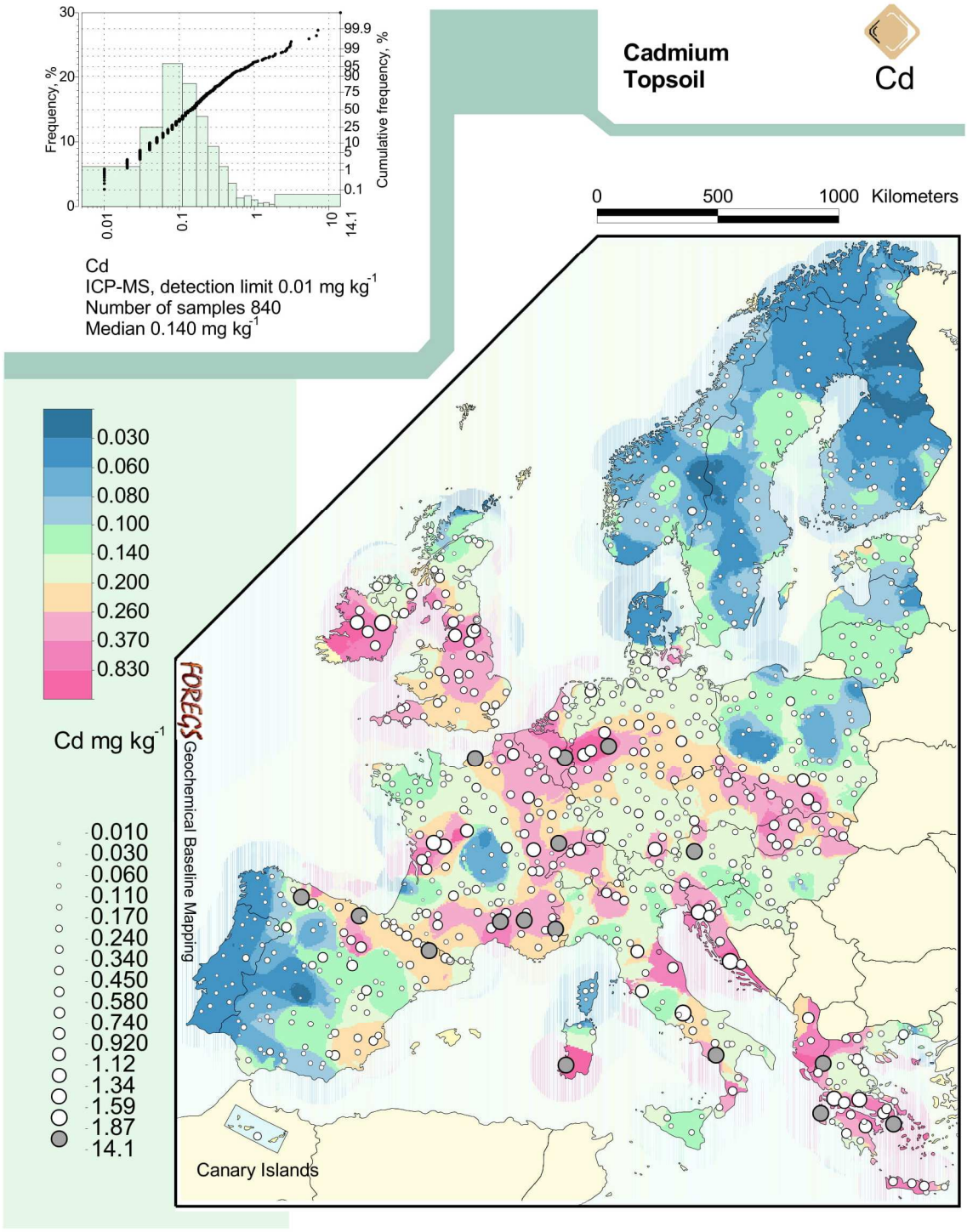


Figure 1.1 European map of Cd concentrations in topsoil in mg kg^{-1}, extracted from Salminen et al. (2005).

1.3.2 *Zinc*

Unlike Cd, Zinc (Zn) is an essential element and nutrient for living organisms, known for several biological functions, especially as an integral part of several enzymes, including the RNA polymerase (Nagajyoti et al. 2010), therefore controlling also cell differentiation and proliferation. In soils, Zn concentrations worldwide are on average 64 mg kg^{-1} (Noulas et al. 2018) but depending on the parent material, environmental conditions and history of human activities, this mean concentration will shift. For instance, 97 mg kg^{-1} is on average the Zn concentration in soils from England and Wales (Alloway 2008), while the overall Zn concentration in soils range from 8 to 100 mg kg^{-1} (Nagajyoti et al. 2010).

Zinc is often associated with Cd in soils (Mulligan et al. 2001; Khan et al. 2017) and, although is not as toxic as Cd, high concentrations of Zn can cause several ecotoxicological effects, mainly to plants, soil dwelling organisms and microorganisms. Data suggest that Zn in soil systems can be more toxic to soil organisms than Pb (Ross and Kaye 1994) and can also decrease bacterial diversity in contaminated lands (Moffett et al. 2003). Zn toxic effects are mainly limited to the lower trophic levels, with the phytotoxicity itself acting as a barrier for the biomagnification of this elements in the food chain, therefore Zn chronic poisoning of wild life and humans via the food chain is rare (Mertens and Smolders 2013).

Zinc deposition in the environment is believed to be on average 227 mg ha^{-1} per year in Europe, with similar rates for England and Wales (Nicholson et al. 2003). Sources of Zn in soils due to human activities include: atmospheric deposition as a result of emissions from coal burning, waste incineration and industrial processes, municipal and industrial wastes, urban runoff, mine activities and drainage, erosion of Zn-containing soil particles, application of fertilisers, agrochemicals, sewage sludge and livestock manures directly into soils (Alloway 2008, Yadav 2010; Mertens and Smolders 2013; Noulas et al. 2018). In central Britain, Zn enrichment of stream sediments have been associated with agriculture pollution through manure spreading (Salminen et al. 2005). Agricultural soils receive constant Zn additions, according to Nicholson et al. (2003), with the annual input of Zn into agricultural lands in England and Wales (in 2000) on average 5000

tons, from which around 49% were from atmospheric deposition, 37% from livestock manures, 7% from sewage sludge and 5% from inorganic fertilisers.

In Europe, Zn concentrations in topsoil can range from a very deficient amount of 3 mg kg^{-1}, especially in northern countries, to around 2,900 mg kg^{-1} in contaminated areas, such as in north-eastern Italy, Sardinia and Calabria (Figure 1.2), while some stream sediments can contain over 10,000 mg kg^{-1} Zn (Salminen et al. 2005). Assessments of 72 mining areas in China revealed Zn contamination in soils with an average around 1,200 mg kg^{-1} and a maximum of nearly 24,000 mg kg^{-1} (Li et al. 2014; data extracted from figures); while reports on soils from a mining region in India showed a Zn concentration of 5,982 mg kg^{-1} (on average) with the presence of elevated Cd (24 mg kg^{-1}), in which a positive correlation (r = 0.86) was found between the two elements (Anju and Banerjee 2011).

In plants, Zn contents generally range from 30 to 100 mg kg^{-1} of dry matter (Noulas et al. 2018) with 300-400 mg kg^{-1} of Zn in leaves being the common threshold for metabolic perturbations or phytotoxicity (Marschner et al. 1995; Kaur and Garg 2017). However, critical levels lower than 200 mg kg^{-1} Zn have been reported in some crops, such as maize, bush beans and cabbage (Mertens and Smolders 2013). Similar to Cd, Zn toxicity effects in plants include growth inhibition, leaf chlorosis and necrosis, oxidative stress and impairment of photosynthesis (Todeschini et al. 2011). Zn toxicity can also decrease seed germination, plant biomass, chlorophyll and carotenoid contents and the efficiency of photosynthetic energy conversion (Chibuike and Obiora 2014).

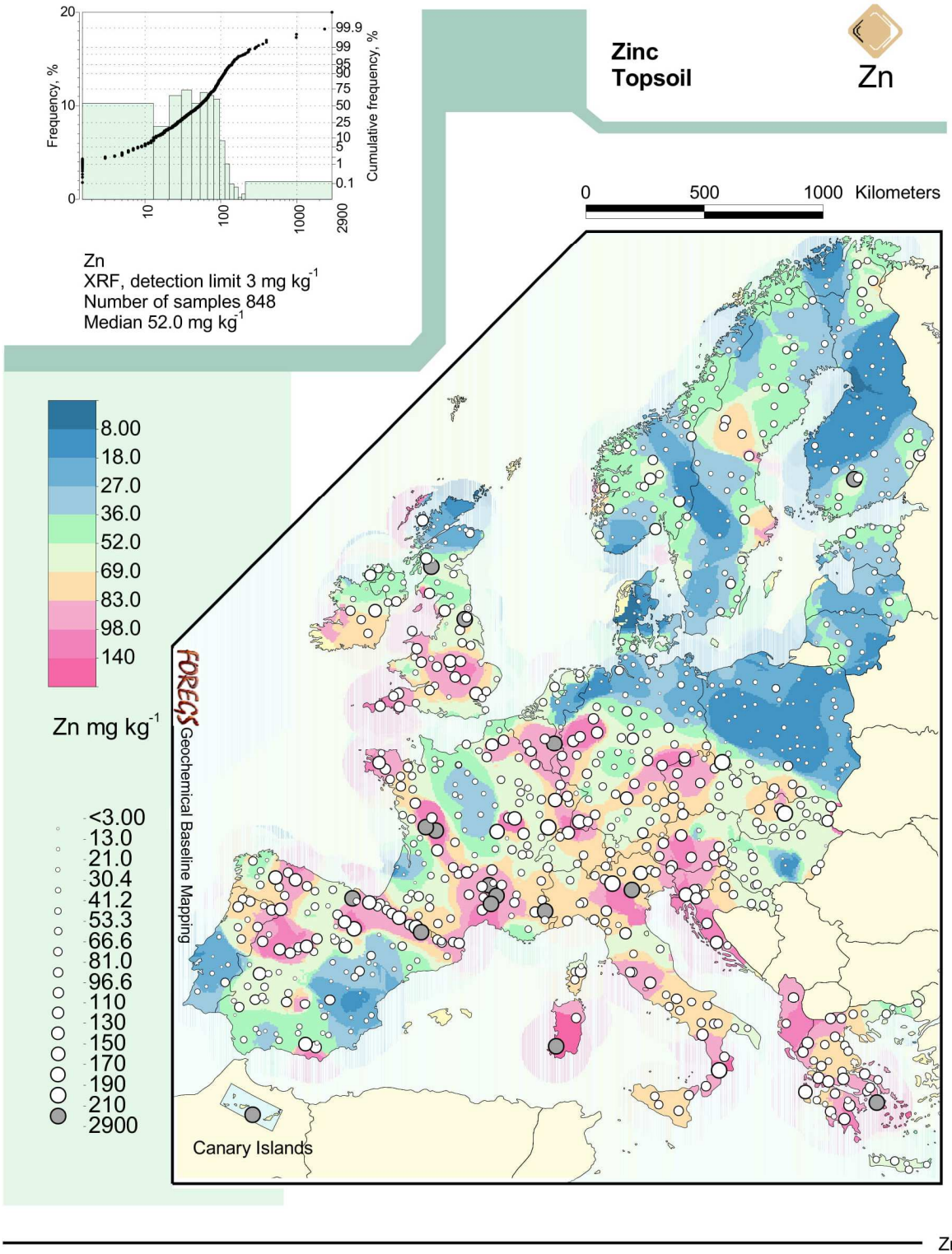


Figure 1.2 European map of Zn concentrations in topsoil in mg kg^{-1}, extracted from Salminen et al. (2005).

1.4 Cd and Zn uptake and transport in plants

1.4.1 Transport proteins

HMs can be taken up by plant root cells mainly by direct interception or diffusion in the soil solution gradient, depending on their bioavailability and solubility (Shah et al. 2010). After translocation to the apoplast, plants have a series of transporters involved in metal uptake and homeostasis, which regulates metal movement into the symplast and further loading into vascular tissues (Palmer and Guerinot 2009; Luo et al. 2016). However the high cation exchange capacity (CEC) from cell walls can strongly limit metal movement in the apoplast (Shah et al. 2010). Metal transporters in plants are diversified and this variation is responsible for the high and low affinity systems necessary to withstand stress conditions or different metal availability in soils, providing enough specificity to meet different cellular requirements within the plant (Guerra et al. 2011).

Transport of metals into the symplast can be carried out by members of numerous transporter families. The main transporter families for HMs are: the heavy metal (Cpx-type) ATPases and the cation diffusion facilitator (CDF) family proteins, which are associated with metal efflux; along with the zinc-iron (ZIP) family proteins and the natural resistance-associated macrophage protein (Nramp) family proteins, which are associated with metal uptake and HM tolerance (Williams et al. 2000; Sheoran et al. 2011; Colangelo and Guerinot 2006; Yang et al. 2005).

The divalent metal ZIP transporters are the most likely to mediate Zn transport in plants and can be found also in bacteria, fungi and humans (Dhankhar et al. 2012), they are named after the Zn-regulated transporters (ZRT) and Fe-regulated transporters, due to their sequence similarities (Zhang et al. 2017). Members of the ZIP family are able to transport not only Zn and Fe, but also several other cations into the cytosol (Pottier et al. 2015; Iori et al. 2016). Because 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+} channels (Guerra et al. 2011; Clemens 2006), but especially through ZIP transporters (Lux et al. 2011; Sheoran et al. 2011; Zhang et al. 2017). While ZIP appears to be mainly responsible for Zn and Cd influx into the cytoplasm, their

efflux into the apoplast are mostly related to another set of proteins, known as heavy metal ATPases (Claus et al. 2013).

The P_{1B}-type ATPases, or heavy metal ATPases (HMAs), have an important role in metal transport in plants and are usually present in the plasma membrane, acting as pump for HM efflux from the cytosol, removing toxic elements from the cytoplasm (Sheoran et al. 2011) or when in the tonoplast membrane it is involved in compartmentalising HMs into the vacuole (Yang et al. 2005). In prokaryotic organisms, several P_{1B}-type ATPases have a specificity for non-essential elements such as Pb^{2+}) and Cd^{2+}, and are also involved in metal efflux (Cobbett et al. 2003). In plants, HMA4 can selectively absorb and transport essential metals as well as HMs, especially Zn^{2+} and Cd^{2+} (Hussain et al. 2004; Adams et al. 2011; Gallego et al. 2012). *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) playing an important role in the long distance transport in plants (Luo et al. 2016; Sarwar et al. 2017). In the hyperaccumulator *Arabidopsis halleri*, *AhHMA4* is also involved in Zn/Cd tolerance by maintaining low concentrations of Zn^{2+} and Cd^{2+} in the cytoplasm (Courbot et al. 2007), and tandem duplication of the *HMA4* gene was found in the Cd/Zn hyperaccumulator *Noccaea caerulescens* (Ó Lochlainn et al. 2011).

Another protein that can mediate Zn^{2+} and Cd^{2+} transport in plants is the Cation Diffusion Facilitator (CDF) family, which are also known as MTP (Metal Tolerance Proteins), and have been associated with metal transport into the vacuoles and other subcellular compartments (Williams et al. 2000; Yang et al. 2005; Clemens 2006; Ricachenevsky et al. 2013). MTPs are $Metal^{2+}/H^{+}$ (or K^{+}) antiporters and generally mediate the efflux of metal cations from the cytoplasm, similar to the heavy metal ATPases (Migeon et al. 2010), hence their correlation with HM tolerance. MTP1, MTP2 and MTP3 are very similar among higher plants, for instance, the *PtMTP1* gene in the woody species *Populus trichocarpa* is closely related to *AtMTP1* from *A. thaliana* (by phylogenetic analyses), with both involved in Zn transport and tolerance by facilitating Zn compartmentalisation into the vacuole (Migeon et al. 2010). The MTP1 gene specifically is believed to create a sink for metals in plant shoots and is known to be highly expressed in Zn

hyperaccumulator species (Gustin et al. 2009; Viehweger et al. 2014). For example, higher expression of *MTP1* was also found in shoots of the Cd/Zn hyperaccumulator *N. caerulescens* than in the closely related non-accumulating species *Thlaspi arvense* (Hammond et al. 2006).

The Nramp gene family are also capable of transporting Cd^{2+} into plant cells (Clemens 2006). Nramps are an integral part of cell membranes of different organisms and in *Arabidosis* the Nramp3 transporter specifically, is known to be localised in the vacuolar membrane (Thomine et al. 2003). Plant Nramps are expressed throughout all plant tissues and transport several different divalent metal cations, such as Zn^{2+}, Mn^{2+} and Fe^{2+} (Palmer and Guerinot 2009; Migeon et al. 2010). Even though Cd is not an essential element, its transport in plant cells is effectively carried out by members of the Nramp family: disruption of *AtNramp3* can increase Cd tolerance, while its overexpression can lead to Cd toxicity in plants (Thomine et al. 2000). Expression patterns of Nramps in poplar trees, which are generally tolerant to metals, showed a high correlation between these genes and heavy-metal ATPases, while Nramp1 in particular was strongly correlated to the accumulation of Cd and Zn, although this protein seems to have a selectivity in favour to the latter (Pottier et al. 2015). Similarly, Nramp1 can also be an important pathway of Cd uptake in Arabidopsis roots (Migeon et al. 2010).

1.4.2 *Phytochelatins and metallothioneins*

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). Two of the main characterised chelators in plant cells are the phytochelatins (PCs) and metallothioneins (MTs) (Clemens 2006). PCs are a class of non-protein structures with increasing repetitions of Gly-Cys terminated by Gly, having the general formula of (γ-Glu-Cys)-n-Gly, and are present in a great variety of plant species, as well as some microorganisms (Garg and Kaur 2013), with a pivotal role in HM detoxification (Kotrba et al. 2009).

PC synthesis and formation of PC-metals complexes are directly related to metal stress in plants and are rapidly induced in cells exposed to a range of HM

ions, such as Cd^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} (Cobbett and Goldsbrough 2002; Yadav 2010; Guerra et al. 2011; Sheoran et al. 2011). For Cd, it is known that deficiency in PC production results in low Cd accumulation (Clemens 2006). PCs are synthesised non-translationally from reduced glutathione (GHS), a reaction catalysed by the enzyme PC synthase, and one of the genes known for encoding this enzyme is the phytochelatin synthetase PCS (Clemens 2001; Yadav 2010; Adams et al. 2011).

PC-metal complexes are possibly redistributed within the plant via apoplast and symplast pathways, initiated by ATP-binding cassette transporters (Kotrba et al. 2009). After being transported to the target tissue, metals are redistributed to sub cellular compartments, where the vacuole is considered to be the main storage compartment for detoxification of HMs (Guerra et al. 2011). The most important mechanism for Cd detoxification in plants is the PC pathway, which involves metal chelation and transport to the vacuole via MTP and CDF proteins (Williams et al. 2000; Yang et al. 2005; Clemens 2006). The role of PCs in HM tolerance has been demonstrated in several plant species for both Cd and Zn (Adams et al. 2011- and references therein).

MTs are proteins with low-molecular weight and rich in cysteine (usually 9-16 Cys residues), which bind metals in metal-thiolate clusters (Cobbett and Goldsbrough 2002; Sheoran et al. 2011). In contrast to PCs, which are enzymatically synthesised, MTs are gene encoded. In plants MTs are considered to be responsible for the homeostasis of essential HMs and the transcription of their genes is controlled by signals from germination to senescence stages (Kotrba et al. 2009). MTs are divided into four subfamilies, from which the Type 2 MTs are considered to be the main group involved in binding Zn and Cd (Hassinen et al. 2011). In humans, around 15% of Zn in cells are bound to MTs (Kimura and Tambe 2016), in other animals, MTs protect against Cd toxicity, while in plants MTs are more associated with copper tolerance and homeostasis (Cobbett and Goldsbrough 2002). However, some plant MT genes can confer Cd tolerance when expressed in transformed yeasts (Kohler et al. 2004; Clemens 2006).

MT expression in plants is not only associated with HM tolerance (Hassinen et al. 2011), but also the metal hyperaccumulation phenotype, which may be

related to a high expression of MT genes, as shown in *N. caerulescens* by Hammond et al. (2006). Plants overexpressing MTs thus, tolerate and accumulate more Cd (Yadav 2010). The expression of some MT genes in plants is usually influenced by the exposure to Cd and Zn in soil (Hassinen et al. 2011; Konlechner et al. 2013), however other factors around the rhizosphere, such as mycorrhizal symbiosis, can up-regulate their expression in plants (Cicatelli et al. 2012; Pallara et al. 2013). Moreover, MTs have also been associated with other roles, such as an antioxidant function and reactive oxygen species (ROS) scavenging and membrane repair (Hall 2002; Wong et al. 2004; Hassinen et al. 2011).

1.5 Phytoremediation

As HMs in soils are well known to be potentially toxic to biota in general, remediation measures must be taken. Although it is very difficult to remove HMs bound to the soil matrix (Thakur et al. 2016), several environmental remediation methods involving physical, chemical, or biological treatments have been developed for reclamation of metal contaminated soils in the past (Mulligan et al. 2001), and their main goal is to create a final solution that is protective of human health and the environment (Wuana and Okieimen 2011). However, treatments like soil washing; acid extraction or electrokinetic remediation can be costly and may irreversibly affect soil properties, destroy biodiversity and even render the soil useless for plant growth (Padmavanthiamma and Li 2007; Meier et al. 2012).

Phytoremediation (or plant-facilitated bioremediation), is a term coined in the 1980s and can be generally defined as the use of plants and their associated microorganisms for environmental cleanup or reclamation (Pilon-Smits 2005; Willey 2007), mostly by removing, destroying or sequestering hazardous contaminants (Prasad 2003). It is an *in situ* technique that can be useful for several contaminants, is solar driven, eco-friendly and cost effective, making it an alternative to the conventional methods (Guerra et al. 2011; Ali et al. 2013). Phytoremediation can preserve the soil structure and protect it from water and wind erosion, therefore reducing the spread of pollutants in the environment. Using plants for remediation can also preserve the soil microbiota and root exudates concentrate microorganisms around the rhizosphere, which may also

participate in the remediation process (Pulford and Watson 2003; Kotrba et al. 2009). Sites subjected to phytoremediation can be aesthetically pleasing, compared to alternatives such as industrial soil washing, and consequently have higher community acceptance than other remediation methods, increasing the likelihood of the successful deployment of this technology.

Among the phytoremediation techniques, phytostabilisation is one of the most important for HMs (Prasad 2003). In phytostabilisation, metal ions can become less available in soil due to absorption, complexation, reduction or precipitation within the roots or the rhizosphere (Ali et al. 2013; Thakur et al. 2016), in which the plant will restrict the transfer of metals to its shoots (Qasim et al. 2016). It does not remove the contaminant from soil, but reduces its inherent hazard to biota (Arthur et al. 2005). By immobilising HMs in soils, plants can reduce their bioavailability and mobility in the environment, therefore preventing their migration to the groundwater or their entry into the food chain (Erakhrumen 2007). For Cd in particular, this technique is more desirable due to the risk this element poses when accumulated in edible plant parts, such as leaves, fruits and seeds.

Phytoextraction is another technique considered to be more efficient for inorganic contaminants in several substrates, such as contaminated soils, sediments and water (Marmiroli et al. 2006). This technique is based on metal removal from soil by plant root uptake and translocation to aboveground parts, where it is accumulated over time. It involves the continuous cropping of plants until the heavy-metal contaminated soil reaches acceptable levels (Sheoran et al. 2011). It is recommended that the biomass produced (with high levels of the contaminant) should be then incinerated or fermented to reduce its volume (Robinson et al. 2009), which can be properly discarded or even used as a biosorbent to remove other contaminants (Arthur et al. 2005). In phytoextraction, plant species with hyperaccumulation capacity are often used. Hyperaccumulators can tolerate and build up high concentrations of metals in comparison to other plants (Gratão et al. 2005) and generally grow naturally in areas with high metal concentrations (Kramer and Chardonnens 2001). These plants can also

accumulate high concentrations of metals that have no known biological functions such as Cd, Hg, Au and Cr (Sheoran et al. 2011).

Phytoextraction efficiency is determined by two key factors: bioconcentration factor and biomass production (Sheoran et al. 2011). Although with time hyperaccumulator plants may eventually decontaminate soils, these species are often of endemic occurrence, slow growth, low root penetration and low biomass production, which may compromise the efficiency of this technique (Bhargava et al. 2012), such as the Zn hyperaccumulator *N. caerulescens* (Ebbs and Kochian 1997). Therefore, it is necessary to search for species and alternatives that enable phytoextraction such as: 1) a plant species capable of producing high biomass, 2) accumulating considerable amounts of metals in aboveground parts, 3) tolerating high levels of the contaminant, 4) rapid growth rates and 5) deep or profuse root systems (Ebbs and Kochian 1997; Sheoran et al. 2011).

1.5.1 Trees in phytoremediation

Non-hyperaccumulator plants can be an alternative if they can tolerate metal stress and have higher biomass production, especially if soil conditions are manipulated to increase metal bioavailability and uptake (Pulford and Watson 2003). Woody plants, such as trees can offer a good solution in terms of phytostabilisation and recovery of degraded environments (French et al. 2006). The use of tree species is an emerging phytoremediation technology and can be sometimes referred to as “Dendroremediation” (Komives and Gullnar 2006). Although generally the metal concentrations in trees do not reach extreme levels, sometimes their greater biomass production may provide a higher metal extraction rate from soils in comparison to herbaceous hyperaccumulators (Luo et al. 2016). In the tropical tree species *Averrhoa carambola* (star fruit), leaf Cd concentrations can indeed reach hyperaccumulator levels of 100 mg kg^{-1} in dry weight (Li et al. 2010), but that is not a common feature.

In comparison to agricultural species, trees can have some advantages for HM remediation, such as deep root systems and site stabilisation, a characteristic that can also be effective to reduce leaching into groundwater (Dos Santos and Wenzel 2007). Control of erosion, litter and vegetation cover and overall addition

of organic matter into soils are also other general advantages of employing trees in soil remediation schemes (Pulford and Watson 2003; Brunner et al. 2008). Trees are also long-lived organisms, which means they could take up contaminants from the environment and store them for a long time (Dominguez et al. 2008), some of which can form mycorrhizal symbiosis with ectomycorrhizal fungi (ECM), capable of accumulating even more metals into its cell walls and fungal vacuoles (Brunner et al. 2008). The most important aspect of trees to make them suitable for phytoremediation is their large biomass production, above and below ground, as well as their high transpiration rates, making them key plants for remediation, with poplar trees having enough advantages to be considered as the first choice for this purpose (Pulford and Watson 2003; Komives and Gullner 2006). Notwithstanding their great potential, limited information is available on the physiological and molecular mechanisms of HMs uptake, transport and sequestration in trees (Konlechner et al. 2013; Luo et al. 2016).

1.5.2 The relevance of Poplars

The genus *Populus* (poplars, cottonwoods and aspens) from the Salicaceae family is considered to be a model tree in forest genetics and biotechnology studies and can be used to assess important plant processes for woody species, much like tomato or *Arabidopsis* are used for herbaceous plants (Guerra et al. 2011), especially after *P. trichocarpa* had its genome sequenced (Tuskan et al. 2006). *Populus* is a genus of deciduous trees, wind-pollinated and diploid (2n = 38), with diffuse-porous and lightweight wood capable reaching 40 m in height in less than 20 years, with production rates from 17 to 30 ton/ha yearly of dry biomass when growing under intensive culture of 6-8 year rotations (Bradshaw et al. 2000; Marmiroli et al. 2011).

Poplars are known to have considerable potential for remediation of contaminated soils, because of their greater biomass, deep root systems (Bhargava et al. 2012), and also for being fast-growing, with high water-use (Robinson et al. 2009). *Populus* species can also rapidly invade disturbed sites, reproduce asexually – by sprouting from the root collar of cut trees or broken branches (Sebastiani et al. 2004; Hamberg et al. 2011) and are not a source of food

for farm animals, therefore reducing the risk of HMs entering the human food chain (Shim et al. 2013).

Although their metal accumulation potential is not extremely high, especially in comparison to the Cd hyperaccumulator *N. caerulescens*, Marmiroli et al. (2011) calculated that in soils with similar levels of Cd contamination (7-8 kg Cd ha^{-1}), poplars could extract more Cd per hectare (250 g Cd ha^{-1}), than *N. caerulescens* (125 g Cd ha^{-1}), mostly due to their greater biomass production. Moreover, some poplar varieties have already presented high accumulation of HMs, especially Cd and Zn (Guerra et al. 2011; Bissonnette et al. 2010; Dominguez et al. 2008; Dos Santos and Wenzel 2007; Robinson et al. 2005; Robinson et al. 2000). However, there is a wide variation in the ability of different *Populus* species and clones to accumulate and allocate metals in aboveground parts (Pietrini et al. 2010).

From the *Populus* genus, *Populus alba* sp. (white poplar) is a species of great interest for phytoremediation of HM contaminated soils, with different studies, either *in vitro* or soil, indicating its potential for the technique (Ciadamidaro et al. 2014; Di Lonardo et al. 2011; Franchin et al. 2007). Nonetheless, *P. trichocarpa* had its genome sequenced and is also able to extract high amounts of HMs from soils (De Oliveira and Tibbett 2018), which makes it a good candidate for molecular studies under HM stress.

According to Bradshaw et al. (2000), *Populus* is regarded as a model system due to the following characteristics:

(1) Abundant genetic variation in natural populations;

(2) Ease of sexual propagation (wind pollinated);

(3) Fast and noticeable physiological responses to environmental variables;

(4) Large database of physiological traits;

(5) Well-characterized molecular physiology and a small genome size (550 million bp);

(6) Ease for cloning individual tree genotypes (vegetative propagation);

(7) Closely related to other model angiosperms;

(8) Easy transformation and regeneration to create transgenic plants; and

(9) Potential for commercial application.

The ability to form mycorrhizal symbiosis is another advantage of *Populus* species compared to hyperaccumulator plants, which are mostly from the Brassicaceae family and generally considered to be non-mycorrhizal (Leyval et al. 1997). Even though recent studies have shown that a few non-Brassicaceae hyperaccumulators can indeed form arbuscular mycorrhizal symbiosis (Vogel-Mikus et al. 2005; Vogel-Mikus et al. 2006), the colonisation rates are very low (Alford et al. 2010).

Populus sp. is one of the few tree genera known to form tripartite symbioses (a three-organism symbiotic association), in combination with both arbuscular and ectomycorrhizal fungi (Ma et al. 2008; Bissonnette et al. 2010; Marmiroli et al. 2011), or in some cases with ectendomycorrhizal fungi (Yu et al. 2001; Siemens and Zwiazek 2008), or even *Pseudomonas* species (Labbe et al. 2014). Fillion et al. (2011), studying phytoremediation strategies in woody species, have demonstrated high colonisation rates of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* in poplars and willows, which resulted in better phosphorus nutrition and biomass increase compared to controls.

A diverse community of ectomycorrhizal (ECM) fungi was also found in poplars growing in metal contaminated soil, 54 species of which 43 were from Basidiomycota phylum (Krpata et al. 2008). Nevertheless, there is a need for more studies involving poplars and ectomycorrhizal fungi in phytoremediation because it is known that woody pioneers species rely greatly on this type of mycorrhizal symbiosis, which can be very important for the primary establishment of these trees in contaminated soil (Colpaert 2008). As for AMF symbiosis, in *P. alba* exposed to 950 mg kg^{-1} Zn, AMF colonisation in roots was similar to uncontaminated controls (Lingua et al. 2012), while in another poplar clone growing in Cd-contaminated soil, Ciadamidaro et al. (2017) reported a 40% increase in plant biomass due to AMF inoculation.

1.5.3 Mycorrhizal fungi in phytoremediation

Other means to improve phytoremediation are by improving plant tolerance to HM toxicity using soil microorganisms, such as plant growth-promoting bacteria (Khan 2006) or mycorrhizal fungi (Vamerali et al. 2010; Saraswat and Rai 2011). Similar to the term Dendroremediation, mycorrhizal-enhanced phytoremediation processes can sometimes be referred to as Mycorrhizoremediation (Garg and Chandel 2010).

Almost all land plants depend on symbiotic mycorrhizal fungi: an integral and functioning part of plant roots, in which the fungi involved provide a direct link between soil and roots (Leyval et al. 1997; Coninx et al. 2017). The main types are Endomycorrhizas, when the fungus colonises the interior of host plant root cells (mostly arbuscular mycorrhizal fungi – AMF, but also ericoid mycorrhizas); and Ectomycorrhizas (ECM), in which the fungus is located outside plant root cells (Smith and Read 2008; Gadd 2010). The establishment of mycorrhizal symbiosis usually allows plants to enhance the uptake of low mobility nutrients, such as phosphate, some nitrogen compounds and metals (Gonzalez-Guerrero et al. 2009). Both AM and ECM symbiosis have a crucial role in alleviating metal stress and facilitating the re-forestation of HM-contaminated areas, mostly by influencing HM availability in the rhizosphere and providing the host plant with water and nutrients in a poor environment (Gherghel and Krause 2012).

Generally, mycorrhization may improve phytoextraction of HMs by at least four mechanisms: (1) promoting plant growth and biomass production; (2) increasing plant tolerance to metal toxicity; (3) increasing soil exploration via extraradical hyphae; and (4) enhancing the bioavailability of elements in the rhizosphere by fungal exudation (Smith and Read 2008; Gonzalez-Guerrero et al. 2009; Gadd 2010; Vamerali et al. 2010; Sheoran et al. 2011). However, the ability of the fungus in surviving in metal-contaminated soils is a prerequisite for its use in phytoremediation (Coninx et al. 2017).

Some of the mechanisms by which mycorrhizal fungi tolerate metal toxicity are by binding toxic cations into their negatively-charged cell walls (containing chitin and melanin or glomalin in AMF), which is believed to account for approximately 50% of the metal ions absorbed (Saraswat and Rai 2011). Chelation

is the first defence mechanism as soon as HMs enter the cytosol, while sequestration into vacuoles or other organelles, scavenging of ROS and pumping metals out of the cytosol are followed in order to prevent cytotoxicity (Luo et al. 2014; Coninx et al. 2017). The way by which mycorrhizal fungi will manage HMs will depend on several factors, such as the particular species, metal, concentration, plant host etc. (Audet and Charest 2007). For instance, in some ectomycorrhizal fungi, Cd is mainly sequestered in the vacuoles, while Zn is expelled from the cytosol via transporters (Bellion et al. 2006). A good representation summarising the mechanisms for HM tolerance in mycorrhizal symbiosis was developed by Gohre and Paszowski (2006) (Figure 1.3), depicting extracellular metal chelation, cell wall binding, chelation in cytosol, metal efflux and vacuole sequestration, for example.

Alleviation of Zn and Cd toxicity in plants by arbuscular mycorrhizal fungi has been reported in the literature (Gaur and Adholeya 2004). Garg and Aggarwal (2012) verified higher tolerance and significant increase of Cd accumulation in roots and shoots of pigeon pea (30% and 16%, respectively) after inoculation with the AMF *Funneliformis (Glomus) mossae*, while Andrade et al. (2008) observed similar results in sunflowers associated with *Rhizophagus irregularis*. Some studies have already reported that plants in association with ectomycorrhizal fungi, especially *Pinus* trees, can resist high concentrations of metals such as Cd, Pb and Zn in soil (Jentschke and Godbold 2000). Inoculation with ectomycorrhizal *Paxillus involutus* in willows also resulted in improvements in Zn tolerance and phytoextraction from soils (Baum et al. 2006). However, no genetic mechanisms were investigated in these studies.

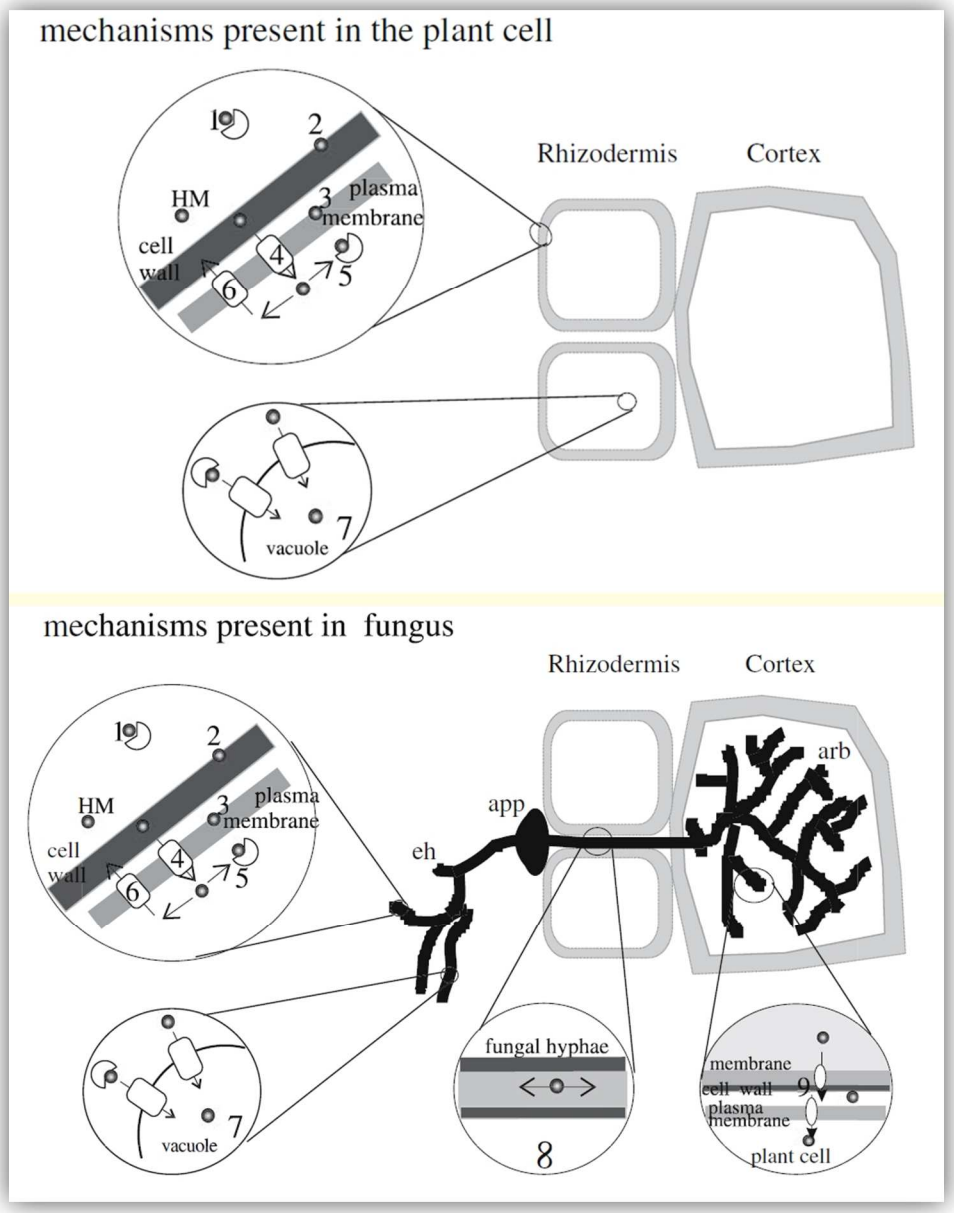


Figure 1.3 Heavy metal detoxification mechanisms of plants and fungi in AMF symbioses. **1** - Chelating agents are secreted, bindings metals in the soil (e.g. organic acids from the plant, glomalin from the fungus). **2** - Binding of HM to cell wall in plants and fungi. **3** - The plasma membrane as a living, selective barrier in plants and fungi. **4** - Specific and nonspecific metal transporters and pores in the plasma membrane of plants and fungi (active and passive import). **5** - Chelates in the cytosol, e.g., metallothioneins (plants and fungi), organic acids and metal-specific chaperons (shown for plants, assumed for AM fungi). **6** - Export via specific or nonspecific active or passive transport from plant/fungal cells. **7** - Sequestration of HM in the vacuole of plant/fungal cells. 8 - Transport of HM in fungal hyphae. **9** - In arbuscules, metal export from the fungus and import into plant cells via active or passive transport (Gohre and Paszowski 2006).

Plant uptake is not always increased by mycorrhizal fungi inoculation (Bissonnette et al. 2010; Chibuike and Obiora 2014). Some ectomycorrhizal fungi, for example, bind HMs into cell-wall components, or store high amounts of these elements in their cytosol as a way of protecting themselves and their plant hosts from metal toxicity (Guerra et al. 2011), which is certainly not favourable for phytoextraction, but it is for phytostabilisation. AMF has been also considered a 'buffer' in protecting plants against HM toxicity, by binding metals into their fungal structures, such as vesicles (Gonzalez-Guerrero et al. 2009; Nayuki et al. 2014), or by producing glomalin (a secreted glycoprotein) which is recalcitrant in soils and can sequester HMs (Bellion et al. 2006; Khan 2006; Gadd 2010; Jia et al. 2016). Overall, AMF symbiosis may improve either phytoextraction or phytostabilisation (Figure 1.4) – or even have no significant effect at all – all will depend on fungal species, plant host, environmental conditions and the HM in question (Garg and Chandel 2010; Coninx et al. 2017). Table 1.1 is a compilation of several of studies on the mycorrhizal effect in plants mostly intended for Cd and Zn phytoremediation.

Considering 59 observations reported in the literature for mycorrhizal plants under Cd exposure (Table 1.1), most cases describe an increase in Cd accumulation in roots - 42% of cases – with some instances of lower Cd accumulation (26%); as for shoots, 41% observed Cd decrease, against 32% of higher Cd accumulation. Thus, in general, most studies verified that mycorrhization decreased Cd in shoots, but increased in roots, the main sink of Cd accumulation. However, depending on the fungal species, this proportion can be entirely different. For example, most studies involving *R. irregularis* report higher Cd in both roots (55% of cases) and shoots (45% of cases), while in the experiments with *F. mossae*, reduction in Cd accumulation is generally observed in both roots and shoots (Table 1.1).

In studies with mycorrhizal plants and Zn (n = 51), reports are more consistent in terms of accumulation and distribution (Table 1.1). On average, 25% of all cases report an increase in Zn accumulation, in either roots or shoots, 25% report a decrease, and generally, half of the studies did not detect any differences in Zn uptake in comparison to non-mycorrhizal treatments. In studies with *F.*

mossae, only 6% of cases verified an increase in shoot accumulation, while in experiments with *R. irregularis*, 24% of cases reported an increase in Zn concentrations in shoots, suggesting that this arbuscular mycorrhizal species might be more suitable in phytoremediation, for either Cd or Zn.

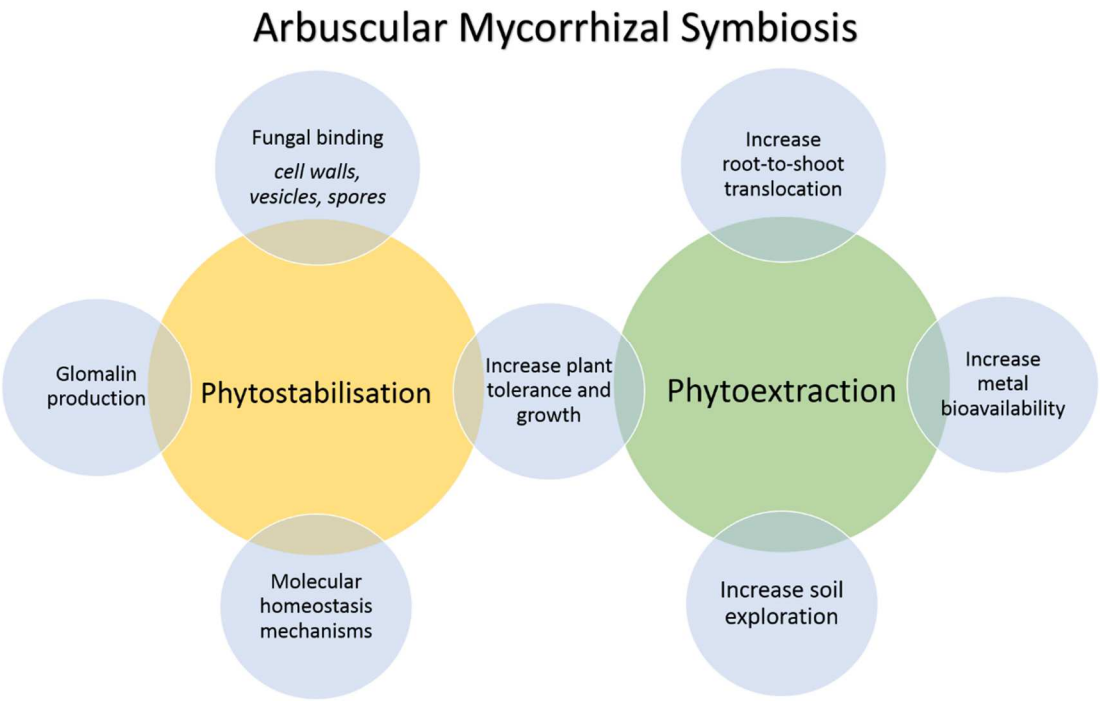


Figure 1.4 – Mechanisms by which arbuscular mycorrhizal fungi can influence or contribute to the phytoremediation of heavy metals in soils.

Moreover, there are not many studies concerning metal toxicity in mycorrhizal fungi, especially compared to the information available on plants and plant communities, which poses a bigger challenge in finding metal-tolerant fungi species (Colpaert 2008). Information about the several molecular and genomic responses in AM fungi in effectively mediating metal stress, especially Cd and Zn, is limited (Kaur and Garg 2017). Therefore, in order to be applied as a phytoremediation enhancing technique, it is necessary to initially understand the effects of HMs in mycorrhizal fungi and mycorrhizal plants, as well as the mechanisms by which these fungi promote HM uptake, tolerance, translocation and/or distribution in host plants.

1.5.4 Mycorrhizal fungi and plant gene expression

Gene expression for HM 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. Some studies have suggested that AMF can down-regulate gene expression for Zn transporters to promote an optimum concentration of this element within the plant (Burleigh and Bechman 2002). In tomatoes (*Lycopersicum esculentum*), Ouziad et al. (2005) observed that inoculation with *R. irregularis* distinctly reduced the level of *Lemt2* transcripts (coding for MTs) when plants were grown in high Cd concentrations, and also reduced the transcripts of *LeNramp1* (for metal transporter) in a soil with high contents of Zn. Similarly, Dabrowska et al. (2014) observed increasing expression of the MT gene *BnMT2* in the leaves of *Brassica napus* L. after inoculation with AM spores. In *P. alba* under Cu and Zn stress, inoculation with AMF lead to an overall induction of several heavy-metal related genes, such as the MTs *PaMT1*, *PaMT2* and *PaMT3* (Cicatelli et al. 2010) or other genes involved in RNA processing and amino acid metabolism regardless of HM stress (Cicatelli et al. 2014).

It is important to highlight that most of these genes encoding HM transporters, MTs or phytochelatins, were mainly characterized for herbaceous plants or species belonging to the genus Arabidopsis (Yang et al. 2005; Dhankhar et al. 2012), whilst the characterization for poplars is still very scarce, despite the release of the *P. trichocarpa* genome (Guerra et al. 2011). Assessing the effects of mycorrhizal fungi 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 symbionts gene expression and transcriptional responses (Liu et al. 2007).

Table 1.1 Compilation of several studies on the effect of mycorrhizal symbiosis (arbuscular and ectomycorrhizal fungi) on Cd and Zn accumulation in different host plants. Increase in uptake due to symbiosis is symbolised by a (+) sign and decrease by (-), while no significant effects are represented by (=). Unclear or absent data are symbolised by "x".

Host plant	Mycorrhizal fungus	Col. rate	Effect in metal accumulation		Main tissue [1]	Ref.
		(%)	Shoots	Roots	S or R	
Allium cepa	*Funneliformis mossae*	0-12	Zn(=)	x	x	1
Bidens pilosa	*Glomus macrocarpum*	52-65	Zn(+)	Zn(=)	S = R	2
Brassica chinensis	*F. mossae*	30	Cd(+)	Cd(+)	R	3
	Rhizophagus irregularis	30	Cd(+)	Cd(+)	R	3
	G. versiforme	25	Cd(+)	Cd(+)	R	3
Cajanus cajan	*F. mossae*	x	Cd(+)	Cd(+)	R	4
	F. mossae	x	Cd(-) Zn(-)	Cd(-) Zn(-)	R	5
	F. mossae	76-88	Cd(-)	Cd(-)	R	6
	F. mossae	65	Cd(-)	Cd(-)	R	7
Chrysopogon zizanioides	*Gigaspora margarita*	1-12	Zn(+)	Zn(-)	S = R	8
	R. clarus	22-35	Zn(=)	Zn(=)	R	8
	Dentiscutata heterogama	1-2	Zn(=)	Zn(-)	R	8
Daucus carota	*R. irregularis*	74-84	x	Zn(-)	x	9
Helianthus annuus	*R. irregularis*	31	Cd(+)	Cd(+)	R	10
	R. irregularis	38-41	Cd(+) Zn(=)	Cd(=) Zn(=)	R	11
	F. mossae	40-43	Cd(-) Zn(-)	Cd(=) Zn(=)	R	11
Helichrysum italicum	*Septoglomus viscosum*	x	Cd(+) Zn(=)	Cd(-) Zn(-)	R	12
Hordeum vulgare	*R. irregularis*	38.1	Zn(=)	x	x	13
Ipomoea aquatica	*G. caledonium* + *G. versiforme*	x	Cd(-)	Cd(-)	S = R	14
	Mixed (AMF) [2]	x	Cd(+)	Cd(+)	R	15
Lonicera japonica	*R. irregularis*	89-96	Cd(-)	Cd(+)	R	16
	G. versiforme	91-96	Cd(-)	Cd(-)	R	16
Medicago sativa	*F. mossae*	33-37	Zn(=)	Zn(+)	R	17
	R. irregularis	30-50	Cd(-)	Cd(+)	R	18
Nicotiana tabacum	*R. irregularis*	15-35	Cd(+)	Cd(+)	S	19
	R. irregularis	95	Cd(-)	Cd(-)	S	20
	R. irregularis (BEG75)	30-60	Cd(-)	Cd(=)	S	21
	R. irregularis (PH5)	10-30	Cd(-)	Cd(=)	S	21
	F. mossae	< 10	Cd(-)	Cd(-)	S	21

Table 1.1 Cont.

Host plant	Mycorrhizal fungus	Col. rate (%)	Metal accumulation effect		Main tissue S or R	Ref.
			Shoots	Roots		
Oryza sativa	*G. versiforme*	50-60	Cd(-) Zn(-)	Cd(+) Zn(=)	R	22
	F. mossae	30-50	Cd(=) Zn(=)	Cd(+) Zn(=)	R	22
	G. diaphanum	30-70	Cd(-) Zn(-)	Cd(+) Zn(=)	R	22
Passiflora foetida	*G. macrocarpum*	65-73	Zn(+)	Zn(+)	R	2
Phragmites australis	*R. irregularis*	13-16	Cd(+) Zn(+)	x	x	23
	F. mossae	15-17	Cd(+) Zn(+)	x	x	23
	R. irregularis	x	Cd (+)	Cd(+)	x	24
	R. irregularis	x	Cd (-)	Cd(+)	R	25
Pinus sylvestris	*Suillus bovinus*	x	Zn (+)	Zn(=)	R	26
	Suillus bovinus	x	Zn(=)	x	x	27
Pisum sativum	*F. mossae*	64-79	Cd(-)	Cd(-)	R	6
Plantago lanceolata	*R. irregularis*	90-99	Cd(=) Zn(=)	Cd(+) Zn(+)	R	28
Populus × generosa	*R. irregularis*	34-59	Cd(=) Zn(=)	Cd(=) Zn(=)	S	29
Populus alba	*F. mossae*	11-35	Cd(=) Zn(=)	x	x	30
	R. irregularis	13-31	Zn(=)	Zn(=)	S	31
	F. mossae	5-17	Zn(=)	Zn(+)	S	31
	R. irregularis	11	Zn(=)	Zn(=)	S	32
	F. mossae	5	Zn(-)	Zn(=)	S	32
	R. irregularis	20	Zn(+)	Zn(=)	S	33
Populus canadensis	*Hebeloma crustuliniforme*	x	Cd(=)	Cd(=)	S	34
	Paxillus involutus	x	Cd(+)	Cd(=)	S	34
	Pisolithus tinctorius	x	Cd(+)	Cd(=)	S	34
Populus deltoides	*R. irregularis*	45-50	Cd(+)	Cd(+)	R	35
Populus nigra	*R. irregularis*	40	Zn(=)	Zn(=)	S	32
	F. mossae	28	Zn(=)	Zn(=)	S	32
	R. irregularis	55-93	Cd(=) Zn(=)	x	x	36
	H. mesophaeum	2-12	Cd(=) Zn(=)	x	x	36
S. lycopersicum	Mixed (AMF)	55	Zn (-)	Zn(-)	R	37
	R. irregularis	68	Cd (+)	Cd(=)	R	38
Salix × dasyclados	*Paxillus involutus*	21-29	Cd(=) Zn(=)	Cd(-) Zn(=)	S	39
Salix alba	*H. mesophaeum*	45-61	Cd(-) Zn(=)	x	x	36
Salix viminalis	*R. irregularis*	0-6	Cd(=) Zn(=)	Cd(=) Zn(=)	S	29
	H. crustuliniforme	x	Cd(-)	Cd(=)	S	34
	P. involutus	x	Cd(=)	Cd(=)	S	34
	P. tinctorius	x	Cd(=)	Cd(-)	S	34
Sesbania cannabina	*F. mossae*	37-47	Zn(=)	Zn(=)	R	17
S. rostrata	*F. mossae*	64-68	Zn(-)	Zn(-)	R	17

Table 1.1 Cont.

Host plant	Mycorrhizal fungus	Col. rate (%)	Metal accumulation effect		Main tissue	Ref.
			Shoots	Roots	S or R	
Solanum melongena	Mixed (AMF)	x	Cd(=)	Cd(=)	R	40
Solanum nigrum	*F. mossae*	67-72	Cd(+)	Cd(+)	S	41
	R. irregularis	3-5	Zn(+)	Zn(=)	R	42
	G. claroideum	2	Zn(+)	Z (=)	R	42
Solanum photeinocarpu	*G. versiforme*	77-94	Cd(+)	Cd(+)	R	43
Sorghum bicolor	*R. irregularis*	33-36	Zn(+)	Zn(+)	R	44
	G. spurcum	33-36	Zn(+)	Zn(+)	R	44
Thlaspi praecox	*Glomus* sp.	20-87	Cd(-) Zn(=)	Cd(-) Zn(-)	S	45
Trifolium pratense	*F. mossae*	50-59	Zn(=)	Zn(+)	R	46
Trifolium repens	Mixed (AMF)	33-50	Zn(-)	Zn(-)	R	47
Trifolium subterraneum	*F. mossae*	0-12.1	Cd(=) Zn(=)	Cd(+) Zn(=)	R	48
	Mixed (AMF)	2.4-6.4	Cd(=) Zn(=)	Cd(+) Zn(+)	R	48
Triticum aestivum	*R. irregularis*	41-47	Zn(-)	Zn(-)	x	49
Vicia faba	*F. mossae*	58	Cd(=) Zn(=)	Cd(=) Zn(+)	R	50
Zea mays	*F. mossae*	40-50	Cd(-)	Cd(=)	R	51
	R. irregularis	40-45	Cd(+)	Cd(+)	R	51
	F. mossae	28	Zn(=)	Zn(+)	R	1
	F. mossae	21-28	Cd(-) Zn(-)	Cd(-) Zn(-)	S	52
	R. irregularis	70	Cd(=)	Cd(=)	R	53
	F. mossae	40	Cd(-)	Cd(-)	R	53
	G. constrictum	35	Cd(-)	Cd(+)	R	53
	Caroideoglomus etunicatum	20-24	Cd(-)	Cd(=)	R	54

[1] Main tissue: plant tissue - roots (R) or shoots (S) - with the highest accumulation of Cd or Zn (mg kg^{-1}; or µg $plant^{-1}$). [2] Native microbiota from collected soil or a mix of inoculum containing different AMF spores, hyphae and/or root fragments.

References (Ref.): Gildon and Tinker 1983 [1] Tseng et al. 2009 [2] Wu et al. 2016 [3] Garg and Aggarwal 2012 [4] Garg and Kaur 2013 [5] Garg et al. 2015 [6] Garg and Chandel 2012 [7] Meyer et al. 2017 [8] Audet and Charest 2009 [9] Andrade et al. 2008 [10] Hassan et al. 2013 [11] Brunetti et al. 2018 [12] Watts-Williams and Cavagnaro 2018 [13] Hu et al 2013 [14] Bhaduri and Fulekar 2012 [15] Jiang et al 2016b [16] Lin et al. 2007 [17] Wang et al. 2012 [18] Wang et al. 2013 [19] Janouskova et al. 2005 [20] Janouskova et al. 2007 [21] Zhang et al. 2005 [22] Zheng et al. 2015 [23] Wang et al. 2017 [24] Huang et al. 2017 [25] Adriaensen et al. 2003 [26] Adriaensen et al. 2006 [27] Orlowska et al. 2012 [28] Bissonnette et al. 2010 [29] Baldantoni et al. 2011 [30] Cicatelli et al. 2010 [31] Lingua et al 2008 [32] Lingua et al. 2012 [33] Sell et al 2005 [34] Chen et al. 2016 [35] Mrnk et al. 2012 [36] Watts-Williams et al 2013 [37] Kumar et al. 2015 [38] Baum et al. 2006 [39] Chaturvedi et al. 2018 [40] Jiang et al. 2016 [41] Marques et al. 2008 [42] Tan et al. 2015 [43] Toler et al. 2005 [44] Vogel-Mikus et al. 2005 [45] Chen et al. 2003 [46] Zhu et al. 2001 [47] Tonin et al. 2001 [48] Khan et al. 2014 [49] Zhang et al. 2006 [50] Aghababaei et al. 2014 [51] Weissenhorn et al. 1995 [52] Liu et al. 2014 [53] Chang et al. 2018 [54].

1.6 Hypotheses, objectives and thesis structure

Considering the literature reviewed above, there is a clear need for more detailed knowledge on (1) the genetic basis for HM accumulation in poplar trees, (2) gene expression of poplar metal transporters, as well as (3) the influence of mycorrhizal fungi on gene regulation of host plants under HM stress. Such information can be critical for genetic improvement and use of biotechnology to design transgenic plants and microorganisms that can be efficiently applied in phytoremediation and bioremediation processes.

Overall aim:

To understand the physiological basis underpinning the potential of poplar trees to be used in Cd and Zn phytoremediation, and to investigate the use of mycorrhizal symbiosis as a method of manipulating metal extraction/sequestration; while presenting additional fundamental knowledge on metal uptake dynamics in mycorrhizal poplars and some of the underlying molecular mechanisms.

General hypotheses:

The hypotheses that set off the entire sequence of experiments in this thesis was that poplar trees would respond differently to Cd and Zn stress under mycorrhizal symbiosis, and that the fungal partner could alter metal uptake dynamics and distribution in the host plant.

Objectives:

- To screen for ectomycorrhizal fungi species tolerant to Cd and Zn *in vitro*.
- To determine Cd/Zn toxicity thresholds in *Populus trichocarpa* (Torr. & A. Gray), and their accumulation capacity in shoots (phytoextraction) and roots (phytostabilisation).
- To evaluate the influence of arbuscular mycorrhizal fungus *Rhizophagus irregularis* [(Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler] in increasing metal tolerance and/or accumulation in poplars.

- To assess the influence of mycorrhizal symbiosis in the expression of poplar genes encoding HM-related proteins stress (e.g. metal transporters, phytochelatins or metallothioneins) under Cd and Zn stress.
- To investigate the bioremediation potential of transgenic yeast carrying a metallothionein gene from *P. trichocarpa*.

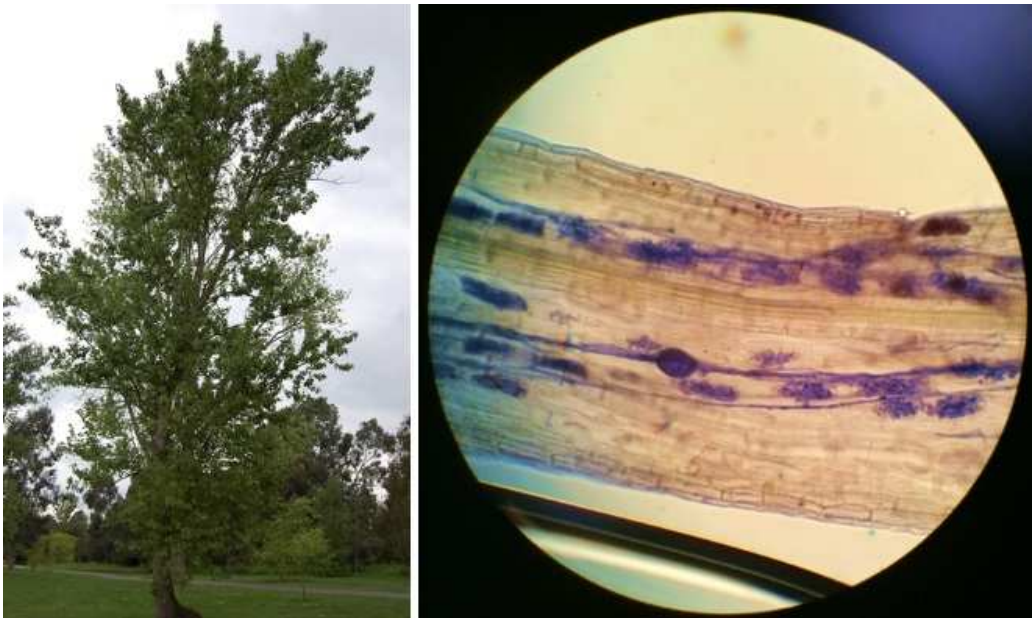


Figure 1.5 Left: Photograph of a *P. trichocarpa* tree at Kew Gardens, London. Source: https://davisla.wordpress.com/; Right: Poplar roots colonised by *R. irregularis*

This thesis is divided into 6 chapters, a brief summary of each chapter is provided below, and a flowchart illustrating the different stages of this project is presented in Figure 1.6.

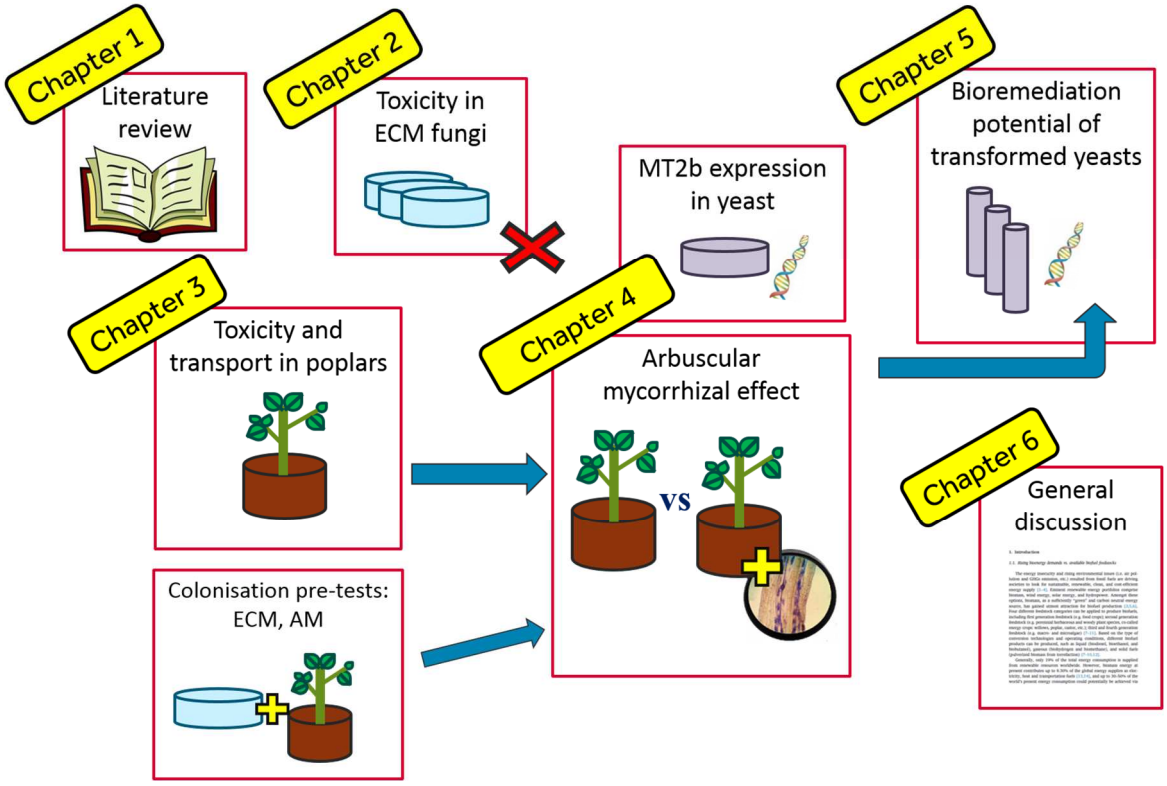


Figure 1.6 Flowchart with all the stages (tests and experiments) in this thesis, and the respective chapters in which they are presented.

Chapter 2

Cd and Zn interactions and toxicity in ectomycorrhizal basidiomycetes in axenic culture;

This chapter aimed to evaluate the response of different ectomycorrhizal (ECM) species *in vitro* to Cd and Zn toxicity. The use of different methods for determining toxicity thresholds in ECM are also explored.

Hypotheses were: i) ECM fungi species respond differently to Cd and Zn toxicity; ii) Zn can alleviate Cd toxic effects due to competition in uptake; iii) Cd and Zn toxicity thresholds differ and are probably higher in solid (agar) media in comparison to liquid media.

This experiment would serve as basis for selecting tolerant species to inoculate poplar trees under HM stress, however, no evidence of mycorrhizal symbiosis was found (data not shown) after several attempts, and therefore the other chapters do not include any ECM work. Chapter 2 has been published at *PeerJ* (2018): https://doi.org/10.7717/peerj.4478

Chapter 3

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

The main objectives of this chapter were to investigate the behaviour of the particular poplar clone chosen for this project (*Populus trichocarpa* Trichobel) under a range of Cd and Zn concentrations. Dry biomass, shoot height, leaf transpiration and Cd/Zn accumulation and distribution were some of the parameters assessed.

Hypotheses were: i) *P. trichocarpa* is tolerant to high Cd/Zn concentrations; ii) metal translocation patterns can vary depending on their concentrations; iii) Zn addition can prevent Cd uptake and toxicity in poplars; and iv) the expression of *PtHMA4* gene is involved in Cd and Zn transport from roots to shoots.

The toxicity thresholds found in this experiment were used for the subsequent experiments. Chapter 3 has been published by *Environmental and Experimental Botany* (2018): https://doi.org/10.1016/j.envexpbot.2018.07.011. This paper also includes one of the results described in Chapter 4 (i.e. expression of the *PtHMA4* gene), due to its relevance in discussing the Cd and Zn translocation patterns found in poplar.

Chapter 4

The influence of mycorrhizal symbiosis in *Populus trichocarpa* under Cd and Zn stress: transcript analyses and phytoremediation potential.

Chapter 4 has the core experiment of this thesis in which poplar trees in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* were assessed under high Cd and Zn concentrations. Besides morphological parameters and metal accumulation patterns, the expression of seven heavy-metal related genes were determined in poplars roots and leaves with and without symbiosis. Selected genes were: *PtMTP1*, *PtHMA4, PtNramp3, PtZIP1, PtPCS1, PtMT2a* and *PtMT2b*. Due to the high correlation found between root colonisation and MT2b expression in poplar roots – especially under Cd exposure - this gene was transformed into yeasts to investigate its involvement in Cd tolerance**.**

Hypotheses were: i) mycorrhizal symbiosis enhances Cd/Zn uptake and influences their distribution in poplars; ii) poplar genes for metal uptake are down-regulated under metal exposure, while genes associated with metal chelation are up-regulated; iii) genes involved in heavy metal transport and chelation are affected by metal exposure as well as by mycorrhizal symbiosis (in roots and leaves); and iv) metallothionein gene *PtMT2b* is involved in heavy metal tolerance and should increase Cd tolerance in transgenic yeast carrying this gene.

Chapter 5

Bioremediation potential of Cd by transgenic yeasts expressing a metallothionein gene from *Populus trichocarpa*.

After verifying that metallothionein gene MT2b confers tolerance to Cd, two versions of this gene were compared by growing transformed *S. cerevisiae* under different Cd doses. One strain contained the original gene sequence, while the other had a single nucleotide substitution, leading to a slightly different protein, with its third amino acid, cysteine, being replaced by a tyrosine (C3Y). Promising results from the mutated MT2b sequence, lead to subsequent assays with this particular transgenic strain (*PtMT2b* 'Y').

Hypotheses were: i) *PtMT2b* increases yeast tolerance to Cd; ii) the mutated gene *PtMT2b* 'Y' is not as efficient in conferring Cd tolerance in yeast due to the lack of one cysteine in the peptide sequence; iii) transformed yeasts can effectively bioremediate Cd from aqueous solutions (by surface biosorption or intracellular accumulation); and iv) Mutant yeast strains have higher growth under nutrient deficiency (Fe, Mn and Zn) if carrying *PtMT2b* 'Y'.

Chapter 6

General discussion.

Main findings are discussed in the wider context of practical phytoremediation as well as the potential of *P. trichocarpa* for other biotechnological applications, such as their use for bioenergy production, metal recovery, carbon sequestration and genetic engineering. Methodological limitations of this thesis are also discussed. Further research that could be carried out by using the data derived

from this work are suggested. Concluding remarks covering the overall thesis complete this chapter.

1.7 References

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

Adriaensen K, van der Lelie D, Laere AV, Vangronsveld J, Colpaert JV. 2003. A zinc-adapted fungus protects pines from zinc stress, *New Phytologist*. **161**: 549-555.

Adriaensen K, Vangronsveld J, Colpaert JV. 2006. Zinc-tolerant *Suillus bovinus* improves growth of Zn-exposed *Pinus sylvestris* seedlings, *Mycorrhiza*. **16**: 553-558.

Adriano DC. 2001. *Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals*, Springer, New York.

Aghababaei F, Raiesi F, Hosseinpur A. 2014. The significant contribution of mycorrhizal fungi and earthworms to maize protection and phytoremediation in Cd-polluted soils, *Pedobiologia*. **57:** 223-233.

Alford ER, Pilon-Smits EAH, Paschke MW. 2010. Metallophytes—a view from the rhizosphere, *Plant and Soil*. **337**: 33-50.

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

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

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.

Anju M, Banerjee DK. 2011. Associations of cadmium, zinc, and lead in soils from a lead and zinc mining area as studied by single and sequential extractions, *Environmental Monitoring and Assessment*. **176**: 67-85.

Arthur EL, Rice PJ, Rice PJ, Anderson TA, *et al.* 2005. Phytoremediation—an overview, *Critical Reviews in Plant Sciences*. **24**: 109-122.

ATSDR, Agency for Toxic Substances and Disease Registry. 2017. CERCLA Priority List of Hazardous Substances. https://www.atsdr.cdc.gov/spl/index.html. 10 Oct. 2018.

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

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

Baldantoni D, Bellino A, Cicatelli A, Castiglione S. **2011.** Artificial mycorrhization does not influence the effects of iron availability on Fe, Zn, Cu, Pb and Cd accumulation in leaves of a heavy metal tolerant white poplar clone, *Plant Biosystems*. **145**: 236-240.

Baum C, Hrynkiewicz K, Leinweber P, Meißner R. 2006. Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (*Salix* × *dasyclados*), *Journal of Plant Nutrition and Soil Science*. **169**: 516-522.

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M. 2006. Extracellularand cellularmechanisms sustainingmetal tolerance in ectomycorrhizal fungi, *FEMS Microbiology Letters*. **254**: 173-181.

Bhaduri AM, Fulekar MH. 2012. Assessment of arbuscular mycorrhizal fungi on the phytoremediation potential of *Ipomoea aquatica* on cadmium uptake, *3 Biotech*. **2:** 193-198.

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.

Bizarro VG, Meurer EJ, Tatsch FRP. 2008. Teor de cádmio em fertilizantes fosfatados comercializados no Brasil, *Ciência Rural*. **38**: 247-250, 2008.

Bradl HB. 2005. *Heavy Metals in the Environment*, *Vol 6*, Elsevier. 2005.

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.

Brunetti G, Ruta C, Traversa A, D'Ambruoso G, *et al.* 2018. Remediation of a heavy metals contaminated soil using mycorrhized and non-mycorrhized *Helichrysum italicum* (Roth) Don, *Land Degradation and Development*. **29:** 91-104.

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

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

Chaney R. 2010. Cadmium and Zinc. In: Hooda PS, ed., *Trace Elements in Soils*. Blackwell Publishing Ltd, UK, p. 410-439.

Chang Q, Diao FW, Wang QF, Pan L, Dang ZH, Guo W. 2018. Effects of arbuscular mycorrhizal symbiosis on growth, nutrient and metal uptake by maize seedlings (*Zea mays* L.) grown in soils spiked with Lanthanum and Cadmium, *Environmental Pollution.* **241:** 607-615.

Chaturvedi R, Favas P, Pratas J, Varuna M, Paula MS. 2018. Assessment of edibility and effect of arbuscular mycorrhizal fungi on *Solanum melongena* L. grown under heavy metal(loid) contaminated soil, *Ecotoxicology and Environmental Safety*. **148**: 318-326.

Chen BD, Li XL, Tao HQ, Christie P, Wong MH. 2003. The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc, *Chemosphere*. **50**: 839-846.

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.

Chibuike GU, Obiora SC. 2014. Heavy metal polluted soils: effect on plants and bioremediation methods, *Applied and Environmental Soil Science*. Article ID 752708, http://dx.doi.org/10.1155/2014/752708.

Ciadamidaro L, Girardclos O, Bert V, Zappelini C, Yung L, Foulon J *et al.* 2017. Poplar biomass production at phytomanagement sites is significantly enhanced by mycorrhizal inoculation, *Environmental and Experimental Botany*. **139**: 48-56.

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

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, 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, 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.

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

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.

Cobbett CS, Hussain D, Haydon MJ. 2003. Structural and functional relationships between type 1_B heavy metal transporting P-type ATPases in *Arabidopsis*, *New Phytologist*. **159**: 315-321.

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.

Colpaert JV. 2008. Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi. In: Avery SV, Stratford M, Van West P, eds, *Stress in yeasts and filamentous fungi*. Academic, Amsterdam, p. 157-174.

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

Courbot M, Willems G, Motte P, Arvidsson S, *et al.* 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase, *American Society of Plant Biologists*. **144**: 1052-1065.

Dabrowska G, Baum C, Trejgell A, Hrykienwicz K. 2014. Impact of arbuscular mycorrhizal fungi on the growth and expression of gene encoding stress protein – metallothionein BnMT2 in the non-host crop *Brassica napus* L, *Journal of Plant Nutrition and Soil Science*. **35:** 49-58.

Das P, Samantaray S, Rout GR. 1997. Studies on cadmium toxicity in plants: a review, *Environmental Pollution*. **98**: 29-36.

De Oliveira VH, Melo LCA, Abreu CA, Coscione AR. 2016. Influences of soil pH on cadmium toxicity to eight plant species, *Ecotoxicology and Environmental Contamination*. **11**: 45-52.

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

Dhankhar R, Sainger PA, Sainger M. 2012. Phytoextraction of Zinc: physiological and molecular mechanism, *Soil and Sediment Contamination*. **21**: 115-133.

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.

Dominguez MT, Maranon T, Murillo JM, Schulin R, Robinson BH. 2008. Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: A large-scale phytomanagement case study, *Environmental Pollution*. **152**: 50-59.

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

Ebbs SD, Kochian LV. 1997. Toxicity of zinc and copper to *Brassica* species: implications for phytoremediation, *Journal of Environmental Quality*. **26**: 776-781.

Erakhrumen AA. 2007. Phytoremediation: an environmentally sound technology for pollution prevention, control and remediation in developing countries, *Educational Research and Review*. **2**: 151-156.

Fillion M, Brisson J, Guidi W, Labrecque M. 2011. Increasing phosphorus removal in willow and poplar vegetation filters using arbuscular mycorrhizal fungi, *Ecological Engineering*. **37:** 199-205.

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

Freitas EVS, Nascimento CWA, Goulart DF, Silva JPS. 2009. Disponibilidade de cádmio e chumbo para milho em solo adubado com fertilizantes fosfatados, *Revista Brasileira de Ciência do Solo*. **33**: 1899-1907.

French CJ, Dickinson NM, Putwain PD. 2006. Woody biomass phytoremediation of contaminated brownfield land, *Environmental Pollution*. **141**: 387-395.

Gadd GM. 2010. Metals, minerals and microbes: Geomicrobiology and bioremediation, *Microbiology*. **156:** 609-643.

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, *et al.* 2012. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms, *Environmental and Experimental Botany*. **83**: 33-46.

Galloway JN, Thornton JD, Norton SA, Volchok HL, McLean RA. 1982. Trace metals in atmospheric deposition: a review and assessment, *Atmospheric Environment (1967)*. **16:** 1677-1700.

Gao X, Flaten DN, Tenuta M, Grimmett MG, Gawalko EJ, Grant CA. 2011. Soil solution dynamics and plant uptake of cadmium and zinc by durum wheat following phosphate fertilization, *Plant and Soil*. **338**: 423-434. 2011.

Garbisu C, Alkorta I. 2001. Phytoremediation of organic contaminants in soils, *Bioresource Technology*. **79:** 273-276.

Garg N, Aggarwal N. **2012.** Effect of mycorrhizal inoculations on heavy metal uptake and stress alleviation of *Cajanus cajan* (L.) Millsp. genotypes grown in cadmium and lead contaminated soils, *Journal of Plant Growth Regulation*. **66:** 9-26.

Garg N, Chandel S. 2010. Arbuscular mycorrhizal networks: process and functions. A review, *Agronomy for Sustainable Development*. **30:** 581-599.

Garg N, Chandel S. 2012. Role of arbuscular mycorrhizal (AM) fungi on growth, cadmium uptake, osmolyte, and phytochelatin synthesis in *Cajanus cajan* (l.) millsp. under NaCl and Cd stresses, *Journal of Plant Growth Regulation*. **31**: 292-308.

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

Garg N, Singla P, Bhandari P. 2015. Metal uptake, oxidative metabolism, and mycorrhization in pigeonpea and pea under arsenic and cadmium stress, *Turkish Journal of Agriculture and Forestry*. **39**: 234-250.

Gaur A, Adholeya A. 2004. Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils, *Current Science*. **86:** 528-234.

Gherghel F, Krause K. 2012. Role of mycorrhiza in re-forestation at heavy metal-contaminated sites. In: Kothe E, Varma A, eds., *Bio-Geo Interactions in Metal-Contaminated Soils*, Springer, Berlin, p. 183-199.

Gildon A, Tinker PB. **1983.** Interactions of vesicular-arbuscular mycorrhizal infection and heavy metal in plants – I. The effects of heavy metals on the development of vesicular-arbuscular mycorrhizas, *New Phytologist*. **95:** 247-261.

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

Gonçalves VC, Meurer EJ, Tatsch FRP, Carvalho SA, Santos Neto OA. 2008. Biodisponibilidade de cádmio em fertilizantes fosfatados, *Revista Brasileira de Ciência do Solo*. **32:** 2871-2875.

Gonzalez-Guerrero M, Benabdellah K, Ferrol N, Azcon-Aguilar C. 2009. Mechanisms underlying heavy metal tolerance in arbuscular mycorrhizas. In: Azcon-Aguilar C, Barea JM, Gianinazzi S, Gianinazzi-Pearson V, eds., *Mycorrhizas: Functional Processes and Ecological Impact*. Springer, Berlin, p. 1-16.

Gratão PL, Polle A, Lea PJ, Azevedo RA. 2005. Making the life of heavy metal-stressed plants a little easier, *Functional Plant Biology.* **32**: 481-494.

Guala SD, Vega FA, Covelo EF. 2010. The dynamics of heavy metals in plant–soil interactions, *Ecological Modelling*. **221:** 1148-1152.

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, p. 387-413.

Gustin JL, Loureiro ME, Kim D, Na G, Tikhonova M, Salt DE. 2009. MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants, *The Plant Journal*. **57**: 1116-1127.

Hall JL. 2002. Cellular mechanisms for heavy metal detoxification and tolerance, *Journal of Experimental Botany*. **53:** 1-11.

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 *et al.* 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 *et al.* 2008. Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of *HMA4*, *Nature*. **453**: 391-395.

Hassan SE, Hijri M, St-Arnaud M. 2013. Effect of arbuscular mycorrhizal fungi on trace metal uptake by sunflower plants grown on cadmium contaminated soil, *New Biotechnology*. **30**: 780-787.

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, p. 135-225.

Ho YS, El-Khaiary MI. 2009. Metal research trends in the environmental field. In: Wang LK, Chen JP, Hung Y, Shammas NK, eds., *Heavy metals in the environment,* Taylor and Francis & CRC Press, New York, p. 1-12.

Hu J, Li J, Wu F, Wu S, Ye Z, Lin X, Wong MH. 2013. Arbuscular mycorrhizal fungi induced differential Cd and P phytoavailability via intercropping of upland kangkong (*Ipomoea aquatica* Forsk.) with Alfred stonecrop (*Sedum alfredii* Hance): post-harvest study, *Environmental Science and Pollution Research*. **20**: 8457-63.

Huang X, Ho SH, Zhu S, Ma F, Wu J, Yang J, Wang L. **2017.** Adaptive response of arbuscular mycorrhizal symbiosis to accumulation of elements and translocation in *Phragmites australis* affected by cadmium stress, *Journal of Environmental Management*. **197:** 448-455

Hussain D, Haydon MJ, Wang Y, Wong E *et al.* 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. *et al*. 2016. Physiology and genetic architecture of traits associated with cadmium tolerance and accumulation in *Populus nigra* L., *Trees*. **30**: 125-139.

Janouskova M, Pavlikova D, Macek T, Vosatka M. 2005. Influence of arbuscular mycorrhiza on the growth and cadmium uptake of tobacco with inserted metallothionein gene, *Applied Soil Ecology*. **29:** 209-214.

Janouskova M, Vosatka M, Rossi L, Lugon-Moulin N. 2007. Effects of arbuscular mycorrhizal inoculation on cadmium accumulation by different tobacco (*Nicotiana tabacum* L.) types, *Applied Soil Ecology*. **35**: 502-510.

Janssen MPM, Ma WC, Van Straalen NM. 1993. Biomagnification of metals in terrestrial ecosystems, *Science of the Total Environment*. **134**: 511-524.

Jentschke G, Godbold DL. **2000.** Metal toxicity and ectomycorrhizas, *Physiologia Plantarum*. **109**: 107-116.

Jia X, Zhao Y, Liu T, Huang S, Chang Y. 2016. Elevated CO_2 increases glomalin-related soil protein (GRSP) in the rhizosphere of *Robinia pseudoacacia* L. seedlings in Pb- and Cdcontaminated soils*, Environmental Pollution*. **218:** 349-357.

Jiang QY, Tan SY, Zhuo F, Yang DJ, Ye ZH, Jing YX. 2016. Effect of *Funneliformis mosseae* on the growth, cadmium accumulation and antioxidant activities of *Solanum nigrum*, *Applied Soil Ecology*. **98**: 112-120.

Jiang QY, Zhuo F, Long SH, Zhao HD. 2016b. Can arbuscular mycorrhizal fungi reduce Cd uptake and alleviate Cd toxicity of *Lonicera japonica* grown in Cd-added soils? *Scientific Reports*. **6:** 21805.

Jones A, Panagos P, Barcelo S, Bouraoui F, *et al*. 2012. *The State of Soil in Europe: A Contribution from JRC to the European Environmental Agency's Environment State and Outlook Report.* SOER 2010. Publications Office, Luxembourg.

Kabata-Pendias A, Pendias H. 2001. *Trace Elements in Soils and Plants*, Florida: CRC press.

Kaur H, Garg N. 2017. Recent perspectives on cross talk between cadmium, zinc, and arbuscular mycorrhizal fungi in plants, *Journal of Plant Growth Regulation*. **37**: 680-693.

Khan A, Sharif M, Ali A, Shah SNM, *et al.* 2014. Potential of AM fungi in phytoremediation of heavy metals and effect on yield of wheat crop, *American Journal of Plant Sciences*. **5**: 1578-1586.

Khan AG. 2006. Mycorrhizoremediation—an enhanced form of phytoremediation, *Journal of Zhejiang University SCIENCE B*. **7**: 503-514.

Khan MA, Khan S, Khan A, Alam M. 2017. Soil contamination with cadmium, consequences and remediation using organic amendments, *Science of the Total Environment*. **601-602**: 1591-1605.

Kimura T, Kambe T. 2016. The functions of metallothionein and ZIP and ZnT transporters: an overview and perspective, *International Journal of Molecular Sciences*. **17**: 336.

Kirkham MB. 2006. Cadmium in plants on polluted soils: effects of soil factors, hyperaccumulation, and amendments, *Geoderma*. **137**: 19-32.

Kohler A, Blaudez D, Chalot M, Martin F. 2004. Cloning and expression of multiple

metallothioneins from hybrid poplar, *New Phytologist*. **164:** 83-93.

Komives T, Gullner G. 2006. Dendroremediation: the use of trees in cleaning up polluted soils. In: Mackova M, *et al*., eds., *Phytoremediation Rhizoremediation*, p. 23-31.

Konlechner C, Turktas M, Langer I, Vaculik M *et al.* 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, Chardonnens AN. 2001. The use of transgenic plants in the bioremediation of soils contaminated with trace elements, *Applied Microbiology and Biotechnology*. **55**: 661-672.

Krpata D, Peintner U, Langer I, Fitz WJ, Schweiger P. 2008. Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site, *Mycological Research*. **112**: 1069-1079.

Kumar P, Lucini L, Rouphael Y, Cardarelli M, *et al.* 2015. Insight into the role of grafting and arbuscular mycorrhiza on cadmium stress tolerance in tomato, *Frontiers in Plant Science.* **6**: 477.

Labbe JL, Weston DJ, Dunkirk N, Pelletier DA, Tuskan GA. 2014. Newly identified helper bacteria stimulate ectomycorrhizal formation in *Populus*, *Frontiers in Plant Science***. 5**: 1-10.

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.

Leyval C, Turnau K, Haselwandter K. 1997. Effect of heavy-metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects, *Mycorrhiza*. **7**: 139-153.

Li JT, Liao B, Lan CY, Ye ZH, Baker AJM, Shu WS. 2010. Cadmium tolerance and accumulation in cultivars of a high-biomass tropical tree (*Averrhoa carambola*) and its potential for phytoextraction, *Journal of Environmental Quality*. **39**: 1262-1268.

Li Z, Ma Z, van der Kuijp TJ, Yuan Z, Huang L. 2014. A reviewof soil heavymetal pollution frommines in China: Pollution and health risk assessment, *Science of the Total Environment*. **468-469**: 843-853.

Lin AJ, Zhang XH, Wong MH, Ye ZH, *et al.* 2007. Increase of multi-metal tolerance of three leguminous plants by arbuscular mycorrhizal fungi colonization, *Environmental Geochemistry and Health*. **29**: 473-481.

Lingua G, Bona E, Todeschini V, Cattaneo C, Marsano F, Berta G, Cavaletto M. 2012. Effects of heavy metals and arbuscular mycorrhiza on the leaf proteome of a selected poplar clone: a time course analysis, *PLoS ONE*. **7**: 1-25.

Lingua G, Franchin C, Todeschini V. *et al*. 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.

Liu L, Gong Z, Zhang Y, Li P. 2014. Growth, cadmium uptake and accumulation of maize (*Zea mays* L.) under the effects of arbuscular mycorrhizal fungi, *Ecotoxicology*. **23**: 1979-1986.

Ó Lochlainn S, Bowen HC, Fray RG, Hammond JP *et al*. 2011. Tandem quadruplication of HMA4 in the Zinc (Zn) and Cadmium (Cd) hyperaccumulator *Noccaea caerulescens,* PLOS One. **6**: e17814.

Long ER, Macdonald DD, Smith SL, Calder FD. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments, *Environmental Management*. **19**: 81-97.

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.

Luo ZB, Wu C, Zhang C, Li H, Lipka U, Polle A. 2014. The role of ectomycorrhizas in heavy metal stress tolerance of hostplants, *Environmental and Experimental Botany*. **108**: 47-62.

Lux A, Martinka M, Vaculik M, White PJ. 2011. Root responses to cadmium in the rhizosphere: a review, *Journal of Experimental Botany*. **62**: 21-37.

Ma L, Wu X, Zheng L. 2008. Mycorrhizal formation of nine ectomycorrhizal fungi on poplar cuttings, *Frontiers of Forestry in China*. **3**: 475-479.

Marmiroli M, Pietrini F, Maestri E, Zacchini M, Marmiroli N, Massacci A. 2011. Growth, physiological and molecular traits in Salicaceae trees investigated for

phytoremediation of heavy metals and organics, *Tree Physiology*. **31:** 1319-1334.

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

Marques APGC, Oliveira RS, Rangel AOSS, Castro PML. 2008. Application of manure and compost to contaminated soils and its effect on zinc accumulation by *Solanum nigrum* inoculated with arbuscular mycorrhizal fungi, *Environmental Pollution*. **151:** 608-620.

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

Marx DH, Bryan WC. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*, *Forest Science*. **21**: 245-254.

Meier S, Borie F, Bolan N, Cornejo P. 2012. Phytoremediation of metal-polluted soils by arbuscular mycorrhizal fungi, *Critical Reviews in Environmental Science and Technology*. **42**: 741-775.

Mertens J, Smolders E. 2013. Zinc. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, p. 465-493.

Meyer E, Londono DMM, Armas RD, Giachini AJ, *et al.* 2017. Arbuscular mycorrhizal fungi in the growth and extraction of trace elements by *Chrysopogon zizanioides* (vetiver) in a substrate containing coal mine wastes, *International Journal of Phytoremediation*. **19**: 113-120.

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

Mirlean N, Roisenberg A. 2006. The effect of emissions of fertilizer production on the environment contamination by cadmium and arsenic in southern Brazil, *Environmental Pollution*. **143**: 335-340.

Mirsal I. 2010. *Soil pollution: origin, monitoring & remediation*, 2nd ed., Springer, Berlin Heidelberg.

Moffett BF, Nicholson FA, Uwakwe NC, Chambers BJ, Harris JA, Hill TCJ. 2003. Zinc contamination decreases the bacterial diversity of agricultural soil, *FEMS Microbiology Ecology*. **43**: 13-19.

Monpetit E, Lachapelle E. 2017. New environmental technology uptake and bias toward the status quo: The case of phytoremediation, *Environmental Technology and Innovation*. **7:** 102-109.

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.

Mulligan CN, Yong RN, Gibbs BF. 2001. Remediation technologies for metal-contaminated soils and groundwater: an evaluation, *Engineering Geology*. **60**: 193-207.

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

Naja GM, Volesky B. 2009. Toxicity and sources of Pb, Cd, Hg, Cr, As, and radionuclides in the environment. In: Wang LK, Chen JP, Hung Y, Shammas NK, ed., *Heavy metals in the environment*, Taylor and Francis & CRC Press, New York, p. 13-61.

Nascimento LFC, Izario Filho HJ, Pereira ML, Baccan N. 2005. Cadmium quantification in Brazilian mothers colostrum: a regional study, *Revista Brasileira de Saúde Materno Infantil*. **5**: 193-198.

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

Nicholson FA, Jones KC, Johnston AE. 1994. Effect of phosphate fertilizers and atmospheric deposition on long-term changes in the cadmium content of soils and crops, *Environmental Science and Technology*. **28:** 2170-2175.

Nicholson FA, Smith SR, Alloway BJ, Carlton-Smith C, Chambersa BJ. 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales, *Science of the Total Environment*. **331**: 205-219.

Nieboer E, Richardson DH. 1980. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions, *Environmental Pollution Series B, Chemical and Physical*. **1**: 3-26.

Noulas C, Tziouvalekas M, Karyotis T. 2018. Zinc in soils, water and food crops, *Journal of Trace Elements in Medicine and Biology*. **49**: 252-260.

Orlowska E, Godzik B, Turnau K. **2012.** Effect of different arbuscular mycorrhizal fungal isolates on growth and arsenic accumulation in *Plantago lanceolata* L., *Environmental Pollution*. **168:** 121-130.

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.

Padmavathiamma PK, Li LY. 2007. Phytoremediation technology: hyper-accumulation metals in plants, *Water Air and Soil Pollution*. **184**: 105-126.

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

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.

Pan J, Plant JA, Voulvoulis N, Oates CJ, Ihlenfeld C. 2010. Cadmium levels in Europe: implications for human health, *Environmental Geochemistry and Health*. **32**: 1-12.

Patra M, Bhowmik N, Bandopadhyay B, Sharma A. 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance, *Environmental and experimental botany*. **52**: 199-223.

Pietrini F, Zacchini M, Iori V, Pietrosanti L, Bianconi D, Massacci A. 2010. Screening of poplar clones for cadmium phytoremediation using photosynthesis, biomass and cadmium content analyses, *International Journal of Phytoremediation*. **12**: 105-120.

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.

Pourret O. 2018. On the necessity of banning the term “heavy metal” from the scientific literature, *Sustainability*. **10**: 2879.

Prasad MNV. 2003. Phytoremediation of metal-polluted ecosystems: hype for commercialization, *Russian Journal of Plant Physiology*. **50**: 764-780.

Pulford ID, Watson C. 2003. Phytoremediation of heavy metal-contaminated land by trees - A review, *Environment International*. **29**: 529-540.

Qasim B, Motelica-Heino M, Bourgerie S, Gauthier A, Morabito D. 2016. Rhizosphere effects of *Populus euramericana* Dorskamp on the mobility of Zn, Pb and Cd in contaminated technosols, *Journal of Soils and Sediments*. **16**: 811-820.

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.

Rivetta A, Negrini N, Cocucci M. 1997. Involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination, *Plant, Cell and Environment*. **20**: 600-608.

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.

Robinson BH, Mills T, Green S, Chancerel B, *et al.* 2005. Trace element accumulation by poplars and willows used for stock fodder, *New Zealand Journal of Agricultural Research*. **48**: 489-497.

Robinson BH, Mills T, Petit D, Fung LE, Green SR, Clothier BE. 2000. Natural and induced cadmium-accumulation in poplar and willow: Implications for phytoremediation, *Plant and Soil*. **227**: 301-306.

Ross SM, Kaye KJ. 1994. The meaning of metal toxicity in soil-plant systems. In: Ross SM, ed., *Toxic Metals in Soil-Plant Systems*. John Wiley & Sons Ltd. West Sussex, p. 28–61.

Salminen R, Batista MJ, Bidovec M, Demetriades A, De Vivo B *et al*. 2005. Geochemical atlas of Europe. Part 1: Background Information, Methodology and Maps. Espoo, Geological Survey of Finland, p. 526.

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, New York, p. 225-240.

Sarwar N, Imran M, Shaheen MR, Ishaque W *et al.* 2017. Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives, *Chemosphere*. **171**: 710-721.

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM. 2002. The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes, *Journal of Experimental Botany.* **53:** 2381-2392.

Schutzendubel A, Polle A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization, *Journal of experimental botany*. **53:** 1351-1365.

Science Communication Unit, University of the West of England, Bristol. 2013. *Science for Environment Policy In-depth Report: Soil Contamination: Impacts on Human Health*. Report produced for the European Commission DG Environment, September 2013.

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.

Sell J, Kayser A, Schulin R, Brunner I. **2005.** Contribution of ectomycorrhizal fungi to cadmium uptake of poplars and willows from a heavily polluted soil, *Plant and Soil*. **277**: 245-253.

Shah FUR, Ahmad N, Masood KR, Peralta-Videa JR. 2010. Heavy metal toxicity in plants, In: Ashraf M, Ozturk M, Ahmad MSA, eds., *Plant Adaptation and Phytoremediation,* 1st ed, Springer, p. 71-97.

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.

Shim D, Kim S, Choi, Y. *et al*. 2013. Transgenic poplar trees expressing yeast cadmium factor 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil, *Chemosphere*. **90**: 1478-1486.

Siemens JA, Zwiazek JJ. 2008. Root hydraulic properties and growth of balsam poplar (*Populus balsamifera*) mycorrhizal with *Hebeloma crustuliniforme* and *Wilcoxina mikolae* var. *mikolae*, *Mycorrhiza*. **18**: 393-401.

Smith SE, Read D. 2008. *Mycorrhizal Symbiosis 3rd edition.* Academic Press, London.

Smolders E, Mertens J. 2013. Cadmium. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, p. 283-311.

Srivastava J, Chandra H, Nautiyal AR, Kalra SJS. 2012. Response of C3 and C4 plant systems exposed to heavy metals for phytoextraction at elevated atmospheric co2 and at elevated temperature, In: Srivastava J, ed., *Environmental Contamination,* 1st ed, InTech, Croatia, p. 3-16.

Tan SY, Jiang QY, Zhuo F, Liu H, Wang YT, Li SS, *et al.* 2015. Effect of inoculation with *Glomus versiforme* on cadmium accumulation, antioxidant activities and phytochelatins of *Solanum photeinocarpum*, *PLoS ONE*. **10**: e0132347.

Templeton DM, Liu Y. 2010. Multiple roles of cadmium in cell death and survival, *Chemico-biological interactions*. **188**: 267-275.

Thakur S, Singh L, Ab Wahid Z, Siddiqui MF, *et al.* 2016. Plant-driven removal of heavy metals from soil: uptake, translocation, tolerance mechanism, challenges, and future perspectives, *Environmental Monitoring and Assessment*. **188**: 206.

Thomine S, Lelievre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H. 2003. AtNramp3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency, *The Plant Journal*. **34**: 685-695.

Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI. 2000. Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes, *PNAS*. **97**: 4991-4996.

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

Tonin C, Vandenkoornhuyse P, Joner EJ, Straczek J, Leyval C. 2001. Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover, *Mycorrhiza*. **10**: 161-168.

Toth G, Hermann T, Da Silva MR, Montanarella L. 2016. Heavy metals in agricultural soils of the European Union with implications for food safety, *Environment International*. **88**: 299-309.

Tseng CC, Wang JW, Yang L. 2009. Accumulation of copper, lead, and zinc by in situ plants inoculated with AM fungi in multicontaminated soil, *Communications in Soil Science and Plant Analysis*. **40**: 3367-3386.

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

Vamerali T, Bandiera M, Mosca G. 2010. Field crops for phytoremediation of metal-contaminated land: a review, *Environmental Chemistry Letters*. **8**: 1-17

Verret F, Gravot A, Auroy P, *et al*. 2004. Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance, *FEBS Letters.* **576**: 306-3012.

Viehweger K. 2014. How plants cope with heavy metals, *Botanical Studies*. **55**: 35.

Vogel-Mikus K, Drobne D, Regvar M. 2005. Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonisation of pennycress *Thlaspi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia, *Environmental Pollution*. **133**: 233-242.

Vogel-Mikus K, Pongrac P, Kump P, Necemer M, Regvar M. 2006. Colonisation of a Zn, Cd and Pb hyperaccumulator *Thlaspi praecox* Wulfen with indigenous arbuscular mycorrhizal fungal mixture induces changes in heavy metal and nutrient uptake, *Environmental Pollution*. **139:** 362-371.

Wang FY, Shi ZY, Xu XF, Wang XG, Li YJ. 2013. Contribution of AM inoculation and cattle manure to lead and cadmium phytoremediation by tobacco plants, *Environmental Sciences: Processes and Impacts*. **15**: 794-801.

Wang L, Huang X, Ma F, Hsin S, Wu J, Zhu S. **2017**. Role of *Rhizophagus irregularis* in alleviating cadmium toxicity via improving the growth, micro- and macroelements uptake in *Phragmites australis*, *Environmental Science and Pollution Research.* **24**: 3593-3607.

Wang Y, Huang J, Gao Y. 2012. Arbuscular mycorrhizal colonization alters subcellular distribution and chemical forms of cadmium in *Medicago sativa* L. and resists cadmium toxicity, *PLoS ONE*. **7**: e48669.

Watts-Williams SJ, Cavagnaro TR. 2018. Arbuscular mycorrhizal fungi increase grain zinc concentration and modify the expression of root ZIP transporter genes in a modern barley (*Hordeum vulgare*) cultivar, *Plant Science*. **274**: 163-170.

Watts-Williams SJ, Patti AF, Cavagnaro TR. 2013. Arbuscular mycorrhizas are beneficial under both deficient and toxic soil zinc conditions, *Plant and Soil*. **371**: 299-312.

Weissenhorn I, Leyval C, Belgy G, Berthelin J. 1995. Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil, *Mycorrhiza*. **5**: 245-251.

Wenzel WW. 2013. Arsenic. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, p. 241-282.

Willey N. 2007. Preface. In: Willey N, ed., *Phytoremediation: Methods and Reviews.* Humana Press Inc. Totowa, New Jersey 07512

Williams LE, Pittman JK, Hall JL. 2000. Emerging mechanisms for heavy metal transport in plants, *Biochimica et Biophysica Acta*. **1465**: 104-126.

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.

Wong MH. 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils, *Chemosphere*. **50**: 775-780.

Wu Z, Wu W, Zhou S, Wu S. 2016. Mycorrhizal inoculation affects pb and cd accumulation and translocation in pakchoi (*Brassica chinensis* L.), *Pedosphere*. **26**: 13-26.

Wuana RA, Okieimen FE. 2011. Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation, *ISRN Ecology*. Article ID 402647 http://dx.doi.org/10.5402/2011/402647.

Yadav SK. 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants, *South African Journal of Botany*. **76:** 167-179.

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.

Yu TJC, Egger KN, Peterson RL. 2001. Ectendomycorrhizal associations – characteristics and functions, *Mycorrhiza*. **11**: 167-177.

Zenk MH. 1996. Heavy metal detoxification in higher plants - a review, *Gene*. **179:** 21-30.

Zhang H, Zhao S, Li D, Xu X, Li C. 2017. Genome-wide analysis of the ZRT, IRT-like protein (ZIP) family and their responses to metal stress in *Populus trichocarpa*, *Plant Molecular Biology Reporter*. **35**: 534-549.

Zhang XH, Lin AJ, Chen BD, Wang YS, Smith SE, Smith AF. 2006. Effects of *Glomus mossae* on the toxicity of heavy metals to *Vicia faba*, *Journal of Environmental Sciences*. **18**: 721-726.

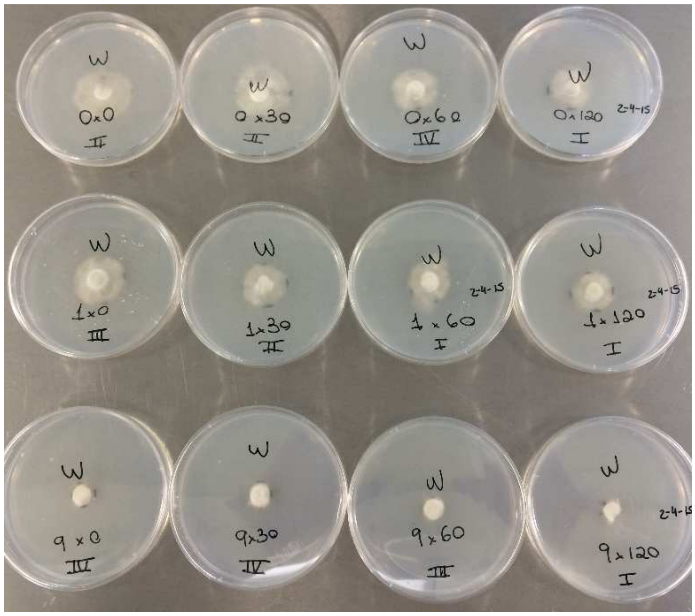
Zhang XH, Zhu YG, Chen BD, Lin AJ, Smith SE, Smith FA. **2005.** Arbuscular mycorrhizal fungi contribute to resistance of upland rice to combined metal contamination of soil, *Journal of Plant Nutrition*. **28**: 2065-2077.

Zheng S, Wang C, Shen Z, Quan Y, Liu X. **2015.** Role of extrinsic arbuscular mycorrhizal fungi in heavy metal-contaminated wetlands with various soil moisture levels, *International Journal of Phytoremediation*. **17**: 208-214.

Zhu YG, Christie P, Laidlaw AS. 2001. Uptake of Zn by arbuscular mycorrhizal white clover from Zn-contaminated soil, *Chemosphere*. **42:** 193-199.

Chapter 2

Cd and Zn interactions and toxicity in ectomycorrhizal basidiomycetes in axenic culture



Left: Some basidiomycetes collected for isolation during the PhD

Right: Axenic cultures of *Hebeloma subsaponaceum under different concentrations of Cd and Zn.*

Abstract

Metal contamination in soils affects both above and belowground communities, including soil microorganisms. Ectomycorrhizal (ECM) fungi are an important component in belowground community and tolerant strains have great potential in enhancing plant-based remediation techniques.

We assessed cadmium and zinc toxicity in five ECM species in liquid media (*Hebeloma subsaponaceum*; *H. cylindrosporum*; *H. crustuliniforme*; *Scleroderma* sp.; *Austroboletus occidentalis*) and investigated the potential of Zn to alleviate Cd toxicity. Due to highly divergent results reported in the literature, liquid and solid media were compared experimentally for the first time in terms of differential toxicity thresholds in Cd and Zn interactions. A wide range of Cd and Zn concentrations were applied to ectomycorrhizal fungi in axenic cultures (in mg L^{-1}): 0; 1; 3; 9; 27; 81; 243 for the Cd treatments, and 0; 1; 30; 90; 270; 810; 2430 for Zn. Combined Zn and Cd treatments were also applied to *H. subsaponaceum* and *Scleroderma* sp. Dry weight was recorded after 30 days, and in case of solid medium treatments, radial growth was also measured.

All species were adversely affected by high levels of Cd and Zn, and *A. occidentalis* was the most sensitive, with considerable biomass decrease at 1 mg L^{-1} Cd, while *Scleroderma* sp. and *H. subsaponaceum* were the most tolerant, which are species commonly found in highly contaminated sites. Cd was generally 10 times more toxic than Zn, which may explain why Zn had little impact in alleviating Cd effects. In some cases, Cd and Zn interactions led to a synergistic toxicity, depending on the concentrations applied and type of media used. Increased tolerance patterns were detected in fungi grown in solid medium and may be the cause of divergent toxicity thresholds found in the literature. Furthermore, solid medium allows measuring radial growth/mycelial density as endpoints which are informative and in this case appeared be related to the high tolerance indices found in *H. subsaponaceum*.

2.1 Introduction

Cadmium (Cd) is one of the most hazardous metals in the environment, ranked seventh in toxicity by the Agency for Toxic Substance and Disease Registry (ATSDR 2017). It lacks any known biological function, it can be toxic to living organisms at relatively low concentrations (Alloway 2013) and has a high mobility in soils (Lei et al. 2010). Cd can be frequently found in zinc (Zn) bearing minerals (Alloway 2013) and due to their similar geochemical characteristics they are often associated in soils (Kabata-Pendias and Pendias 2001). Although Zn is a micronutrient, high concentrations in the environment can be extremely harmful to biota. Data suggest that Zn can be more toxic to soil organisms than Pb (Ross and Kaye 1994) and decrease bacterial diversity in contaminated lands (Moffett et al. 2003).

In metal contaminated soils, symbiotic fungi such as ectomycorrhizal fungi (ECM) may improve plant fitness and metal tolerance, such as by promoting better growth or nutrition, preventing metal uptake and protecting against other abiotic and biotic stresses (Krznaric et al. 2009; Rodriguez and Redman 2008; Zheng et al. 2009), being crucial for plant survival in such environments (Saraswat and Rai 2011). Almost all land plants depend on symbiotic mycorrhizal fungi (Leyval et al. 1997), with woody pioneers species relying mostly on phenotypic plasticity and ectomycorrhizal assossiations to withstand metal-polluted soils (Colpaert 2008; Krpata et al. 2008). However, the extent of the ameliorating effects of the symbiosis is difficult to demonstrate and depends on the fungal species, plant genotype (Krznaric et al. 2009) and the differential toxicity of metals (Fomina et al. 2005).

Several studies focus on assessing metal toxicity in different ECM fungi *in vitro* in order to identify tolerant species and strains (Blaudez et al. 2000b; Fomina et al. 2005). However, comparisons are difficult with a variety of methods employed, different fungal strains, and a range of metal concentrations and endpoints considered (e.g. radial growth or biomass production). The types of media used can also vary, as well as their physical states: liquid or solid agar (Tam, 1995; Colpaert et al. 2004; Zheng et al. 2009). This appears to be responsible for variation in bioavailability and therefore causes a distinct difference in the toxicity thresholds for Cd and Zn (Table 2.1). Interactions between metals are also responsible for variation

in toxicity responses, for instance, in some cases it has been observed that Zn is able to reduce Cd toxicity in certain ECM fungi, often attributed to the ionic competition for binding sites (Hartley et al. 1997b).

Table 2.1. Toxicity thresholds for Cd and Zn in ectomycorrhizal fungi grown in either liquid or solid media. Toxic concentrations were considered as the minimum concentration reported to cause any adverse effects or as the only concentration value reported by the author(s).

	Toxic concentrations (mg L^{-1})	
	Solid	Liquid
Zn		
mean	309	123
median	292	22
maximum	975	500
Cd		
mean	12	2.2
median	2.0	0.9
maximum	50	10
ECM species tested	17	12
References consulted	11[a]	5[b]

[a] (Blaudez et al. 2000b; Brown and Wilkins 1985; Colpaert and Van Assche 1987; Colpaert and Van Assche 1992; Colpaert et al. 2000; Colpaert et al. 2004; Colpaert et al. 2005; Denny and Wilkins 1987; Krznaric et al. 2009; Ray et al. 2005; Willenborg et al. 1990) [b] (Colpaert and Van Assche 1987; Courbot et al. 2004; Grazzioti et al. 2001; Hartley et al. 1997; Tam 1995).

Given the ambiguities across published dataset, we aimed to elucidate our current understanding of metal toxicity by addressing specific issues such as: the possible Zn and Cd antagonistic/synergistic interactions in ectomycorrhizal fungi, the ability of Zn in alleviating Cd toxicity effects; and the different toxicity thresholds arising from using either liquid or solid media under the same range of concentrations.

2.2 Materials and Methods

2.2.1 Assessing Cd and Zn toxicity

Toxicity trials were performed *in vitro* using five ECM species originated from non-polluted environments: *Hebeloma subsaponaceum* (from a Boreal Forest, Norway); *H. cylindrosporum* (from under pine trees, France); *H. crustuliniforme* (from Sitka spruce, Brown Earth); *Scleroderma* sp. (woodlands, Western Australia) and *Austroboletus occidentalis* (Western Australia), a species recently found to be a non-colonizing fungal partner (Kariman et al. 2014). These species were selected from our in-house collection due to their growth rates observed previously in agar medium. Methods were based on a previous study by Chen and Tibbett (2007). Four circular plugs (1 mm) were cut out from the edges of actively growing colonies (5 weeks old) and transferred to Petri dishes with 25 mL of Melin-Norkrans liquid medium (MMN). The medium composition was: 6.51 mM NH_4NO_3, 0.57 mM $MgSO_4 \cdot 7H_2O$, 0.23 mM $CaCl_2$, 0.015 mM $ZnSO_4$, 0.3 mM Thiamine, 5.55 mM d-glucose, 2 mM KH_2PO_4, 0.035 mM Ferric EDTA; pH was adjusted to 5.5. No Zn ($ZnSO_4$) was added to the initial MMN medium used for the Zn treatments, as this metal was added later to make up the desired range of concentrations.

Cd and Zn concentrations were added via $CdCl_2$ and $ZnSO_4$ solutions to the final medium, and the final concentrations were (in mg L^{-1}): 0; 1; 3; 9; 27; 81; 243 for the Cd treatments, and 0; 1; 30; 90; 270; 810; 2430 for the Zn treatments. Such concentrations were selected based on similar toxicity experiments with mycorrhizal fungi found in the literature (Blaudez et al. 2000b; Colpaert and Van Assche 1992; Colpaert et al. 2004; Ray et al. 2005; Tam 1995; Willenborg et al. 1990).

The fungal cultures were incubated in the dark at 20°C for 30 days, each treatment had four replicates. The mycelial mats were then removed from the medium, placed on small aluminum envelopes (weighed previously) and oven-dried overnight at 60°C. The dry weight (DW) was assessed gravimetrically. The Tolerance Index (TI %) was used to express the tolerance results (Fomina et al. 2005), calculated by the equation:

$$TI(\%) = \frac{DWtreated}{DWcontrol}\ x\ 100 \qquad (1)$$

In which DW is the dry weight obtained from the fungal biomass.

Statistical analysis was performed on the dry weight data using STATISTICA 12®. To attain normal distribution (Shapiro-Wilk), box-cox transformation was applied. However, the data did not meet the assumption of homogeneity of variances (Levene's test). Thus, analysis of variance was carried out using Welch's test (Zar 2010), followed by Dunnett's test to determine the LOAEC values (Lowest Observed Adverse Effects Concentration), which also does not require equal variances (Quinn and Keough 2002). The Dunnett's for Zn toxicity considered the treatment of 1 mg L^{-1} Zn as the control.

2.2.2 Cd and Zn interactions

To verify the effect of Zn in preventing Cd toxicity in ECM fungi, a second experiment was carried out using the same methods described above, except no basal Zn was added to the basic MMN medium in all treatments, and was added later to make up the desired range of concentrations; growth period of 21 days. However, because *H. cylindrosporum* had lower or similar performance as *H. subsaponaceum*, the former was excluded from this experiment. In this case, ECM species were exposed to Cd and Zn together, with concentrations added in different combinations: 0, 1 and 9 mg L^{-1} for Cd, and 0, 1, 9 and 30 mg L^{-1} for Zn. Therefore, this assay was comprised of 12 treatments (Cd × Zn: 0×0, 0×1, 0×9, 0×30, 1×0, 1×1,1×9, 1×30, 9×0, 9×1, 9×9, 9×30 mg L^{-1}).

Relative dry weight was calculated with equation (1), and ANOVA followed by Tukey's test were performed to verify significant differences among the Zn treatments (0; 1; 9 and 30 mg L^{-1}). For attaining normality and homoscedasticity in two variables (1 mg L^{-1} Cd in *H. crustuliniforme* and 0 mg L^{-1} Cd in *Scleroderma* sp.), data were transformed by the equation: 1/x.

Due to the high Cd toxicity observed, this experiment was repeated subsequently with only *Scleroderma sp.* and *Hebeloma subsaponaceum*, but using another range of concentrations (0; 1; 9 mg L^{-1} Cd and 0; 30; 60; 120 mg L^{-1} Zn) and two types of MMN media, a solid medium containing 2% agar, and a liquid medium as described previously, with four replicates. Plates were incubated in the dark, at 20 ± 2°C for 30 days. By the end of the growth period, treatments with solid media were measured for radial growth (a mean between vertical and horizontal diameters, in centimeters). After which the agar was cut and removed from the plates and melted in a microwave in short 15 seconds burst for no more than one minute in total (Karaduman et al. 2012); the mycelium was removed and blotted dry with absorbent paper until it was free of all agar medium, the mycelium was then washed with deionized water, oven-dried overnight (60°C) and weighed. Liquid media treatments were handled as described previously. Statistical analyses were performed following the same steps as the previous experiments. Contour plots were achieved by linear interpolation (using SigmaPlot®) of the fungal Tolerance Indexes (TI%, but in this case considering 100% as the treatment with the highest biomass production: i.e. Cd x Zn (0 x 30 mg L^{-1} in liquid cultures and Cd x Zn (1 x 30 mg L^{-1}) in solid cultures); using 12 Zn × Cd co-ordinates, based on publications by Hartley et al. (1997b) and Krznaric et al. (2010).

2.3 Results

All species assessed were negatively affected by either Cd or Zn, depending on the concentration, although lower Zn concentrations had a positive effect on all strains (Figure 2.1). Biomass decreased in all species exposed to Cd, and a critical effect was observed in *A. occidentalis*, *H. cylindrosporum* and *H. crustuliniforme* in concentration as low as 1 mg L^{-1}, highlighting Cd pronounced toxicity. There was no visible growth at highest Cd and Zn concentrations, thus the dry weight detected in these cases, i.e. < 2 mg (Figure 2.1) were considered as being from the four circular agar plugs (1 mm) initially used for inoculation. Reduced biomass due to Cd and Zn toxicity was a common consequence observed in ECM fungi, regardless of the species. Cadmium, for being an element with no known biological function, is

considerably more toxic than Zn and its toxic effects began at concentrations at least 30 times lower than the concentrations necessary for Zn to display toxicity (Figure 2.1). Nonetheless, Zn toxicity was observed in lower concentrations than expected, three species had LOAEC values of 90 mg L^{-1} (*H. crustuliniforme* and *H. subsaponaceum*) or lower (*A. occidentalis*) (Figure 2.1). From the LOAEC values determined, the most sensitive species to metal toxicity considering both Cd and Zn, were *A. occidentalis* and *H. cylindrosporum*, while *Scleroderma* sp. and *H. subsaponaceum* were the most tolerant.

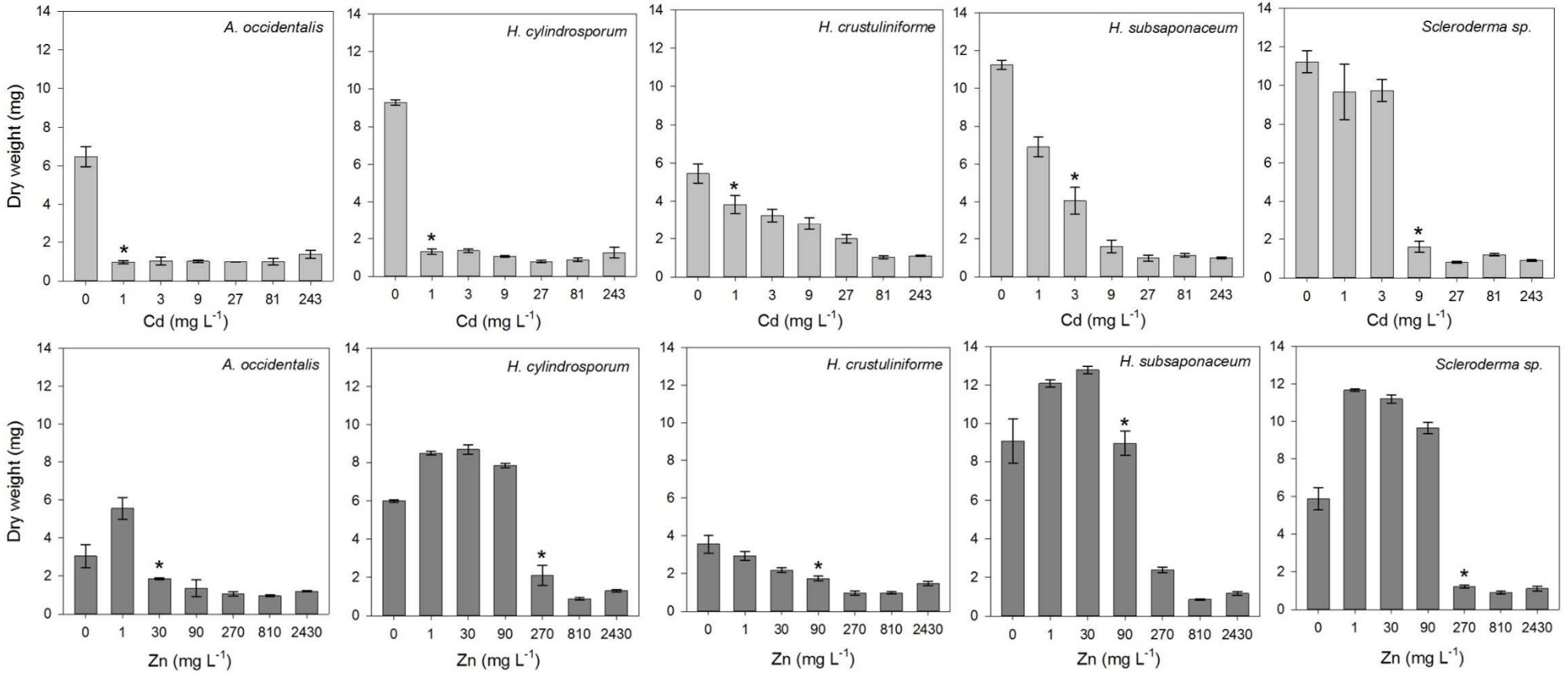


Figure 2.1 Dry weight of five ECM species (*Austroboletus occidentalis*, *Hebeloma cylindrosporum, H. crustuliniforme, H. subsaponaceum*, *Scleroderma* sp.) after 30 days under a range of Cd or Zn concentrations in liquid media (n = 4; standard error bars). Asterisks represent the first concentration from which fungal growth starts to be adversely affected, LOAEC, determined by Dunnett's test ($p < 0.05$). LOAEC for Cd and Zn (in mg L^{-1}) were, respectively, 1 and 30 in *A. occidentalis*; 1 and 270 in *H. cylindrosporum*; 1 and 90 in *H. crustuliniforme*; 3 and 90 in *H. subsaponaceum*; 9 and 270 in *Scleroderma* sp.

Almost all species had higher growth under low concentrations of Zn, except for *H. crustuliniforme*, which was the only species not to show any growth improvement even at the lowest Zn treatment, of 1 mg L^{-1} (Figure 2.2B), a concentration long considered to be beneficial and typically part of the basic formulation of fungal growth media (Marx and Bryan 1975; Pridham and Gottlieb 1948; Tibbett et al. 1999).

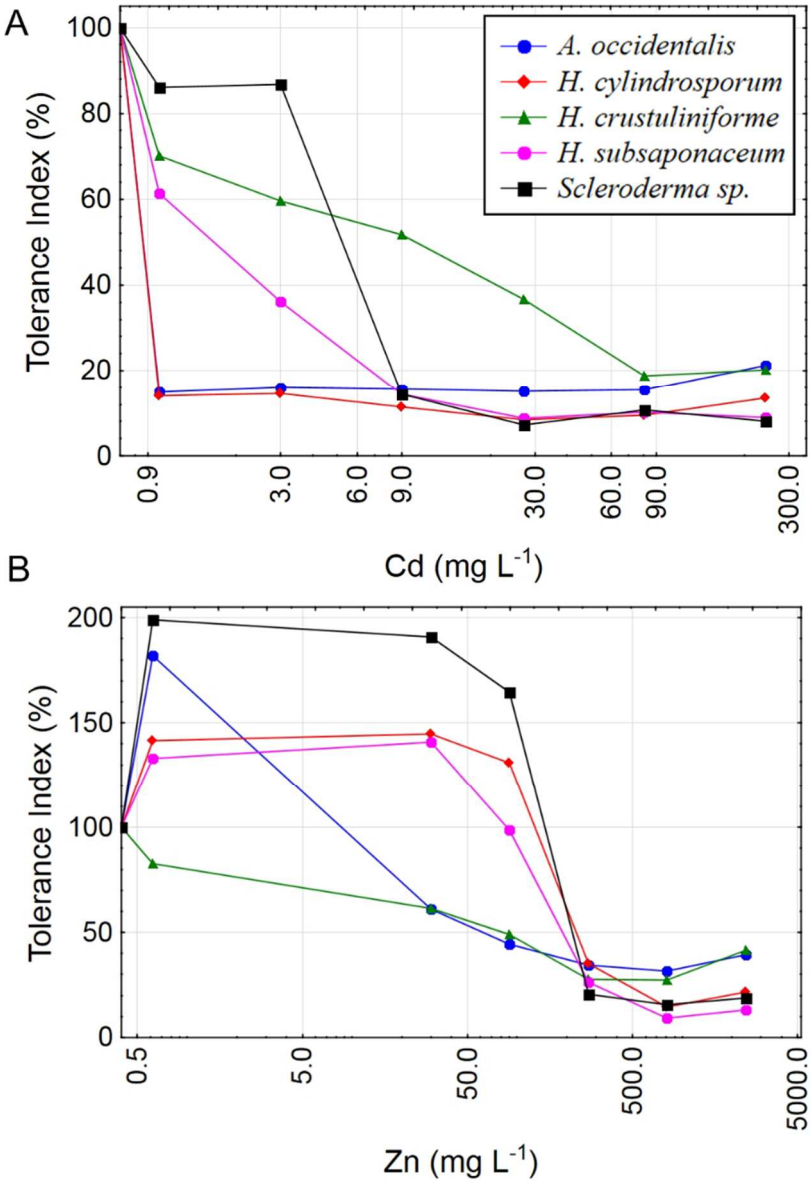


Figure 2.2 Metal tolerance indices (TI%) for five ECM species under increasing concentrations of Cd (0; 1; 3; 9; 27; 81; 243 mg L^{-1}) or Zn (0; 1; 30; 90; 270; 810; 2430 mg L^{-1}) in liquid media. X axes are in logarithmic scale. *TI% = DW treated/DW control × 100*.

In the second experiment, in which the ECM fungi were exposed to mixed concentrations of Cd and Zn, it was observed that Zn addition had little effect on the dry weight of all species, regardless of the Cd concentration, except for *Scleroderma* sp. and *H. subsaponaceum*: the only species in which Zn addition promoted biomass increase at both non-contaminated media (Cd: 0 mg L^{-1}) and highest Cd concentration, of 9 mg L^{-1} (Figure 2.3).

Because both *A. occidentalis* and *H. crustuliniforme* had poor biomass production and suffered highly from Cd and Zn toxicity (data not shown); Based on previous results (Figure 2.1), their responses were entirely predictable.

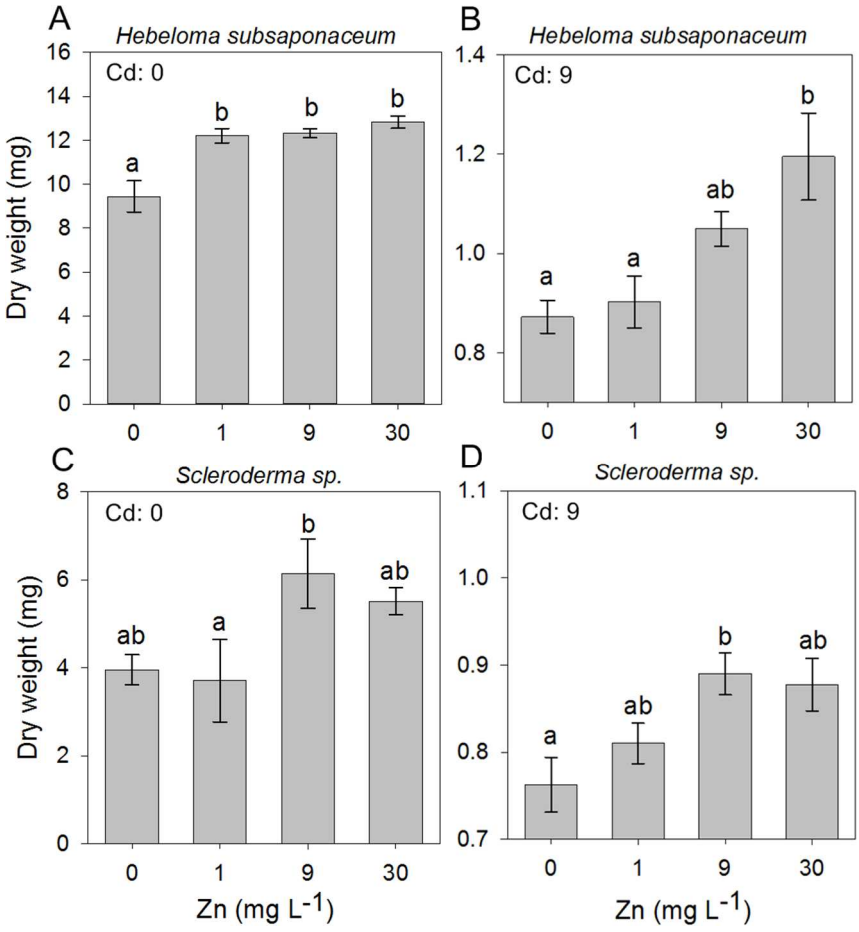


Figure 2.3 Effects of Zn concentrations on dry weights (mean, n = 4; standard error bars) of *Hebeloma subsaponaceum* and *Scleroderma* sp. under two Cd concentrations (0 and 9 mg L^{-1}). Data for other species were not significantly different and therefore are not shown. Different letters represent significant differences by Tukey test ($p<0.05$).

In a concluding experiment *H. subsaponaceum* and *Scleroderma* sp. were exposed to Cd along with higher Zn concentrations, in both solid and liquid media. Dry weight and radial growth (in solid media only) were evaluated (Table 2.2). In general, Zn addition did not alleviate Cd toxicity effects in both species, however there were a few exceptions: at 1 mg L^{-1} Cd, the addition of Zn (30 mg L^{-1}) promoted a dry weight increase in *Scleroderma* sp. (from 2.7 to 10.5 mg) in liquid media. However, this effect was not significant in solid media (Table 2.2). In *H. subsaponaceum,* 30 mg L^{-1} of Zn was beneficial at the highest Cd concentration (9 mg L^{-1}), in solid media, but the same was not observed in liquid media.

In a few instances, toxicity was even more acute in the presence of both Cd and Zn, such as the dry weight decrease in *Scleroderma* sp. at 120 mg L^{-1} Zn, but only in the presence of Cd, suggesting a synergistic toxicity. Similar effect was also observed in the radial growth of *H. subsaponaceum* (Table 2.2), in which there was a decrease in the radial growth at 120 mg L^{-1} Zn in *H. subsaponaceum* for all Cd treatments, however, the dry weight was not affected in these cases. As for *Scleroderma* sp., radial growth was not negatively affected despite either Cd or Zn additions.

Contour plots were created using the Tolerance Index of the dry weight of *Scleroderma* sp. and *H. subsaponaceum* in order to visualize the different responses between the cultures grown in solid and liquid media (Figure 2.4). *Scleroderma* sp. was very sensitive to increasing Cd and Zn concentrations, but around 30 mg L^{-1} Zn it exhibited distinct tolerance (≥ 70%), even in the presence of 1 mg L^{-1} Cd and in both types of media. Despite this increment in the tolerance index caused by Zn, it is clear that higher Zn concentrations were extremely toxic to this species at higher Cd doses (Figure 2.4C). Tolerance indices were in general considerably higher in solid media, for instance, in *H. subsaponaceum* tolerance index was mostly over 50% in solid media, while in liquid media it was mainly around 40% or lower (Figure 2.4A and B).

Table 2.2. Fungal dry weight (mg) and radial growth (cm) of *Hebeloma subsaponaceum* and *Scleroderma* sp. grown in liquid and solid media containing different Cd and Zn concentrations (mean ± SE).

		H. subsaponaceum			*Scleroderma* sp.		
	Zn (mg L^{-1})	Cd (mg L^{-1})					
		0	1	9	0	1	9
Liquid media dry weight (mg)	0	11.0 ± 0.1	10.4 ± 1.1	2.7 ± 0.3	4.4 ± 1.6	2.7 ± 0.8	1.6 ± 0.1
	30	21.1 ± 3.9 a	12.0 ± 0.3	2.8 ± 0.2	9.8 ± 0.6 a	10.5 ± 0.3 a	1.3 ± 0.1
	60	11.5 ± 0.2	12.9 ± 0.3	3.4 ± 0.3	6.0 ± 0.4	2.0 ± 0.3	1.3 ± 0.1
	120	9.2 ± 1.1	9.1 ± 1.2	2.6 ± 0.2	1.6 ± 0.2	1.5 ± 0.1	1.1 ± 0.1 b
Solid media dry weight (mg)	0	10.8 ± 0.6	10.2 ± 0.8	4.5 ± 0.4	17.0 ± 2.7	16.9 ± 1.3	14.8 ± 1.3
	30	9.2 ± 0.6	10.7 ± 1.0	5.6 ± 0.1 a	18.5 ± 1.6	19.8 ± 2.9	12.2 ± 1.5
	60	9.1 ± 0.1	8.2 ± 0.2	5.5 ± 0.1	19.4 ± 1.7	14.4 ± 0.4	12.8 ± 1.0
	120	8.2 ± 0.5	7.9 ± 0.5	4.1 ± 0.3	17.3 ± 1.0	11.7 ± 0.8 b	7.9 ± 2.5 b
Solid media radial growth (cm)	0	3.1 ± 0.1	2.6 ± 0.0	1.3 ± 0.1	6.0 ± 0.1	5.9 ± 0.2	4.3 ± 0.1
	30	2.8 ± 0.2	2.5 ± 0.0	1.2 ± 0.0	6.1 ± 0.2	6.5 ± 0.2	5.0 ± 0.2
	60	2.7 ± 0.1	2.4 ± 0.1 b	1.2 ± 0.0	6.5 ± 0.1	6.4 ± 0.2	5.9 ± 0.2 a
	120	2.4 ± 0.1 b	2.3 ± 0.0 b	1.0. ± 0.0 b	6.8 ± 0.2 a	6.3 ± 0.1	4.3 ± 0.4

a - Mean values higher than the control (Zn: 0 mg L^{-1}) in each Cd treatment;

b - Mean values lower than the control; all by Dunnett's test ($p<0.05$).

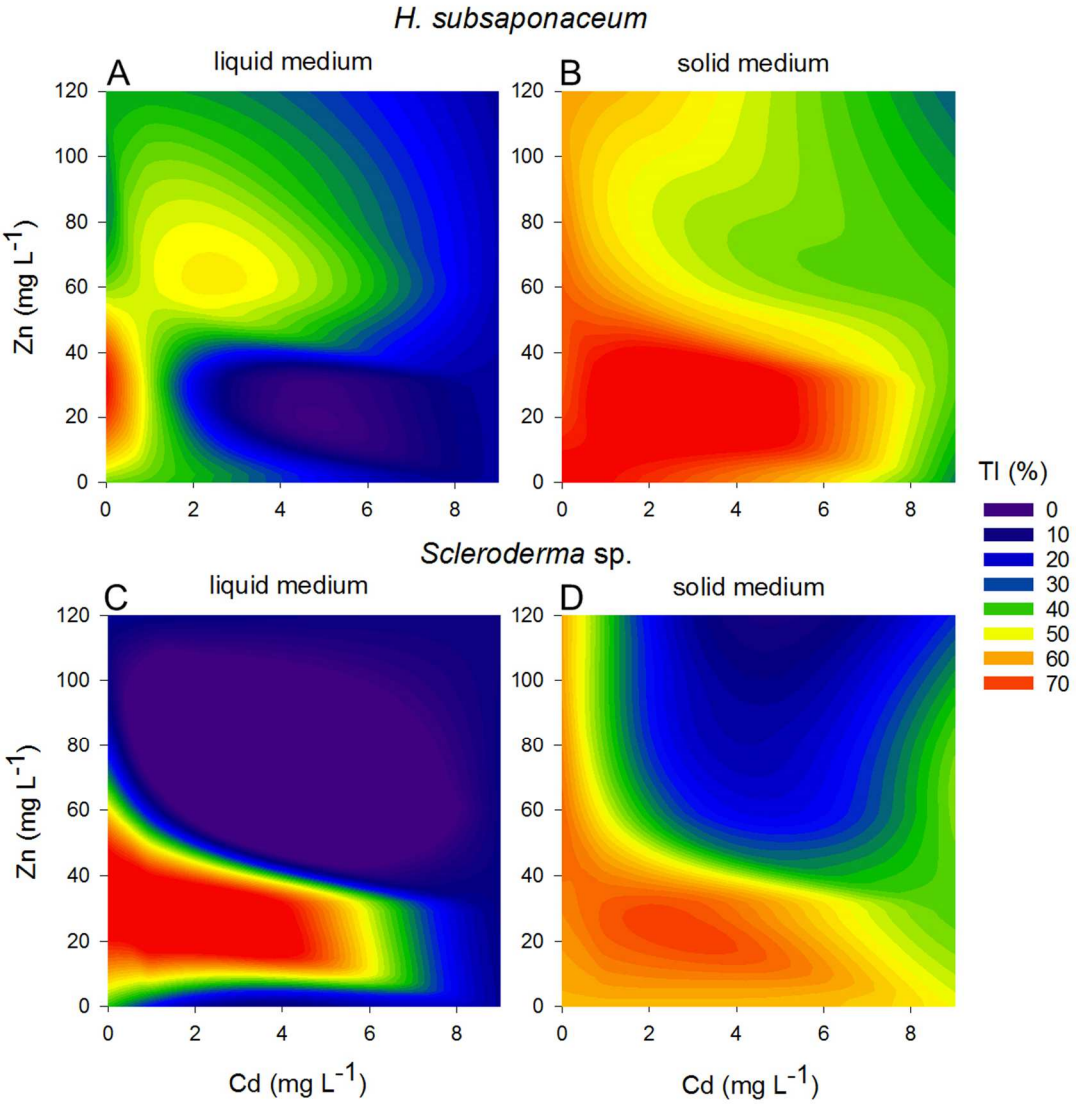


Figure 2.4 Contour plots: Tolerance indices (TI%) for *H. subsaponaceum* and *Scleroderma* sp. exposed to Cd and Zn *in vitro* in two types of Modified Melin-Norkrans media, liquid (left) and solid (right). *TI% = DW treated/DW control × 100*. The reference value (100%) was considered as the treatment which produced the most biomass (dry weight). Contour plots produced by linear interpolation. High TI% (orange and red) are associated with lower toxicity, while low TI% (purple and blue) with higher toxicity.

2.4 Discussion

Reports show that there is a great variation in Cd tolerance among ECM fungal species but generally Cd causes toxicity at around 1 mg L^{-1} *in vitro* (Colpaert and Van Assche 1992; Tam 1995; Ray et al. 2005). Our data is in keeping with this general tenet, which applies to a number of different genera, such as *Laccaria*, *Scleroderma*,

Suillus, *Pisolithus, Cenococcum, Thelephora* and *Paxillus* (Colpaert and Van Assche 1992; Tam 1995; Colpaert et al. 2000; Ray et al. 2005; Krznaric et al. 2009;). Nonetheless, in some cases Cd effects are only evident at higher concentrations, such as 50 mg L^{-1} verified in *Amanita muscaria* growing in solid MMN media (Willenborg et al. 1990), although this species is commonly known to have a high Cd tolerance (Colpaert and Van Assche 1992; Colpaert 2008). Here the highest LOAEC values for Cd were observed for *H. subsaponaceum* (3 mg L^{-1}) and *Scleroderma* sp. (9 mg L^{-1}) (Figure 2.1), both basidiomycetes frequently found on highly polluted soils in the environment (Colpaert 2008).

The low LOAEC value for *H. crustuliniforme* might be interpreted as a high sensitivity to Cd, however the Tolerance Index (TI %) clearly showed that this species had the most gradual decline in biomass of all Cd treated fungi, indicating less sensitivity to elevated Cd concentrations (Figure 2.2). For instance, at 9 mg L^{-1} Cd or more, *H. crustuliniforme* was the only species with a TI equal or higher than 20%. This fact emphasizes the importance of using more than one index for interpretations of toxicity data.

Unlike Cd, the range of Zn toxic concentrations is highly variable (generally from 10 to 500 mg L^{-1}) depending on the species, strains, or even the type of growth media (Colpaert and Van Assche 1987; Tam 1995). Blaudez et al. (2000b) verified Zn toxicity on *Suillus luteus* in solid MMN media at a concentration of 25 mg L^{-1}, while for the same species Colpaert et al. (2000) found toxicity only at 300 mg L^{-1}, but using a different growth media (solid Fries). In an experiment with ECM fungi *in vitro*, Cd^{2+} and Zn^{2+} were also considered the most toxic metals compared to Pb^{2+} and Sb^{3-} (Hartley et al. 1997b). Nonetheless, Hoiland (1995), who also tested metal toxicity in Basidiomycota, found Cd to be very toxic, but Zn only moderately toxic. Most of the species in the current study presented considerable growth at 1 mg L^{-1} Zn, however *H. crustuliniforme* had an unexpected reduction on the tolerance index, suggesting that its growth may have been influenced by other factors, such as the media itself. MMN medium usually offers effective results for ECM fungi tests, however some species display different responses to growth media depending on aspects such as nutrient composition or pH (Islam and Ohga 2013) . For example, Willenborg et al.

(1990) also found poor development of *H. crustuliniforme* in MMN media, which was almost half the growth reached by the same strain in malt extract media.

High metal concentrations exert several toxic effects in fungi and may affect almost all aspects of their metabolism and differentiation, with the cellular membrane being the initial point of action of toxicity if there is a direct contact between the metal and the cellular components (Gadd 1993). Other common effects are the inhibition of enzymes, disruption of membranes, and growth inhibition (Gadd et al. 2012). Exposure to Cd^{2+} resulted in the collapse of mitochondrial membranes in yeasts (Wang et al. 2017).

Several mechanisms of tolerance may act on alleviating metal stresses in fungi, such as increasing metal efflux; reduction of uptake, metal chelation and intracellular sequestration. Ramesh et al. (2009) identified two metallothionein genes in *H. cylindrosporum* capable of restoring the growth of transformed yeasts under Cd toxicity. Cell wall adsorption was also an important contribution in conferring tolerance, especially in the case of Cd (Galli et al. 1994; Frey et al. 2000; Bellion et al. 2006;). Sequestration into cytosolic vesicles has been shown to be a possible mechanism for Zn tolerance in *H. cylindrosporum* under sub-toxic concentrations (27 mg L^{-1} $ZnCl_2$), representing the main pool of free Zn ions in this species (Blaudez and Chalot 2011).

Yet, when exposed to solutions containing high concentrations of metals, such as in this experiment, binding sites in cell walls can be quickly saturated and become an inefficient strategy in preventing toxicity (Colpaert et al. 2011). A study in *Lentiluna edodes* showed high accumulation of Cd in mycelia after only 24h of exposure in liquid medium (Zhao et al. 2015). Therefore, the physical state of the growth media may have also been responsible for the high Cd sensitivity found in these ECM fungi. Willenborg et al. (1990), for instance, verified Cd toxicity in *H. crustuliniforme* only at 50 mg L^{-1}, but using solid MMN media, while in our study, with liquid MMN solutions, this species suffered toxicity at 1 mg L^{-1} (Figure 2.1).

When Cd and Zn were added together, the concentrations of 30 and 9 mg L^{-1} Zn resulted in biomass increase in *H. subsaponaceum* and *Scleroderma* sp., respectively, exposed to the highest Cd concentration (9 mg L^{-1}). However the

Tolerance Index (a percentage of the control biomass) was lower or the same for all Zn treatments in both species (around 80% less, compared to the control – Table S2.1). This means that although some Zn concentrations promoted fungal growth, they were not able to effectively alleviate Cd toxicity, which suggests that these metals are not sharing the same uptake pathways entirely and/or not competing for the same bonding sites in fungal tissues. However, Cd and Zn toxicity varies depending on the tolerance capacity of different species and strains (Colpaert and Van Assche 1992). Thus, another explanation for the lack of a pronounced Zn ameliorating effect is that all strains used in this assay were highly sensitive to both metals added to the media, considering they were all originated from non-contaminated land.

Despite causing negative effects in certain concentrations, Zn can also be beneficial by acting antagonistically against Cd toxicity in some ECM fungi. Krznaric et al. (2010) reported that tolerance to Cd increased significantly due to Zn additions (80-325 mg L^{-1}) in a *S. luteus* strain isolated from contaminated soil. Similar ameliorating effects were observed in other ECM fungi isolates from non-polluted areas by Hartley et al. (1997b), however a synergistic toxic effect between Cd and Zn was also described by the authors in *S. granulatus*, showing that the interactions between these metals in ectomycorrhizal fungi may occur differently inter or intra-specifically. Even ECM strains originally from polluted areas, which are regarded as more tolerant to toxicity, can suffer from combined effects of Cd and Zn toxicity (Krznaric et al. 2010).

Zn addition led to a few ameliorating effects in both species, mostly at concentrations up to 30 mg L^{-1}, however, most treatments were either unaffected by Zn, or caused toxicity in conjunction with Cd, especially at 120 mg L^{-1}. It is believed that Zn tolerance mechanisms may increase Cd tolerance when both metals are in excess (Krznaric et al. 2010); thus, if Zn tolerance is not a present trait in the ectomycorrhizal species, it is most likely that the two metals will cause synergistic toxicity instead of alleviating adverse effects. Such results support the affirmation that the toxic effects from multiple metals cannot be predicted from their individual toxicity, as the interactions between them influence their relative toxicity to ECM

fungi (Hartley et al. 1997b). Moreover, tolerance and detoxification of Zn and Cd can happen via different mechanisms. In *Pisolithus tinctorius*, Zn tolerance was conferred by binding the metal to extrahyphal slime (Tam 1995), while for Cd, vacuole compartmentation and cell wall binding were considered the main metal-detoxification mechanisms in *Paxillus involutus* (Blaudez et al. 2000a). Further investigations are still necessary to elucidate the mechanisms responsible for a possible antagonistic effect.

The fact that radial growth decreased in *H. subsaponaceum* when exposed to high Zn concentrations, but its dry weight did not differ, indicates an increase in mycelial density, which is regarded as an important mechanism to withstand metal toxicity (Hartley et al. 1997a). Such mechanism was not observed in *Scleroderma* sp. growing in solid medium, wherein radial growth was unaffected or sometimes increased in response to toxic concentrations. Although this is just one of several mechanisms governing Cd and Zn tolerance in ECM fungi, it is believed that higher density under metal stress is likely to be a significant trait in polluted soils, also affecting the degree of exposure of the plant symbiont (Colpaert et al. 2000). Furthermore, it highlights the importance of using both endpoints (dry weight and radial growth) when screening ECM fungi for metal tolerance.

As suggested earlier, the physical state of growth media can provide different results in terms of toxicity assessment. An advantage of using liquid media, is that it allows a more accurate regulation of the metal concentrations to which the organisms are exposed and is does not depend on growth form (Hartley et al. 1997a). However, screenings on solid media allow the assessment of both biomass and radial growth, which can provide more information regarding tolerance aspects, such as the increase in mycelial density observed here in *H. subsaponaceum* (Table 2.2). In addition, solid media are more likely to reflect mycelial growth in soils, for instance, basidiomycetes do not completely differentiate in liquid substrates, and this may affect their tolerance to metal toxicity (Hartley et al. 1997a). Agar media may offer lower metal bioavailability when compared to liquid media, as it is possible that complexation of metals within agar substrate occurs, masking mycelial response to toxicity (Colpaert et al. 2000), however it is also useful to avoid acute toxicity due the

exposure of highly available metals, as found in liquid media. This experiment clearly demonstrated that the patterns in Cd and Zn sensitivity changed between liquid and solid media and both *H. subsaponaceum* and *Scleroderma* sp. presented higher tolerance indices in agar (Figure 2.4). Similar effects were also reported by (Colpaert et al. 2000). The high availability of Cd^{2+} in liquid media may have been responsible for a rapid saturation of the binding sites in hyphal cell walls, which can be happen within minutes in these cases (Colpaert et al. 2011), leading to an acute Cd toxic effect.

Despite all the implications, the decision of choosing either liquid or solid media is not often addressed in metal toxicity assessments for ECM fungi in the literature. Out of 16 articles on Cd and/or Zn toxicity in ECM fungi in the past three decades, only five used liquid growth media, for which the Cd and Zn concentrations considered toxic were, in average, 2.2 mg L^{-1} and 123 mg L^{-1} (Colpaert and Van Assche 1987; Courbot et al. 2004; Grazzioti et al. 2001; Hartley et al. 1997; Tam 1995), while for the ones that utilized solid media, toxic concentrations were notably higher: in average 12 mg L^{-1} for Cd and 309 mg L^{-1} for Zn (Table 2.1).

2.5 Conclusions

In the present study, all five ECM species (*A. occidentalis, H. cylindrosporum, H. subsaponaceum, H. crustuliniforme* and *Scleroderma* sp.) tested exhibited high metal sensitivity *in vitro* conditions (liquid media), and Cd was at least 10 times more toxic than Zn, which by itself may explain why Zn had no alleviating effects in Cd toxicity. *H. subsaponaceum* and *Scleroderma* sp. were more tolerant to elevated Cd when grown in solid media compared to liquid, although in both cases higher Zn concentrations were detrimental to these species (synergism) with only a few signs of alleviating Cd toxicity (antagonism). Further research on the mechanisms underlying Zn and Cd antagonistic or synergistic interactions is needed. Additionally, Cd and Zn interactions were also affected by the type of media used, leading to different tolerance patterns, which may help explain the hitherto baffling range of previously recorded results.

A great advantage of using solid media in metal toxicity assays is that it allows the measurement of biomass as well as radial growth and, therefore, the mycelia density, which in this case appears to be a mechanism behind the higher tolerance indices found for *H. subsaponaceum* in contrast to *Scleroderma* sp. Overall, mycorrhizal symbiosis with these species could possibly lead to a better fitness of a host plant exposed to Cd or Zn in contaminated soil, and could be interesting candidates for further investigations.

2.6 References

Alloway BJ. 2013. *Heavy metals in soils*. Dordrecht: Springer.

ATSDR, Agency for Toxic Substances and Disease Registry. 2017. CERCLA Priority List of Hazardous Substances. https://www.atsdr.cdc.gov/spl/index.html. 10 Oct. 2018.

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M. 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi, *FEMS Microbiology Letters*. **254**: 173-181.

Blaudez D, Botton B, Chalot M. 2000. Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*, *Microbiology*. **146**: 1109-1117.

Blaudez D, Chalot M. 2011. Characterization of the ER-located zinc transporter ZnT1 and identification of a vesicular zinc storage compartment in *Hebeloma cylindrosporum*, *Fungal Genetics and Biology*. **48**: 496-503.

Blaudez D, Jacob C, Turnau K, Colpaert JV, Ahonen-Jonnarth U, Finlay R, Botton B, Chalot M. 2000. Differential responses of ectomycorrhizal fungi to heavy metals in vitro, *Mycological Research*. **104**: 1366-1371,

Brown MT, Wilkins DA. 1985. Zinc tolerance of *Amanita* and *Paxillus, Transactions of the British Mycological Society*. **84**: 367-369.

Chen SH, Tibbett M. 2007. Phosphate supply and arsenate toxicity in ectomycorrhizal fungi, *Journal of Basic Microbiology*. **47**: 358-362.

Colpaert JV. 2008. Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi. In: Avery SV, Stratford M, Van West P, eds., *Stress in yeasts and filamentous fungi*. Amsterdam: Academic Press, p. 157-174.

Colpaert JV, Adriaensen K, Muller LAH, Lambaerts M, Faes C, Carleer R, Vangronsveld J. 2005. Element profiles and growth in Zn-sensitive and Zn-resistant Suilloid fungi, *Mycorrhiza* **15**: 628-634.

Colpaert JV, Muller LAH, Lambaerts M, Adriaensen K, Vangronsveld J. 2004. Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi, *New Phytologist* **162**: 549-559.

Colpaert JV, Van Assche JA. 1987. Heavy metal tolerance in some ectomycorrhizal fungi, *Functional Ecology* **1**: 415-421.

Colpaert JV, Van Assche JA. 1992. The effects of cadmium and the cadmium-zinc interaction on the axenic growth of ectomycorrhizal fungi, *Plant and Soil*. **145**: 237-243.

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J. 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*, *New Phytologist*. **147**: 367-379.

Colpaert JV, Wevers JHL, Krznaric E, Adriaensen K. 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution, *Annals of Forest Science* **68**: 17-24.

Courbot M, Diez L, Ruotolo R, Chalot M, Leroy P. 2004. Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*, *Applied and Environmental Microbiology.* **70**: 7413-7417.

Denny HJ, Wilkins DA. 1987. Zinc tolerance in *Betula* spp. III Variation in response to zinc among ectomycorrhizal associates, *New Phytologist*. **106**: 545-553.

Fomina MA, Alexander IJ, Colpaert JV, Gadd GM. 2005. Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi, *Soil Biology and Biochemistry* **37**: 851-866.

Frey B, Zierold K, Brunner I. 2000. Extracellular complexation of Cd in the Hartig net and cytosolic Zn sequestration in the fungal mantle of *Picea abies-Hebeloma crustuliniforme* ectomycorrhizas, *Plant, Cell and Environment*. **23**: 1257-1265.

Gadd GM. 1993. Interactions of fungi with toxic metals, *New Phytologist*. **124**: 25-60.

Gadd GM, Rhee YJ, Stephenson K, Wei Z. 2012. Geomycology: metals, actinides and biominerals, *Environmental Microbiology Reports*, **4**:270-296.

Galli U, Schüepp H, Brunold C. 1994. Heavy metal binding by mycorrhizal fungi, *Physiologia Plantarum*. **92**: 364-368.

Grazzioti PH, Siqueira JO, Moreira FM, Carvalho D. 2001. Effect of Zn, Cd and Cu on the

behavior of ectomycorrhizal fungi in culture media, *Revista Brasileira de Ciência do Solo*. **25**: 831-837 (in Portuguese).

Hartley J, Cairney JWG, Meharg AA. 1997. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? *Plant and Soil*. **189**: 303-319.

Hartley J, Cairney JWG, Sanders FE, Meharg AA. 1997. Toxic interactions of metal ions (Cd^{2+}, Pb^{2+}, Zn^{2+} and Sb^{3-}) on in vitro biomass production of ectomycorrhizal fungi, *New Phytologist*. **137**: 551-562.

Hoiland K. 1995. Reaction of some decomposer basidiomycetes to toxic elements, *Nordic Journal of Botany*. **15**: 305-318.

Islam F, Ohga S. 2013. Effects of media formulation on the growth and morphology of ectomycorrhizae and their association with host plant, *ISRN Agronomy 2013*: 317903.

Kabata-Pendias A, Pendias H. 2001. *Trace elements in soils and plants*. Boca Raton: CRC Press.

Karaduman AB, Atli B, Yamac M. 2012. An example of comparison of storage methods of macrofungus cultures: *Schizophyllum commune*, *Turkish Journal of Botany*. **36**: 205-212.

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.

Krpata D, Peintner U, Langer I, Fitz WJ, Schweiger P. 2008. Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site, *Mycological Research.* **112**: 1069-1079.

Krznaric E, Verbruggen N, Wevers JHL, Carleer R, Vangronsveld J, Colpaert JV. 2009. Cd-tolerant *Suillus luteus*: a fungal insurance for pines exposed to Cd, *Environmental Pollution*. **157**: 1581-1588.

Krznaric E, Wevers JHL, Cloquet C, Vangronsveld J, Vanhaecke F, Colpaert JV. 2010. Zn pollution counteracts Cd toxicity in metal-tolerant ectomycorrhizal fungi and their host plant, *Pinus sylvestris*, *Environmental Microbiology*. **12**: 2133-2141.

Lei M, Zhang Y, Khan S, Qin PF, Liao BH. 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.

Leyval C, Turnau K, Haselwandter K. 1997. Effect of heavy-metal pollution on mycorrhizal

colonization and function: physiological, ecological and applied aspects, *Mycorrhiza*. **7**: 139-153.

Marx DH, Bryan WC. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*, *Forest Science*. **21**: 245-254.

Moffett BF, Nicholson FA, Uwakwe NC, Chambers BJ, Harris JA, Hill TCJ. 2003. Zinc contamination decreases the bacterial diversity of agricultural soil, *FEMS Microbiology Ecology*. **43**: 13-19.

Pridham TG, Gottlieb D. 1948. The utilization of carbon compounds by some actinomycetales as an aid for species determination, *Journal of Bacteriology*. **56**: 107-114.

Quinn GP, Keough MJ. 2002. *Experimental design and data analysis for biologists*. Cambridge: Cambridge University Press.

Ramesh G, Podila GK, Gay G, Marmeisse R, Reddy MS. 2009. Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*, *Applied and Environmental Microbiology*. **75**: 2266-2274.

Ray P, Tiwari R, Reddy UG, Adholeya A. 2005. Detecting the heavy metal tolerance level in ectomycorrhizal fungi in vitro, *World Journal of Microbiology & Biotechnology*. **21**: 309-315.

Rodriguez R, Redman R. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis, *Journal of Experimental Botany*. **59**: 1109-1114.

Ross SM, Kaye KJ. 1994. The meaning of metal toxicity in soil-plant systems. In: Ross SM, ed., *Toxic metals in soil-plant systems*. West Sussex: John Wiley & Sons Ltd, p. 28-61.

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*. New York: Springer, p. 225-240.

Tam PCF. 1995. Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*, *Mycorrhiza*. **5**: 181-187.

Tibbett M, Sanders FE, Cairney JWG, Leake JR. 1999. Temperature regulation of extracellular proteases in ectomycorrhizal fungi (*Hebeloma* spp.) grown in axenic culture, *Mycological Research*. **103**: 707-714.

Wang X, Yi M, Liu H, Han Y, Yi H. 2017. Reactive oxygen species and Ca^{2+} are involved in cadmium-induced cell killing in yeasts cells, *Canadian Journal of Microbiology* **63**: 153-159.

Willenborg A, Schmitz D, Lelley J. 1990. Effects of environmental stress factors on ectomycorrhizal fungi in vitro, *Canadian Journal of Botany*. **68**: 1741-1746.

Zar JH. 2010. *Biostatistical analysis*. Upper Saddle River: Prentice Hall.

Zhao L, Ye JG, Li HB, Yang H, Ke LQ, Liang QL. 2015. Identification of early response genes involved in cadmium resistance in shiitake mushrooms (*Lentinula edodes*), *Mycological Progress*. **14**: 114.

Zheng W, Fei Y, Huang Y. 2009. Soluble protein and acid phosphatase exuded by ectomycorrhizal fungi and seedlings in response to excessive Cu and Cd, *Journal of Environmental Sciences*. **21**: 1667-1672.

2.7 Supplementary Information

Table S2.1 - Average tolerance index (%) based on the dry weight (DW) of four ectomycorrhizal fungi grown in liquid media containing different combinations of Cd and Zn doses (n = 4).

	Austroboletus occidentalis			*Hebeloma crustuliniforme*			*Hebeloma subsaponaceum*			*Scleroderma* sp.		
Zn (mg L^{-1})	---------- Cd (mg L^{-1}) ----------											
	0	1	9	0	1	9	0	1	9	0	1	9
0	100	20	16	100	91	84	100	27	9	100	85	19
1	90	14	16	104	84	82	129	28	10	94	89	20
9	74	16	14	95	86	84	131	20	11	155	89	23
30	62	18	16	63	77	69	136	36	13	139	99	22

TI(%) = (DW-treated / DW-control) x 100

Chapter 3

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

Early development of P. Trichocarpa cuttings under Cd and Zn stress

Abstract

Metal inputs to terrestrial ecosystems are of great concern due to their toxicity to biota, especially for elements with no biological function such as cadmium. Fast-growing trees such as poplars may have potential in phytoremediation schemes.

We assessed accumulation, metal partitioning, gene expression (*Pt-HMA4*) and overall tolerance to, and interaction between, cadmium (Cd) and zinc (Zn) in *Populus trichocarpa* 'Trichobel'. We predicted that Zn would have an antagonistic effect in Cd accumulation and anticipated some level of tolerance to these metals. Poplars were grown in sandy substrate under different metal applications, ranging from 1 to 243 mg kg^{-1} Cd; or 30 to 7,290 mg kg^{-1} Zn; and also two combined treatments: 27 mg kg^{-1} Cd with 90 or 270 mg kg^{-1} Zn. Growth parameters and metal contents in shoots and roots were determined. Transcriptional levels of the *Pt-HMA4* gene were assessed in roots and leaves.

P. trichocarpa showed a surprisingly high tolerance to Cd, with root biomass being affected only at the highest doses applied. Metals accumulated mainly in roots (up to 6,537 mg kg^{-1} Cd and 21,500 mg kg^{-1} Zn), root-to-shoot translocation peaked at the 9 mg kg^{-1} dose for Cd (53%) and 90 mg kg^{-1} for Zn (40%). At high Cd/Zn applications, expression of *Pt-HMA4* in roots decreased significantly. Contrary to the initial presumption, Zn addition increased Cd uptake, reaching hyperaccumulator-like concentrations in shoots (≥ 100 mg kg^{-1} Cd).

Differential root-to-shoot partitioning has a major role in Cd tolerance in *P. trichocarpa*; partly by down-regulating the *Pt-HMA4* gene in roots. Zn addition promoted high Cd uptake without any detriment to plant growth. *P. trichocarpa* was tolerant to extreme Cd concentrations, offering a great potential to be used in phytoremediation techniques for stabilization/extraction of Cd from soils contaminated by both Cd and Zn.

3.1 Introduction

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

Phytoremediation is the use of plants and their associated microorganisms for environmental decontamination (Pilon-Smits 2005), from which phytoextraction is considered to be useful for inorganic contaminants (Marmiroli et al. 2006). It is an *in situ* technique that preserves soil structure and microbial activity, offering protection against erosion (Pulford and Watson 2003; Guerra et al. 2011). Poplars (*Populus* sp.) are trees widely considered for phytoextraction of several metals, such as Cd, Zn, Pb and Cu (Castiglione et al. 2009; Zacchini et al.

2009; Guerra et al. 2011; Dai et al. 2013; Luo et al. 2016), mainly due to their biomass production, deep root systems (Bhargava et al. 2012), tolerance to high metal concentrations and fast growth (Robinson et al. 2009). *Populus* species can also rapidly invade disturbed sites, reproduce asexually (Sebastiani et al. 2004; Hamberg et al. 2011) and are not a source of food for farm animals, reducing the risk of heavy metals entering the human food chain (Shim et al. 2013).

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

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

3.2 Materials and Methods

3.2.1 Plant material and pre-growth

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

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

3.2.2 Treatments and Experimental Design

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

solutions, and all pots contained only one poplar cutting. Metals were added in a single dose and each treatment had four replicates arranged in blocks.

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

3.2.3 Harvest and Phytotoxicity assessment

After exposure to the toxic metals for five weeks, all plants had their living expanded leaves counted and stems measured (before and after exposure to metals). Visual toxicity symptoms recorded using the method described by Kariman et al., (2016), in which leaf areas with symptoms such as discoloration, chlorosis or necrosis were ranked into 6 classes (0 to 5), in which 0 represents no toxicity symptoms, 1 is up to 20% of symptomatic leaf tissue area (SLTA), 2 from 20 to 40%, 3 from 40 to 60%, 4 from 60 to 80% and 5 for symptomatic area greater than 80%. Two mature leaves were assessed for each plant, and the final scoring was the average between those leaves.

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

3.2.4 Acid Digestion and Metal Determination

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

3.2.5 Bioconcentration Factor, Translocation Factor and Tolerance Index

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

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

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

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

3.2.6 PtHMA4 expression in roots and leaves

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

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

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

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

The qPCR reactions were performed in duplicates for each sample using PowerUp™ SYBRGreen™ (Applied Biosystems, UK) with the following the parameters: 1 cycle of 2 min at 50^{o}C followed by 2 min at 95^{o}C (DNA polymerase activation), then 40 cycles of 95^{o}C for 3 seconds (denaturation) and 60^{o}C for 30

seconds (annealing/extension). Primer specificity was verified by electrophoresis and confirmed by melt curve analyses. The qPCR run, data collection and analyses were performed using StepOne™ Real-Time PCR System (Applied Biosystems). Results were analysed by the standard curve method, and gene expression was normalised using *UBQ* as the house keeping gene.

3.2.7 Statistical Analyses

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

3.3 Results

3.3.1 Growth, biomass production and transpiration rate

Both Cd and Zn caused toxicity in *P. trichocarpa* plants after only five weeks of exposure, and the visual effects are evident in shoots and roots (Figure 3.1 and 3.2), especially in Zn treatments.

P. trichocarpa exhibited a considerable tolerance to Cd toxicity, and negative effects were significantly different from control only at the extreme concentration of 243 mg kg^{-1}, except for leaf biomass, which was also affected at 81 mg kg^{-1} Cd. Nonetheless, the total biomass produced (leaves + stems + roots) was similar in all

Cd treatments except for the highest dose of 243 mg kg^{-1} Cd (Table 3.1). Zn toxic effects were detected at the lowest dose applied, of 30 mg kg^{-1}, which reduced leaf and shoot biomass (Table 3.1), although root biomass was unaffected in this treatment. Zn concentrations from 30 to 270 mg kg^{-1} caused comparable toxicity in *P. trichocarpa*, as seen in the total plant biomass produced, but further toxicity was observed at higher concentrations.

Figure 3.1 - Phytotoxic effects of Cd and Zn in *Populus trichocarpa* at different soil concentrations, after five weeks of exposure.

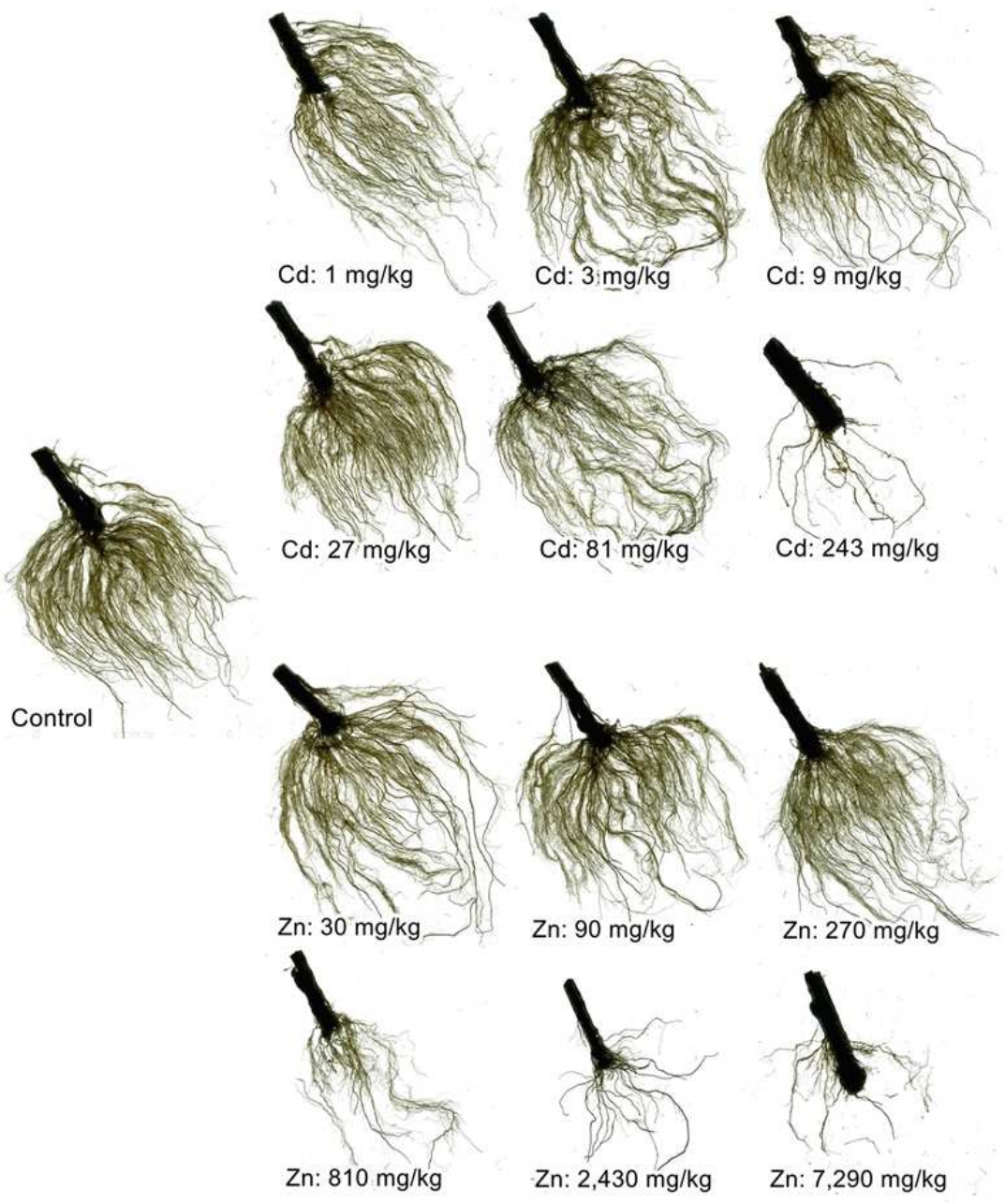


Figure 3.2 - Root scans of *Populus trichocarpa* exposed to different Cd and Zn concentrations during five weeks. Images were used for length, area, volume and diameter analyses.

Table 3.1 Dry biomass production, resulting pH and translocation index (TI) of *P. trichocarpa* exposed to different Cd or Zn concentrations during five weeks.

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

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

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

Initial pH: 6.9;

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

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

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

Foliar symptoms of phytotoxicity were more evident in Cd treatments than in Zn treatments, when compared to control at lower concentrations, 30 to 270 mg kg^{-1} Zn (Figure 3.3). All treatments displayed marginal necrosis in the leaves assessed (older leaves), including the control, but chlorosis and discoloration were present only in Cd-treated plants. Although necrosis and chlorosis were both

considered for the toxicity scoring, chlorosis symptoms were predominantly in Cd treatments. At the highest Zn concentrations (2430 and 7290 mg kg^{-1}) all leaves were scored as a 5, due to extensive foliar necrosis (Figure 3.1).

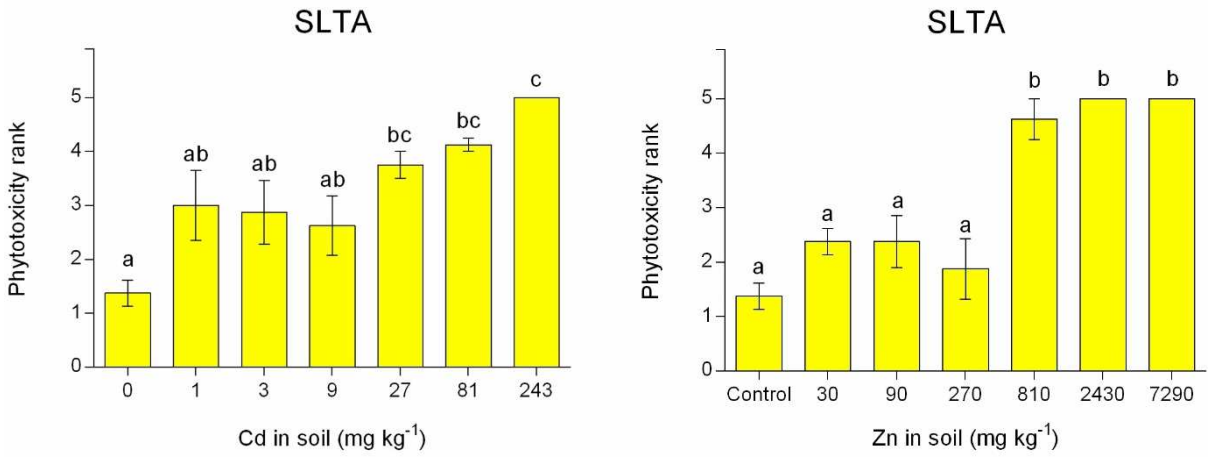


Figure 3.3 - Toxicity ranks of *P. trichocarpa* exposed to different Cd and Zn concentrations. Symptomatic leaf tissue area (SLTA) was assessed visually and scored from 0 to 5 (each score represent 20% of leaf area). Significant differences are represented by different letters by Tukey test ($p < 0.05$) in Cd treatments and Dunn's test ($p < 0.05$) in Zn treatments.

Root scanning allowed the determination of root total length, area, volume and diameter for *P. trichocarpa* grown in different Cd and Zn concentrations. Results for root morphology parameters, leaf transpiration and stomatal conductance can be found in Table S3.1. Roots under Cd treatments displayed a similar response as the other parameters assessed, with evident toxicity effects only at the highest concentration of 243 mg kg^{-1}. In the case of Zn, length, area and volume reduction of roots was caused mainly at 810 mg kg^{-1} or higher concentrations. As for the analyses of stomatal conductance (gs) and transpiration rate (E), there were no significant differences among Zn treatments (Control – 810 mg kg^{-1}) or among Cd treatments, except for the highest concentration of 243 mg kg^{-1}, in which there was a reduction in the transpiration rate (E) in comparison to the control, from 2.65 to 0.48 mmol m^{-2} s^{-1}, and in stomatal conductance (gs), from 0.084 to 0.008 mol m^{-2} s^{-1} (Tukey test, $p = 0.0009$ and $p = 0.0004$, respectively).

3.3.2 Cadmium and zinc uptake, accumulation and translocation

Cd uptake in poplar roots increased almost exponentially and was at least 10 times the concentration applied in some treatments (1 to 9 mg kg^{-1} Cd) (Figure 3.4; Table S3.4). In leaves, an increasing uptake is observed only until the concentration of 9 mg kg^{-1} Cd, after which there is a plateau and Cd concentration is maintained around 50 mg kg^{-1} (Figure 3.4). However, in the treatment with 243 mg kg^{-1}, Cd accumulation surpasses the plateau concentration by more than 10 times (from an average of 45 to 681 mg kg^{-1}). The bioconcentration factor (BCF) shows a decrease in Cd accumulation in roots as concentrations in substrate increases (Table 3.2), except at the highest concentration which had a BCF of 47.6 in poplar roots (tissue concentration of 6,537 mg kg^{-1} Cd), suggesting a loss of regulation in Cd uptake and excessive metal accumulation (Figure 3.4). Overall the concentration of 9 mg kg^{-1} Cd appears to be the threshold in Cd translocation from roots to shoots (T*f* = 53%, the highest in this study), after which the ratio between root and leaf concentration was reduced almost by half (T*f* = 27% at 27 mg kg^{-1} Cd). At the applied dose of 81 mg kg^{-1} Cd, root biomass was not affected despite tissue concentrations reaching nearly 500 mg kg^{-1} Cd (Table 3.1 and Figure 3.4).

Unlike with Cd, Zn content in roots did not differ significantly at lower soil concentrations (≤ 90 mg kg^{-1}), increasing only after 270 mg kg^{-1} Zn (Figure 3.4). Zn content in leaves was a direct result of the concentration applied, although only a slight increase was observed between treatments of 810 and 2430 mg kg^{-1} Zn.

Zn accumulation in roots varied across all treatments, and the highest BCF was found at 30 mg kg^{-1}, and lowest at 7290 mg kg^{-1} (Table 3.2). Considering the tolerance indexes, 90 mg kg^{-1} Zn was the threshold for toxicity in both poplar roots and shoots (Table 3.1). Interestingly, this treatment showed a translocation factor of 40%, nearly the same factor found at the Cd threshold concentration of 9 mg kg^{-1}.

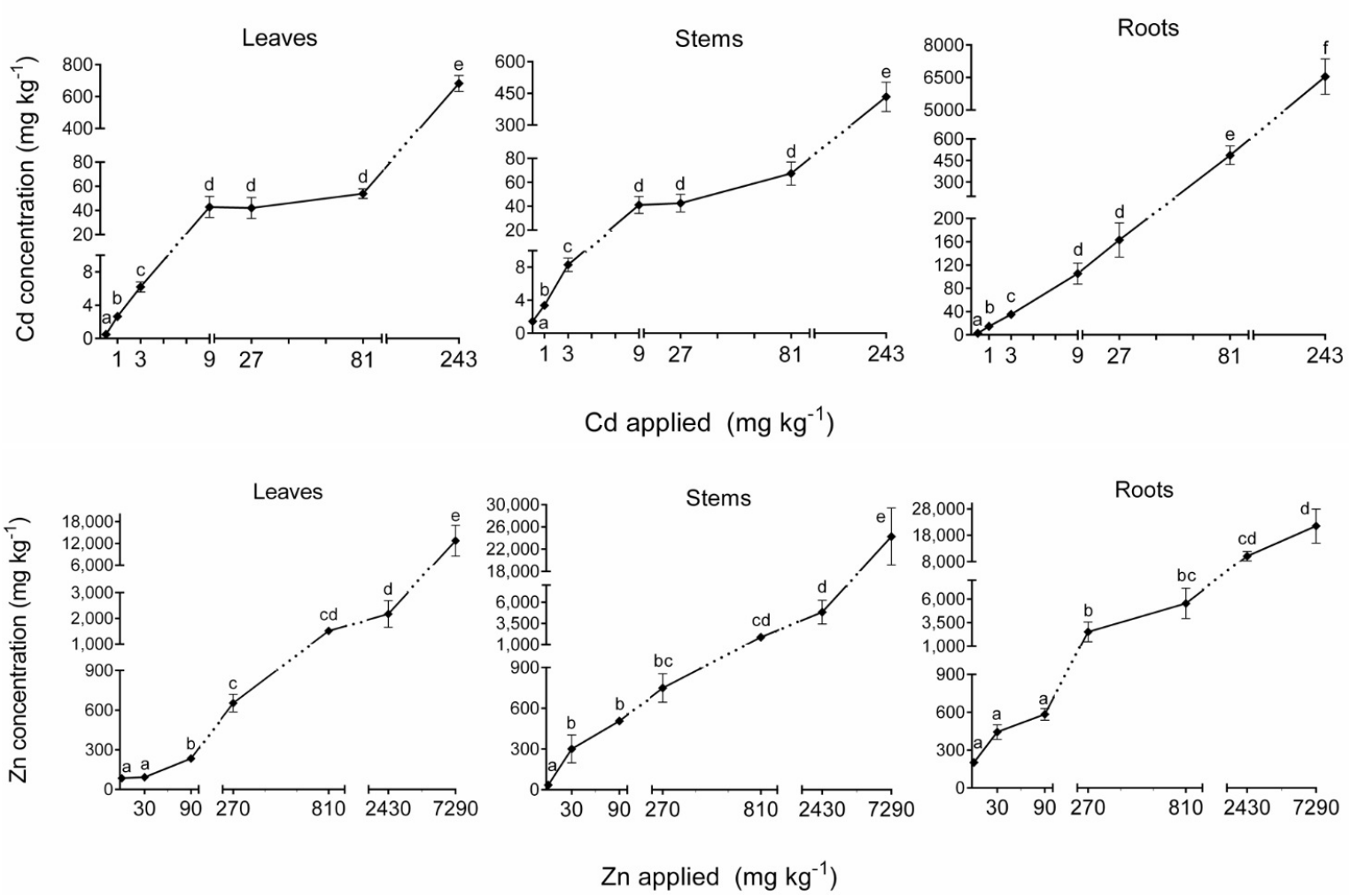


Figure 3.4 - Cd and Zn concentrations (mg kg^{-1}) in leaves, stems and roots of *P. trichocarpa* grown for five weeks in sandy substrate at different Cd or Zn doses. Error bars indicate standard error of the mean (n = 4).
Different letters correspond to significant differences between doses applied (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 S3.4 and S3.5.

Table 3.2 Total metal uptake, translocation factor (*Tf*: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* 'Trichobel' grown for five weeks under different Cd and Zn doses.

Cd (mg kg^{-1})	Cd uptake (µg $plant^{-1}$)	T*f*	BCF	
			Leaf	Root
Control	3.2 ± 0.3	21 ± 6	---	---
1	14.8 ± 3.1	20 ± 3	2.6	14.4
3	31.0 ± 5.2	18 ± 3	2.1	11.7
9	119 ± 11	53 ± 24	4.8	11.7
27	167 ± 22	27 ± 6	1.6	6.0
81	267 ± 52	11 ± 1	0.7	6.0
243	629 ± 157	6 ± 1	2.8	47.6
Zn (mg kg^{-1})	Zn uptake (mg $plant^{-1}$)	T*f*	BCF	
			Leaf	Root
Control	0.3 ± 0.01	33 ± 6	---	---
30	0.5 ± 0.1	21 ± 2	3.1	14.8
90	0.9 ± 0.1	41 ± 6	2.6	6.5
270	2.0 ± 0.2	26 ± 12	2.4	9.3
810	2.5 ± 0.5	33 ± 10	1.9	6.9
2,430	3.5 ± 1.1	21 ± 1	0.9	4.2
7,290	17.9 ± 2.2	59 ± 26	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.

Cd concentration in leaves and roots had an inverse relationship with all other variables. Stomatal conductance (gs) and transpiration rates (E) had a lower correlation to almost all other parameters assessed (especially root parameters), however both variables were highly correlated (r_s > 0.70) to the number of leaves (NL) and shoot growth (SG) (Table S3.2). Overall Zn treatments had a similar correlation among all the parameters assessed to Cd treatments with almost no correlations between E and gs and other variables (Table S3.3).

3.3.3 Expression of PtHMA4 under Cd and Zn stress

Specific amplification of *Pt-HMA4* (POPTR_0006s07650g) was obtained from the designed primers (product length: 130 bp). In the control, *Pt-HMA4* expression was five times higher in roots than in leaves (t-test, p = 0.043), but this variation between tissues were not observed in contaminated treatments. Exposure to either Cd or Zn down-regulated *Pt-HMA4* expression in roots by 2.9-fold and 2.6-fold respectively (Figure 3.5). No differences in transcript levels were found in leaves. Ubiquitin (*UBQ*) was used for normalisation of HMA4 results due to their homogeneous expression across treatments (Control, Cd and Zn): ANOVA, p = 0.768 (leaves) and p = 0.781 (roots).

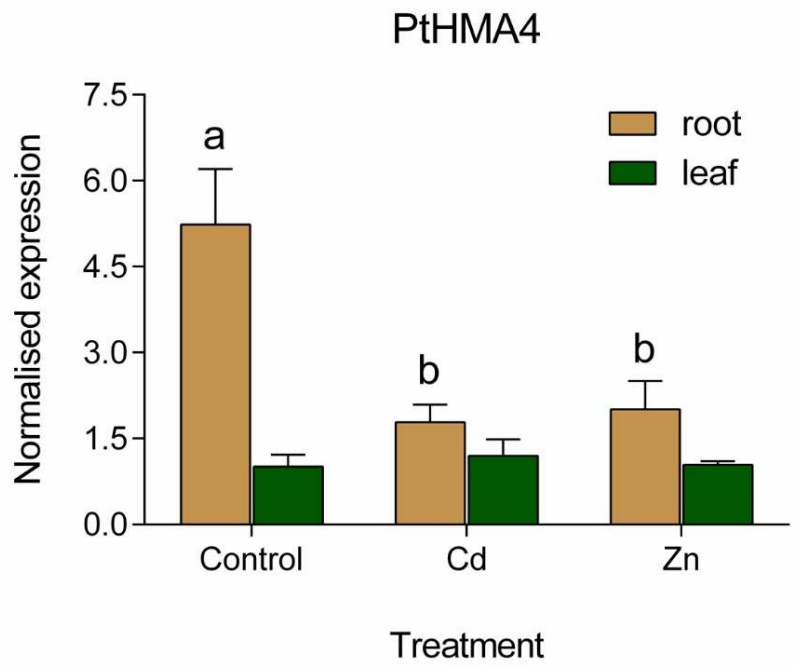


Figure 3.5 Transcript levels of the PtHMA4 gene in roots and leaves of *P. trichocarpa* after growing for eight weeks under Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) stress, and without any metal addition (Control). The mRNA levels were quantified by real-time qPCR and normalised in relation to Ubiquitin (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 (error bars: standard error), determined by Tukey test after ANOVA (p = 0.0167). There were no differences among treatments in leaf tissues.

3.3.4 Cd and Zn interactions and uptake

Biomass production in treatments with combined metal applications did not significantly change from the control nor their corresponding single metal treatments: 27 mg kg^{-1} Cd; or 90 and 270 mg kg^{-1} Zn. For instance, the Tolerance Index (TI) for total biomass was 100% for 27 + 90 mg kg^{-1} Cd Zn, and 83% for 27 + 270 mg kg^{-1} Cd Zn; percentages are related to the non-contaminated control. The same results were observed for root morphology, leaf transpiration and stomatal conductance (data not shown). Despite exhibiting the same tolerance patterns, Zn addition increased Cd uptake, for instance, leaf concentration was of 123 mg kg^{-1} in Cd_{27} + Zn_{90}, almost three times higher than the concentration found when Cd was added singly (Cd_{27}), of 42 mg kg^{-1} (Figure 3.6). Stems and roots also presented higher Cd contents after Zn addition, regardless of Zn concentration. Zn uptake was not affected in the presence of Cd: leaf, stem and root concentrations were not different from when Zn was added separately (Zn_{90} and Zn_{270}) (Figure 3.6).

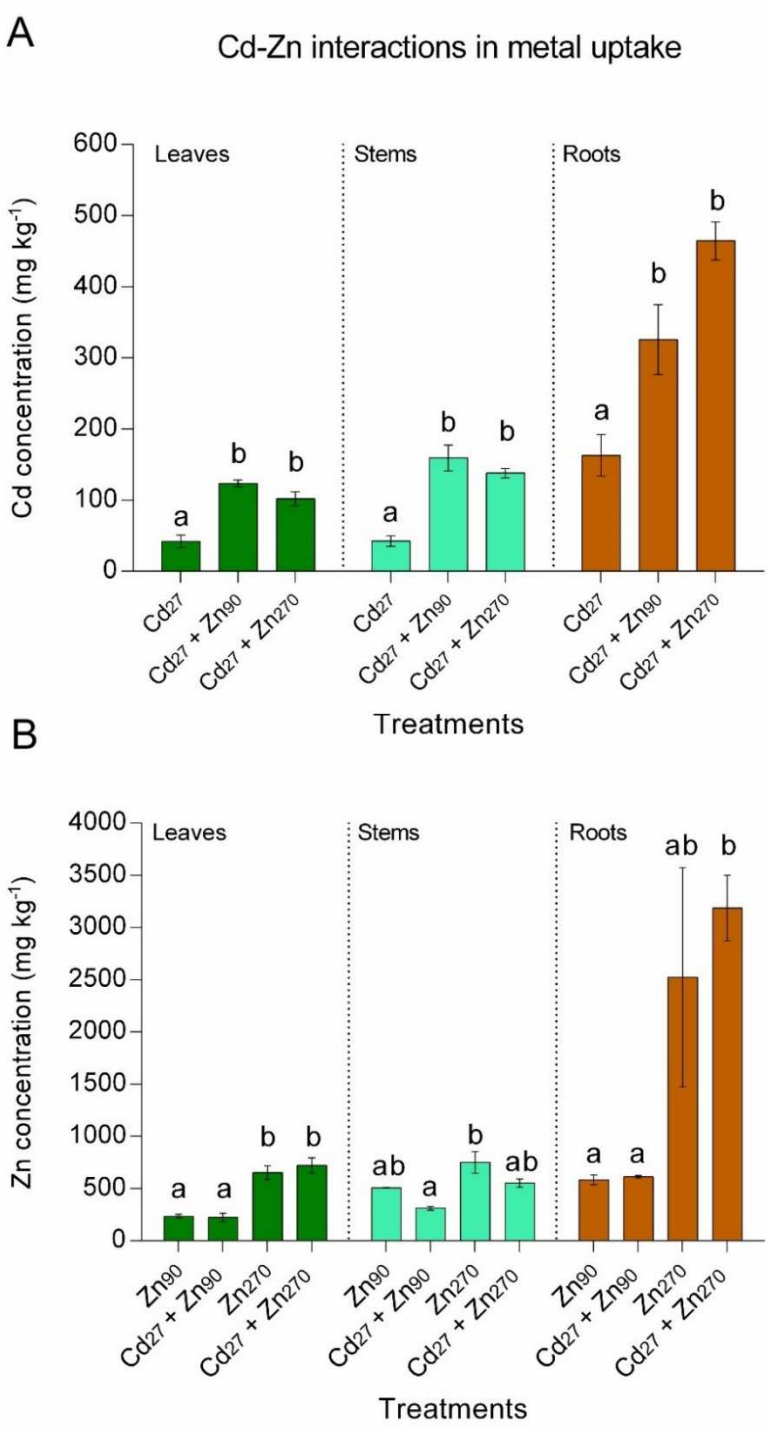


Figure 3.6 Concentrations of Cd (A) and Zn (B) in leaves, stems and roots of *Populus trichocarpa* exposed to different metal combinations: 27 mg kg^{-1} Cd, 90 or 270 mg kg^{-1} Zn. Different letters correspond to significant differences among treatments for the same plant tissue, Tukey test, $p < 0.05$ (A) and Dunn's test, $p < 0.05$ (B).

3.4 Discussion

3.4.1 Cadmium accumulation, distribution and toxicity

Exposure to Cd often leads to oxidative stress and phytotoxicity (Benavides et al. 2005) as a result of Cd replacing other essential elements (e.g. Ca, Fe, Mg and Zn) in enzymes, which usually lose their function (Clemens 2006; Verbruggen et al. 2009; Kupper and Andresen 2016). Growth impairment is a typical effect of

Cd toxicity (Pal et al. 2006), biomass decrease in roots and shoots are commonly reported (Tran and Popova 2013), as well as foliar chlorosis and necrosis (Das et al. 1997); although usually shoots are more sensitive to Cd toxicity than roots (Table 3.3). In the current experiment, despite *P. trichocarpa* showing symptoms of toxicity in leaves under Cd exposure, particularly at soil concentrations higher than 27 mg kg^{-1} Cd, loss of biomass was not evident in most of the treatments. Only at the highest concentration did all roots, stems and leaves present obvious toxic effects, indicating a remarkable tolerance to Cd in comparison to other published studies which often report Cd toxicity ranging from 2.2 to 50 mg kg^{-1} (L^{-1}) Cd (Table 3.3). According to Audet and Charest (2008), plants from the Brassicaceae family, known for their high tolerance to metals, tend to maintain a constant biomass allocation to roots despite exposure to higher metal concentrations in soils, similar to that observed for poplars exposed to Cd in the present study, suggesting that in both cases metal partitioning plays a larger role in tolerance than does biomass plasticity.

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

Cadmium accumulated mainly in the roots, as it is reported in most studies on poplars (Dos Santos Utmazian et al. 2007; Zacchini et al. 2009; Di Lonardo et al. 2011) or other plant species (Obata and Umebayashi 1997; Green and Tibbett 2008; Lux et al. 2011); while stems and leaves had generally the same

concentrations. Despite much higher Cd accumulation, the roots of *P. trichocarpa* were as tolerant as its aboveground parts for most treatments (TI of 102-74%).

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

At 9 mg kg^{-1} the high translocation of Cd to aboveground parts (T*f:* 53%) is considered to be a common mechanism of hyperaccumulators, in which the metal is detoxified by chelation, vacuole storage and rapidly translocation to shoots via the xylem (Tran and Popova 2013). However, at 81 kg kg^{-1}, there is a much higher Cd uptake in roots, which is a reflection of the non-specific mechanisms by which Cd enters the plant system (Lux et al. 2011), thus in order to avoid toxicity in the photosynthetic apparatus, there is a limited transport of Cd to the shoots (T*f*: 11%). Restricting root-to-shoot translocation is a strategy typical of woody species that may contribute to metal tolerance (Arduini et al. 1996) since the first important barrier against Cd toxicity is the immobilization in cell walls in roots (Sanita di Toppi and Gabbrielli 1999). Lower translocation of Cd to shoots can be due to different mechanisms, such as down-regulation of transporter proteins (i. e. heavy metal ATPases and ABC transporters) responsible for Cd loading in the xylem or increasing production of metal chelators (Lux et al. 2011). Lignification of cortical cells, sclerenchyma walls and vascular tissues can also be triggered by Cd

(Luković et al. 2012; Kupper and Andresen 2016; Tylova et al. 2017), which may contribute to the thickening of the Casparian bands in the root apex (Schreiber et al. 1999; White 2001) where high influx of Cd^{2+} occurs (He et al. 2011).

Table 3.3 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. The "lowest observed adverse effect concentration" (LOAEC) shows the lowest Cd or Zn concentration to significantly cause toxicity (in some cases data was extracted from figures). The letter 'x' corresponds to cases in which no toxicity was detected.

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

Table 3.3 Cont.

Populus species	Growth substrate	Metal concentration	Phytotoxicity	LOAEC	Ref.
P. euramericana	soil	8.14 Cd	photosynthesis	8.14 Cd	16
	inert clay	0 – 650 Zn	overall biomass	327 Zn	18
	inert clay	0 – 650 Zn	overall biomass	327 Zn	19
	vermiculite	65 and 650 Zn	biomass, leaf area	65 Zn	20
	nutr. sol.	0, 0.1 and 11 Cd*	root biomass	0.1 Cd	21
	soil + waste	10,300 Zn; 5.5 Cd	x	x	17
P. nigra	soil	1,760 Zn; 32.7 Cd	x	x	22
	soil	300 Zn	shoot height, root biomass	300 Zn	5
	nutr. sol.	5.6 Cd	leaf biomass	5.6 Cd	23
	nutr. sol.	5.6 Cd	overall biomass	5.6 Cd	24
	nutr. sol.	5.6 Cd	root length, leaf area	5.6 Cd	25
P. pyramidalis	soil	0 – 100 Cd	leaf biomass	25 Cd	26
P. tremula	soil	1,760 Zn; 32.7 Cd	x	x	22
	nutr. sol.	2.24 Cd	overall biomass	2.24 Cd	27
	nutr. sol.	2.24 Cd	shoot growth	2.24 Cd	28
	soil	3,000 Zn	x	x	29
P. trichocarpa	nutr. sol.	5.6 Cd	x	x	25
	sand + vermic.	0 – 243 Cd	leaf biomass	81 Cd	current study
	sand + vermic.	0 – 7,290 Zn	leaf, stem biomass	30 Zn	current study
Populus sp.	soil	60 – 486 Zn; 0.05 – 1.6 Cd	x	x	30

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

3.4.2 Zn accumulation, distribution and toxicity

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

Zn toxicity varies considerably among poplar species, but generally a decrease in biomass starts at soil concentrations of 300 mg kg^{-1} Zn (Table 3.3). Di Lonardo et al., (2011) found no effects from 130 mg L^{-1} on shoot biomass of three different *P. alba* varieties *in vitro*, although root biomass in one case decreased by 85% at only 65 mg L^{-1}. In our study, the shoots of *P. trichocarpa* were more sensitive to Zn than the roots, which only presented biomass loss at higher concentrations (≥ 810 mg kg^{-1} Zn). Root tolerance is an important feature in plants exposed to toxic metals, for it implies preservation of cell membranes selectivity properties, the initial step in uptake and xylem loading (Zacchini et al. 2009). Roots accumulated more Zn than the leaves, which is in accordance to some studies in poplars (Dos Santos Utmazian and Wenzel 2007; Romeo et al. 2014), although other poplar species have demonstrated significantly higher Zn contents in leaves

(Lingua et al. 2008; Castiglione et al. 2009; Cicatelli et al. 2010; Todeschini et al. 2011).

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

3.4.3 PtHMA4 is down-regulated in roots under Cd and Zn stress

The significant decrease in root-to-shoot translocation of Cd and Zn observed at the doses applied of 81 mg kg^{-1} Cd and 270 mg kg^{-1} Zn, led us to investigate if the ATPase HMA4, which plays a pivotal role in metal detoxification and long distance transport in plants (Luo et al. *20*16; Sarwar et al. 2017). *Pt-HMA4* was expressed highly in roots, similar to what has been observed for other members of the HMA family in poplar, specifically around xylem vessels (Migeon et al. 2010). In *A. halleri*, exposure to Zn clearly showed an abundance of *HMA4* transcripts in the root xylem adjacent to the pericycle layer, which emphasises HMA4 involvement in xylem loading and justifies its high expression in root tissues (Hanikenne et al. 2008).

Both Cd and Zn amendments resulted in down-regulation of *Pt-HMA4* in poplar roots, which places this gene in the same subgroup of HMAs transporting Zn/Cd/Co/Pb as found in *A. thaliana* (*At-HMA1-4*) and *Oryza sativa* (*Os-HMA1-3*) (Takahashi et al. 2012). Transport proteins such as HMA, can contribute to Cd efflux to the apoplast, sequestration into the vacuoles and directly affect Cd uptake and localisation (Iori et al. 2016; Hasan et al. 2017). Similarly, at high concentrations of Zn, *P. nigra* down-regulated *Pt-HMA4* expression in just 48 hours (Adams et al. 2011), but in the present study we showed that after eight weeks of exposure to Cd or Zn the expression of *Pt-HMA4* was still much lower than uncontaminated control. Small variations in the expression of *HMA4* in *A. thaliana* were demonstrated to have large effects in the Zn gradient in roots (Claus

et al. 2013). Thus we can hypothesize that the down-regulation of *Pt-HMA4* expression under Cd and Zn stress (Figure 3.5) is one of the mechanisms by which *P. trichocarpa* maintains the metal partitioning pattern observed previously, in which a drastic decrease in translocation occurs as metal concentration reaches its toxicity threshold.

3.4.4 Cd and Zn interactions in poplar

Decrease in Cd uptake in plants due to elevated Zn supply has been commonly shown and is often associated with competitive interactions during root uptake, in which Cd is believed to enter the plant via transport processes inherent to Zn (Marschner 1995; Hart et al. 2002; Garg and Kaur 2013). The opposite can also be observed, for instance in wheat, a decrease in Zn translocation was attributed to competition with high Cd concentrations in soil (Green et al. 2010). We predicted similar outcomes, in which Zn would be preferentially taken up by the roots, therefore reducing Cd accumulation in the plant. However Zn had the opposite effect in *P. trichocarpa* under our experimental conditions and caused an overall increase in Cd uptake and accumulation (Figure 3.6).

A pH decline in the substrate due to high cationic concentration (Zn^{2+}) may have played an important part in increasing Cd uptake, which is known for the inverse relationship with soil pH (Chuan et al. 1996; Smolders and Mertens 2013). But substrate pH was unaffected by the addition of 90 mg kg^{-1} Zn compared to when Cd was added singly (pH of 6.1 in both cases), yet it still lead to a significant increase in Cd concentrations in all plant parts: for instance Cd concentration in leaves increased from 42 mg kg^{-1} under single metal treatment (Figure 3.4) to 123 mg kg^{-1} under the combined treatment (Figure 3.6). Similar effect was observed in *Nocceae caerulescens*, in which combined treatments of Zn (500 μM) and Cd (200 μM) in hydroponic cultures resulted in increasing Cd^{2+} influx into root tissues and higher accumulation in shoots (Papoyan et al. 2007), and this response has been associated with hyperaccumulator phenotypes (Lasat et al. 1998; Papoyan and Kochian 2004). Moreover, the hyperaccumulator *Brassica juncea* had an increase in Cd uptake after Zn addition, leading also to a higher tolerance in comparison to

plants exposed to Cd and Zn separately (Kutrowska et al. 2017). In field conditions, positive correlation between Zn and Cd accumulation in shoots was also reported in Cacao trees (Arévalo-Gardini et al. 2017). Such response might be related to an up-regulation of genes encoding some metal transporters in roots triggered by the exposure to Zn^{2+}, through which Cd^{2+} could have been actively transported. For instance, in *Salix caprea* the combined treatment of Cd and Zn induced the expression of transporters ZIP6 and HMA1 (Konlechner et al. 2013). Another reason for higher Cd uptake can be attributed to the direct competition between Zn and Cd for the soil adsorption sites (Lu and Xu 2009), for these elements have similar atomic characteristics and are both affected by electrostatic interactions (Moreira and Alleoni 2010). Considering that the concentrations of Zn added were at least three times higher than Cd, it is likely that Zn caused a displacement of Cd into the solution, increasing its availability for plant uptake.

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

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

Cherif et al. 2011).

3.5 Conclusions

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

3.6 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.

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.

ATSDR, Agency for Toxic Substances and Disease Registry. 2017. *CERCLA Priority List of Hazardous Substances*. https://www.atsdr.cdc.gov/spl/index.html . 10 Oct. 2019.

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

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.

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

Burgess PJ, Incoll LD, Corry DT, Beaton A, Hart BJ. 2005. Poplar (*Populus spp*) growth and crop yields in a silvoarable experiment at three lowland sites in England, *Agroforestry Systems.* **63**: 157-169.

Cakmak I. 2000. Possible roles of zinc in protecting plant cells from damage by reactive oxygen species, *New Phytologist*. **146**: 185–205.

Castiglione S, Franchin C, Fossati T, Lingua G, Torrigiani P, Biondi S. 2007. High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white poplar (*Populus alba* L. cv. Villafranca), *Chemosphere*, **67**: 1117–1126.

Castiglione S, Todeschini V, Franchin C, et al. **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.

Cherif J, Mediouni C, Ammar WB, Jemal F. 2011. Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solanum lycopersicum*), *Journal of Environmental Sciences*. **23**: 837–844.

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

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

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, 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.

Claus J, Bohmann A, Chavarria-Krauser A. 2013. Zinc uptake and radial transoirt ub roots of *Arabidopsis thaliana*: a modelling approach to understand accumulation, *Annals of Botany*. **112**: 369-380.

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

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

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

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

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

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

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.

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

Dos Santos Utmazian MN, Wieshammer G, Vega R, Wenzel WW. 2007. Hydroponic screening for metal resistance and accumulation of cadmium and zinc in twenty clones of willows and poplars, *Environmental Pollution*. 148, 155–165.

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

Durand TC, Hausman JF, Carpin S, Alberic P, Baillif P, et al. **2010a**. Zinc and cadmium effects on growth and ion distribution in *Populus tremula* x *P. alba*, *Biologia Plantarum*. **54**: 191-194.

Durand TC, Sergeant K, Planchon S. et al. **2010b.** Acute metal stress in *Populus tremula* x *P. alba* (717-1B4 genotybe): Leaf and cambial proteome changes induced by cadmium^{2+}, *Proteomics*. **10**: 349-368.

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

Franchin C, Fossati T, Pasquini E, Lingua G, et al. **2007.** High concentrations of zinc and copper induce differential polyamine responses in micropropagated white poplar (*Populus alba*), *Physiologia Plantarum*. **130**: 77–90.

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

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

1309–1318.

Green CE, Chaney RL, Bouwkamp J. 2003. Interactions between cadmium uptake and phytotoxic levels of zinc in hard red spring wheat, *Journal of Plant Nutrition*. **26**: 417–430.

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

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

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.

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

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.

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.

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

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

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

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

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

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

He S, Yang X, He Z, Baligar VC. 2017. Morphological and physiological responses of plants to cadmium toxicity: a review, *Pedosphere*. **27**: 421–438.

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

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.

Iori V, Gaudet M, Fabbrini, F. et al. 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.

Jun R, Ling T. 2012. Increase of Cd accumulation in five poplar (*Populus* L.) with different supply levels of Cd, *International Journal of Phytoremediation*. **14**: 101–113.

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

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.

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.

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

Kirkham MB. 2006. Cadmium in plants on polluted soils: effects of soil factors, hyperaccumulation, and amendments, *Geoderma*. **137**: 19–32.

Konlechner C, Turktas M, Langer I. et al. **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.

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

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

Langer I, Krpata D, Fitz WJ, Wenzel WW, Schweiger PF. 2009. Zinc accumulation potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone in a dose–response study, *Environmental Pollution*. **157**: 2871–2877.

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

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.

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.

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

Ó Lochlainn S, Bowen HC, Fray RG. et al. **2011.** Tandem quadruplication of HMA4 in the Zinc (Zn) and Cadmium (Cd) hyperaccumulator *Noccaea caerulescens*, *PLOS One*. **6**: e17814.

Lu SG, Xu QF. 2009. Competitive adsorption of Cd, Cu, Pb and Zn by different soils of Eastern China, *Environmental Geology*. **57**: 685–693.

Luković J, Merkulov L, Pajevic S. et al. **2012.** Quantitative assessment of effects of cadmium on the histological structure of poplar and willow leaves', *Water, Air and Soil Pollution*. **223**: 2979–2993.

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.

Lux A, Martinka M, Vaculik M, White PJ. 2011. Root responses to cadmium in the rhizosphere: a review, *Journal of Experimental Botany*. **62**: 21–37.

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.

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

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

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

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

Mirck J, Zalesny RS. 2015. Mini-review of knowledge gaps in salt tolerance of plants applied to willows and poplars, *International Journal of Phytoremediation*. **17**: 640–650.

Moreira CS, Alleoni LRF. 2010. Adsorption of Cd, Cu, Ni and Zn in tropical soils under competitive and non-competitive systems, *Scientia Agricola*. **67**: 301–307.

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

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

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

Papoyan A, Kochian LV. 2004. Identification of *Thlaspi caerulescens* genes that may be

involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase, *Plant Physiology*. **136**: 3814–3823.

Papoyan A, Piñeros M, Kochian LV. 2007. Plant Cd^{2+} and Zn^{2+} status effects on root and shoot heavy metal accumulation in *Thlaspi caerulescens*, *New Phytologist*. **175**: 51–58.

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

Polle A, Klein T, Kettner C. 2013. Impact of cadmium on young plants of *Populus euphratica* and *P.* × *canescens*, two poplar species that differ in stress tolerance, *New Forest*. **44**: 13–22.

Pulford ID, Watson C. 2003. Phytoremediation of heavy metal-contaminated land by trees - A review, *Environment International*. **29**: 529–540.

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.

Reeves RD. 2006. Hyperaccumulation of trace elements by plants. In: Morel JL, Echevarria G, Goncharova N, eds. *Phytoremediation of Metal-contaminated Soils*. Netherlands: Springer, 25–52.

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.

Romeo S, Francini A, Ariani A, Sebastiani L. 2014. Phytoremediation of Zn: Identify the diverging resistance, uptake and biomass production behaviours of poplar clones under high zinc stress, *Water, Air and Soil Pollution*. **225**: 1-12.

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

Sanita di Toppi L, Gabbrielli R. 1999. Response to cadmium in higher plants, *Environmental and Experimental Botany*. **41**: 105–130.

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.

Sarwar N, Imran M, Shaheen MR. et al. **2017.** Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives, *Chemosphere*. **171**: 710-721.

Schreiber L, Hartmann K, Skrabs M, Zeier J. 1999. Apoplastic barriers in roots: chemical

composition of endodermal and hypodermal cell walls, *Journal of Experimental Botany*. **50**: 1267–1280.

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.

Sergeant K, Kieffer P, Dommes J, Hausman JF, Renaut J. 2014. Proteomic changes in leaves of poplar exposed to cadmium and low-temperature, *Environmental and Experimental Botany*. **106**: 112-123.

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.

Shim D, Kim S, Choi, Y. et al. 2013. Transgenic poplar trees expressing yeast cadmium factor 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil, *Chemosphere*. **90**: 1478–1486.

Smolders E, Mertens J. 2013. Cadmium. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, 283–311.

Takahashi R, Bashir K, Ishimaru Y, Nishizawa NK, Nakanishi H. 2012. The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice, *Plant Signaling and Behaviour*. **7**: 1605-1607.

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.

Tran TA, Popova LP. 2013. Functions and toxicity of cadmium in plants: Recent advances and future prospects, *Turkish Journal of Botany*. **37**: 1–13.

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

Tylova E, Peckova E, Blascheova Z, Soukoup A. 2017. Casparian bands and suberin lamellae in exodermis of lateral roots: an important trait of root systems response to abiotic stress factors, *Annals of Botany*. **120**: 71-85.

Verbruggen N, Hermans C, Schat H. 2009. Mechanisms to cope with arsenic or cadmium excess in plants, *Current Opinion in Plant Biology*. **12**: 364–372.

White PJ. 2001. The pathways of calcium movement to the xylem, *Journal of Experimental Botany*. **52**: 891–899.

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.

Zar JH. 2010. *Biostatistical analysis*, 5th edn. New Jersey: Prentice Hall.

3.7 Supplementary Information

Table S3.1 Root morphologic parameters, leaf transpiration (E) and stomatal conductance (gs) of *Populus trichocarpa* exposed to different Cd and Zn concentrations for five weeks.

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

Significant differences among treatments (for each metal) are represented by different letters.
Cd treatments: Tukey test: $p < 0.05$, n = 4;
Zn treatments: Dunn test, $p < 0.05$, n = 3.
x's represent dead leaves and measurements were not recorded.

Table S3.2 Spearman correlation (r_s) matrix between 14 different variables from *Populus trichocarpa* grown under different Cd concentrations. Variables were considered monotonic correlated for $p < 0.05$.

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

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

Table S3.3 Spearman correlation (r_s) matrix between 14 different variables from *Populus trichocarpa* grown under different Zn concentrations. Variables were considered monotonic correlated for $p < 0.05$.

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

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

Table S3.4 Cadmium concentration, total uptake, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* 'Trichobel' grown for five weeks under different Cd doses.

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

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

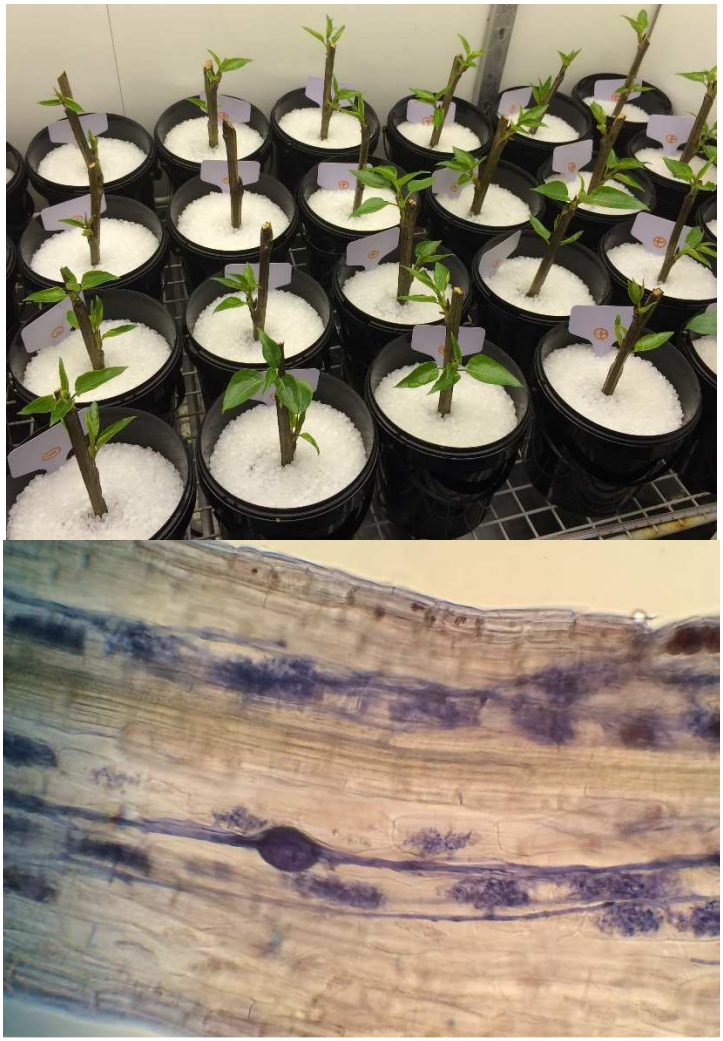
Table S3.5. Zinc concentration, total uptake, translocation factor (Tf: roots-to-leaves) and bioconcentration factor (BCF) in *Populus trichocarpa* 'Trichobel' grown for five weeks under different Zn doses.

Zn ($mg\ kg^{-1}$)	Zn concentration ($g\ kg^{-1}$)			Zn uptake ($mg\ plant^{-1}$)
	Leaves	Stems	Roots	
Control	0.09 ± 0.01 aB	0.04 ± 0.01 aA	0.2 ± 0.01 aC	0.3 ± 0.01
30	0.1 ± 0.01 aA	0.3 ± 0.1 bAB	0.4 ± 0.1 aC	0.5 ± 0.1
90	0.2 ± 0.02 bA	0.5 ± 0.01 bB	0.6 ± 0.1 aB	0.9 ± 0.1
270	0.7 ± 0.1 cA	0.8 ± 0.1 bcA	2.5 ± 0.9 bA	2.0 ± 0.2
810	1.5 ± 0.1 cdA	1.9 ± 0.2 cdAB	5.6 ± 1.3 bcB	2.5 ± 0.5
2,430	2.2 ± 0.4 dA	4.8 ± 1.1 dA	10.1 ± 1.5 cdB	3.5 ± 1.1
7,290	12.8 ± 3.5 eA	24.3 ± 4.2 eA	21.5 ± 5.3 dA	17.9 ± 2.2

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).

Chapter 4

The influence of mycorrhizal symbiosis in *Populus trichocarpa* under Cd and Zn stress: transcript analyses and phytoremediation potential



Top: *Poplar cuttings in growth cabinet. Bottom: Microscopy image of a poplar root colonised by* R. irregularis *with arbuscules and vesicles.*

Abstract

Metal inputs in terrestrial ecosystems are highly concerning due their toxicity to biota. Among soil remediation techniques, phytoremediation is considered to be less harmful to the environment. Alternatives to enhance phytoremediation efficiency are the use of tree species with metal-accumulating capacity and mycorrhizal symbiosis associations as a way of increasing plant growth and tolerance.

We investigated some of the mechanisms by which mycorrhizal fungi can promote tolerance in poplars under Cd and Zn stress. *Populus trichocarpa* cuttings were grown for seven weeks in a clay:sand substrate contaminated by Cd (81 mg kg^{-1}) or Zn (300 mg kg^{-1}) with or without inoculation by arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Growth, transpiration, metal accumulation (leaves, stems and roots) and root colonisation were assessed. The expression of genes involved in metal transport (*PtHMA4*, *PtMTP1*, *PtZIP1* and *PtNramp3*) and chelation (*PtMT2a*, *PtMt2b* and *PCS1*) were quantified by qPCR in roots and leaves. *PtMT2b* function was verified by heterologous expression in yeast under different Cd doses.

P. trichocarpa was highly tolerant to both Cd and Zn, and growth was not different from non-contaminated Control. Mycorrhizal symbiosis increased Zn concentrations by 32 and 37% in leaves (1,200 mg kg^{-1} Zn) and roots (1,100 mg kg^{-1} Zn), respectively. Overall, Cd uptake was not affected by mycorrhization, however shoots of mycorrhizal plants accumulated 41% less Cd when compared to non-mycorrhizal treatments, indicating a strong restriction in translocation. Exposure to either high Zn or Cd down-regulated *PtHMA4* in roots, and up-regulated *PtZIP1* in leaves, suggesting that these genes are involved in both Zn and Cd transport. *PtMT2b* was highly up-regulated in mycorrhizal roots regardless of Cd addition, which may be linked to the restriction of Cd transport in all inoculated plants. Yeasts expressing *PtMT2b* were very tolerant to high Cd (50 µM) in comparison to non-transformed strains. Inoculation of *P. trichocarpa* with *R. irregularis* can promote Zn phytoextraction and Cd phytostabilisation. Differential gene expression patterns under symbiosis appear to be one of the mechanisms in the uptake/translocation dynamics observed. This work highlights the importance of AM symbiosis in phytoremediation studies, and offers candidate genes for future investigations and biotechnological applications.

4.1. Introduction

Soil contamination by heavy metals (HMs) 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 (Nagajyoti et al. 2010; Ali et al. 2013).

Phytoremediation is the use of plants and associated microbiota for environmental decontamination (Pilon-Smits 2005) and it is considered to be less problematic than the physical and chemical remediation processes due to its low environmental impact and overall costs (Gomes et al. 2016). Phytoextraction (uptake and translocation of metals to aboveground parts) and phytostabilisation (immobilisation of contaminants in roots reducing their mobility and availability in soils) are the most common processes for remediation of inorganic contaminants such as HMs (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 (Zacchini et al. 2009; Guerra et al. 2011; Dai et al. 2013; Luo et al. 2016; Redovnikovic et al. 2017), due to their high biomass production, deep root systems (Bhargava et al. 2012), tolerance to elevated metal concentrations (De Oliveira and Tibbett 2018) and rapid growth (Robinson et al. 2009). *Populus* species can promptly invade disturbed sites, reproduce asexually and are not a source of food for farm animals, reducing the risk of heavy metals entering the food chain (Sebastiani et al. 2004; Hamberg et al. 2011; Shim et al. 2013). Symbiotic fungi such as arbuscular mycorrhizal fungi (AMF) are known to improve plant tolerance to biotic and abiotic stresses such as heavy metal toxicity (Miransari 2010) and are considered the most important type of mycorrhiza for phytoremediation (Coninx et al. 2017). However, the mechanisms by which they confer tolerance to HMs have not been clarified (Cicatelli et al. 2014). Inoculation of poplar trees with AMF can significantly increase their biomass production (Ciadamidaro et al. 2017), enhance Cd accumulation (Chen et al. 2016) and

phytostabilisation of HMs such as Cu and Zn (Cicatelli et al. 2014). Nonetheless, 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).

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 zinc-iron proteins (ZIP) and the natural resistance-associated macrophage proteins (NRAMP) (Yang et al. 2005; Colangelo and Guerinot 2006; Sheoran et al. 2011). 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 heavy metals (Kotrba et al. 2009). In order to understand heavy metal 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 HM 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). Some studies have suggested that AMF can

down-regulate gene expression for Zn transporters to promote an optimum concentration of this element within the plant (Burleigh and Bechman 2002). In *P. alba* clones inoculated with AMF, higher uptake and tolerance to Cu and Zn were linked to up-regulation of MT genes (Cicatelli et al. 2010; Pallara et al. 2013).

The genome of *P. trichocarpa* has been completely sequenced (Tuskan et al. 2006) and offers great opportunities for identifying candidate genes for heavy metal uptake in the presence or the absence of AM fungi (Göhre and Paszkowski 2006). Assessing the effects of mycorrhizal fungi 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). Therefore, the objectives of this work were to investigate the influence of mycorrhizal symbiosis (*Rhizophagus irregularis*) in *P. trichocarpa* under Cd and Zn stress. Initial hypotheses were that: i) mycorrhizal symbiosis enhances Cd/Zn uptake and increases plant tolerance to toxicity; ii) poplar genes for metal uptake are down-regulated under metal exposure, while genes associated with metal chelation are up-regulated; and iii) depending on the mycorrhizal effect in metal partitioning, symbiosis could influence the expression of transporter and chelation genes.

4.2. Materials and Methods

4.2.1 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. *Rhizophagus irregularis* inoculum was obtained the University of Reading mycorrhizal collection, which is cultured using Plantago (*Plantago lanceolata* L.) as the host plant. In order to spread the inoculum evenly around the pot, a previously disinfected plastic pipe (9 cm diameter) was placed in the centre of the pot and filled with sandy substrate; the mycorrhizal inoculum was added around the pipe, which was then removed. 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 & 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).

4.2.2 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 contamination (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 stress the application was split into three consecutive days (27 mg Cd/day). For the Zn treatments, a stock 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). Each treatment had six replicates and they were set up in a completely randomised design. Both mycorrhizal and non-mycorrhizal controls received deionised water instead of metal solutions.

4.2.3 Transpiration rate, harvest and pH

After exposure to the toxic metals for four weeks, the two youngest expanded leaves from each plant (including control treatments) 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). This assessment took place in the growth chamber room under constant lighting.

Eight weeks after contamination, plants were harvested and split into leaves, stems and roots (original cutting was discarded). The 9th 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 separately 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).

4.2.4 Mycorrhizal colonisation

Poplar root sub-samples were cleared in KOH solution (10% w/v) at room temperature for 10 days, and 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).

4.2.5 Acid digestion and determination of metal content

Leaf, stem and root samples were ground and 50 mg of material was digested for 8 hours in 5 mL of 70% HNO_3 (≥69% TraceSELECT® for trace analysis) in closed glass vessels on heating blocks at 110^{o}C (Huang et al. 2004). Every digestion run was performed in duplicate, and a blank and a plant certified reference material (IAEA-359 cabbage leaves) were included for quality control. Extracts were then diluted in a solution of 2% HNO_3 + 5 ppb Rh, and filtered. Cd and Zn concentrations were

determined by inductively coupled plasma mass spectrometry (Thermo Scientific™ iCAP™ Q ICP-MS), using rhodium as an internal standard.

The translocation factor (T*f*) is an index used to assess the plant's capacity to translocate heavy metals from roots to aboveground parts (Rafati et al. 2011), and is the ratio between the metal concentrations in shoots (stems + leaves) and roots (Saraswat and Rai 2009; Zacchini et al. 2009); In this case, shoot concentration (mg kg^{-1}) was calculated based on the total amount of Cd accumulated in both leaves and stems (mg) in relation to the total shoot dry biomass (leaves + stems) as represented by the equations below:

$$Shoot\ (conc) = \frac{(Cd\ in\ leaves) + (Cd\ in\ stems)}{Shoot\ biomass} \times 1000 \quad (1)$$

$$Tf = \frac{Shoot\ (conc)}{Root\ (conc)} \times 100 \quad (2)$$

4.2.6 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 65°C (instead of 10 min), LiCl addition was 1/3 of total extract volume (instead of 1/4) and after overnight precipitation at 4°C, 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, using the SensiFAST cDNA synthesis kit (Bioline, UK) and following the manufacturer's instructions. Both DNA and RNAs were quantified using a NanoDrop 2000 Spectrophotometer.

4.2.7 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) available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Table 4.1). 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, with stable expression throughout the experimental treatments (*UBQ*). A schematic representation of the genes selected for qPCR can be found in Figure 4.1.

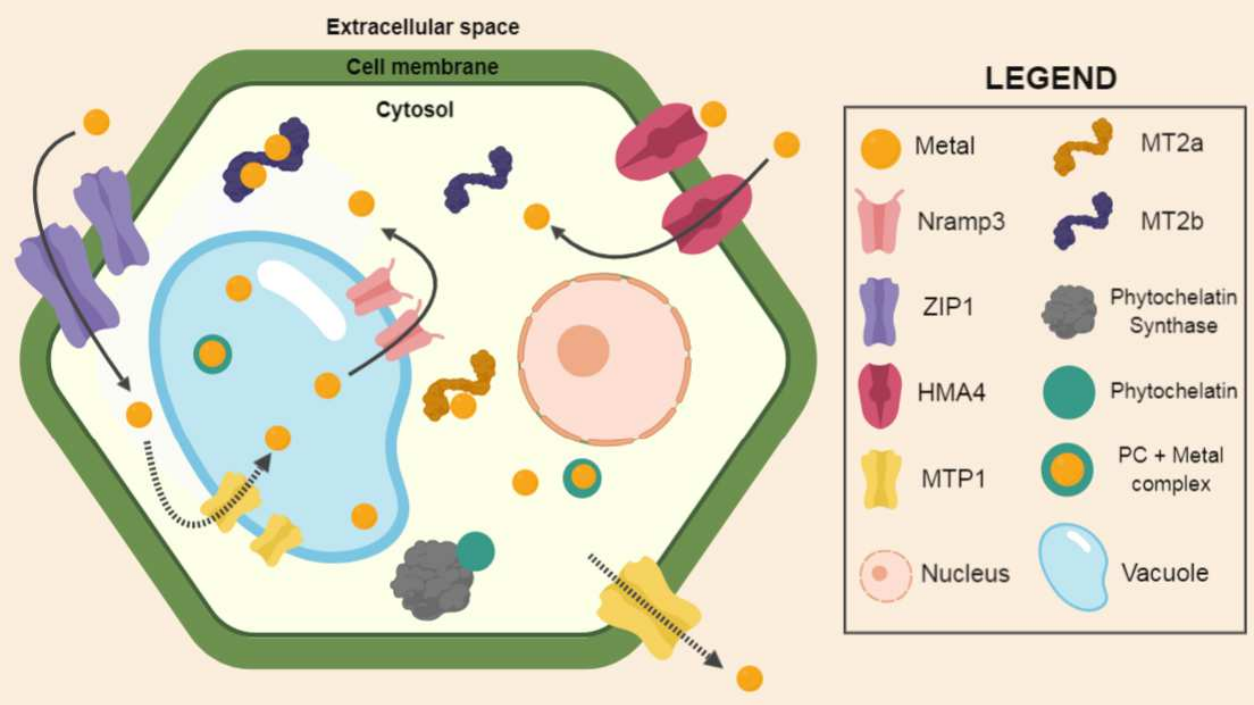


Figure 4.1. Schematic representation of a plant cell with the heavy metal transporters and chelators assessed in *P. trichocarpa* roots and leaves via qPCR (gene expression). Solid arrows represent metal influx into cytosol, while dashed arrows represent metal efflux from the cytosol.

Table 4.1: Primer pairs (and general gene function) used for real-time q-PCR

Gene	Function	Accession	Primer sequence
	Metal transport		
MTP1	Metal tolerance protein Cation diffusion facilitator, cation efflux	XM_002320198	F: AATAGGCAAGGACAGATACGC R: GCTGAGGATTTTGTGCTTCCA
HMA4 [a]	Heavy metal ATPase	XM_006381101	F: ACCAACGTTCTTATGCTTATTGC R: CACTGGCCTTGTGGCTT
ZIP1	Zinc Iron transporter protein	XM_006368992	F: ATGGAGTCTTTCGCCACAGG R: CAAGACCTTACTGTTCTCCTCGT
NRAMP3	Natural resistance-associated macrophage protein	XM_006380608	F: GATTTTGAGTAATGGGGTTTTGCCT R: AATCCCAAAAGCAGCCTCCAATTTC
	Metal chelation		
MT2a [b]	Metallothionein	XM_002308120	F: GAAACTGTGGGTGTGGCTCT R: TGAGGAGCTCATGTCAGGGT
MT2b [c]	Metallothionein	XM_002314146	F: AGCTCCAGTTAGGATGTTCTACG R: TGCGGCGACGTTTTCTCATT
PCS1 [d]	Phytochelatin synthase	XM_002320590	F: TGCACAACGAAGAGGGTTCAT R: GCAACACCAACCCAACTCTC
	Reference gene		
UBQ	Ubiquitin	XM_006373777	F: AGATGGCAGAACTTTGGCTGA R: CGCCAAAGCCATCAAAGAAC

a - Homology to *P. tremula* x *P. alba* P-type heavy metal ATPase (HMA4) (99% nucleotide sequence identity); KM245948. b - Homology to *P. trichocarpa* x *P. deltoides* metallothionein 2a (MT2a) (93% nucleotide seq. identity); c – Homology to *P. trichocarpa* x *P. deltoides* metallothionein 2b (MT2b) (98% nucleotide seq. identity); d – *P. trichocarpa* Cadmium sensitive 1 family protein (POPTR_0014s18420g), based on the homology to *Thlaspi caerulescens* TcPCS1, as verified by Adams et al. 2011.

The qPCR was performed in duplicate for each sample, in roots and leaves using PowerUp™ SYBRGreen™ (Applied Biosystems, UK). Parameters for the qPCR reactions were as follows: 1 cycle of 2 min at 50°C followed by 2 min at 95°C (DNA polymerase

activation), then 40 cycles of 95°C for 3 sec (denaturation) and 60°C for 30 seconds (annealing/extension). Primer specificity was verified by electrophoresis and confirmed by melt curve analyses. The qPCR run, data collection and analyses were performed using StepOne™ Real-Time PCR System (Applied Biosystems). Results were analysed by the standard curve method, and gene expression was normalised using UBQ as the house keeping gene.

4.2.8 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; Figures 4.4 and 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: 58°C), with sequences (5' – 3'):

F - GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAT GTCTTGCTGTGGAGGAAA;

R - GGGGACCACTTTGTACAAGAAAGCTGGGTCTCA TTTGCAGGAGCATGGAT.

The gene was introduced into a Gateway® donor vector pDONR221 (containing the kanamycin resistance gene – Figure S4.1) 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 37°C. Plasmids were isolated from transformed *E. coli* and introduced into destination vector pDR195 (Figure S4.2) 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 30°C (250 rpm) in SC liquid media (5 mL). Cells were then pelleted by centrifugation, and re-suspended in 5 mL of sterile water. 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 30°C for 72 hours in the dark (three replicates).

4.2.9 Statistical analyses

Statistical analyses were performed for all parameters assessed using R software. Dry weight (DW), leaf transpiration (E), and stomatal conductance (gs) were analysed by two-way ANOVA and further Tukey tests ($p < 0.05$). Colonisation percentage was compared among only the mycorrhizal treatments by One-Way ANOVA followed by Tukey test ($p < 0.05$) was used to determine the differences in colonisation percentage of mycorrhizal roots, and the differences in metal contents between leaves, stems and roots. The effects of both mycorrhizal symbiosis and metal additions (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; $p < 0.05$), followed by Tukey contrast analyses. The overall extraction (in µg Cd or mg Zn per plant) in mycorrhizal and non-mycorrhizal poplars was compared by t-test ($p < 0.05$).

Differences in gene expression were performed by ANOVA, followed by a Tukey test when significance was detected ($p < 0.05$). Gene expression comparisons were performed between Control treatments and Cd treatments, or between Control and Zn treatments. Two variables (*MTP1*-root and *NRAMP3*-root) were transformed to attain the ANOVA’s normality and homoscedasticity assumptions, by log(x). To compare 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 in roots and leaves, as well as other parameters assessed such as metal concentrations and colonisation (untransformed data).

4.3 Results

4.3.1 Biomass production, transpiration rate and mycorrhizal 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 4.2), 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; $p = 0.001$).

Table 4.2. 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 (*Rhizophagus irregularis*; + M) or without (autoclaved inoculum).

Treatment	Biomass (g)		E	Colonisation
	Shoot	Root	mmol m^{-2} s^{-1}	%
Control	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	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	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.

No colonisation was detected in non-inoculated poplar roots. 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 mycorrhizal inoculation.

4.3.2 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 increased from ~ 1.3 to ~ 2.6 mg kg^{-1} (Table 4.3), where root-to-shoot translocation (T*f*) decreased sharply from 96% to 34% in mycorrhizal poplars. Under Cd exposure the opposite effect was observed; in this case, root concentrations were similar, but in shoots 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 4.3), where roots were the main sink for Cd storage.

Table 4.3. Cd concentration (mg kg^{-1}) and translocation factor (T*f*: root-to-shoot) in *Populus trichocarpa* under Cd stress (81 mg kg^{-1}) with (+ M) or without inoculation of *Rhizophagus irregularis*.

Treatment	---------- Cd concentration ----------			T*f* (%)
	Leaf	Stem	Root	
Control	0.99 ± 0.3 aA	1.23 ± 0.3 aA	1.34 ± 0.3 aA	96
Control + M	0.76 ± 0.2 aA	0.96 ± 0.1 aA	2.57 ± 0.7 bB	34
Cd	8.47 ± 2.4 bA	48.0 ± 11 bAB	725 ± 240 cB	4
Cd + M	5.02 ± 1.7 cA	26.2 ± 6.6 cAB	871 ± 248 cB	2

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 tissues within the same treatment (rows), by ANOVA and Tukey test ($p < 0.05$).

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

Unlike with Cd, AMF did not affect Zn accumulation, partitioning or translocation in poplars growing in the non-contaminated soil (Table 4.4), 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 4.4). 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 mycorrhizal symbiosis (Table 4.4), and no interactive effects were detected by GLM analyses ($p > 0.2$ for all plant tissues).

Table 4.4. Zn concentration (mg kg^{-1}) and translocation factor (T*f*: root-to-shoot) in *Populus trichocarpa* under Zn stress (300 mg kg^{-1}) with (+ M) or without inoculation of *Rhizophagus irregularis*.

Treatment	---------- Zn concentration ----------			T*f* (%)
	Leaf	Stem	Root	
Control	21.0 ± 8.5 aA	40.0 ± 5.4 aAB	72.2 ± 41 aB	64
Control + M	21.4 ± 10 aA	39.4 ± 8.5 aAB	84.3 ± 57 aB	63
Zn	926 ± 225 bA	426 ± 68 bB	780 ± 102 bA	90
Zn + M	1227 ± 214 bA	472 ± 142 bB	1071 ± 63 bA	79

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 tissues within the same treatment (rows), by ANOVA and Tukey test ($p < 0.05$).

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

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 (Figure 4.2A). Yet, inoculation with *R. irregularis* clearly affected Cd partitioning, which increased Cd percentage content in roots from 64 to 78%. Under Zn contaminated soil, mycorrhizal poplars extracted overall more Zn (in mg per plant) than their non-mycorrhizal counterparts, although metal allocation followed the same pattern of: leaves > stems > roots (Figure 4.2B), with only 8% of Zn being sequestered in roots for both cases.

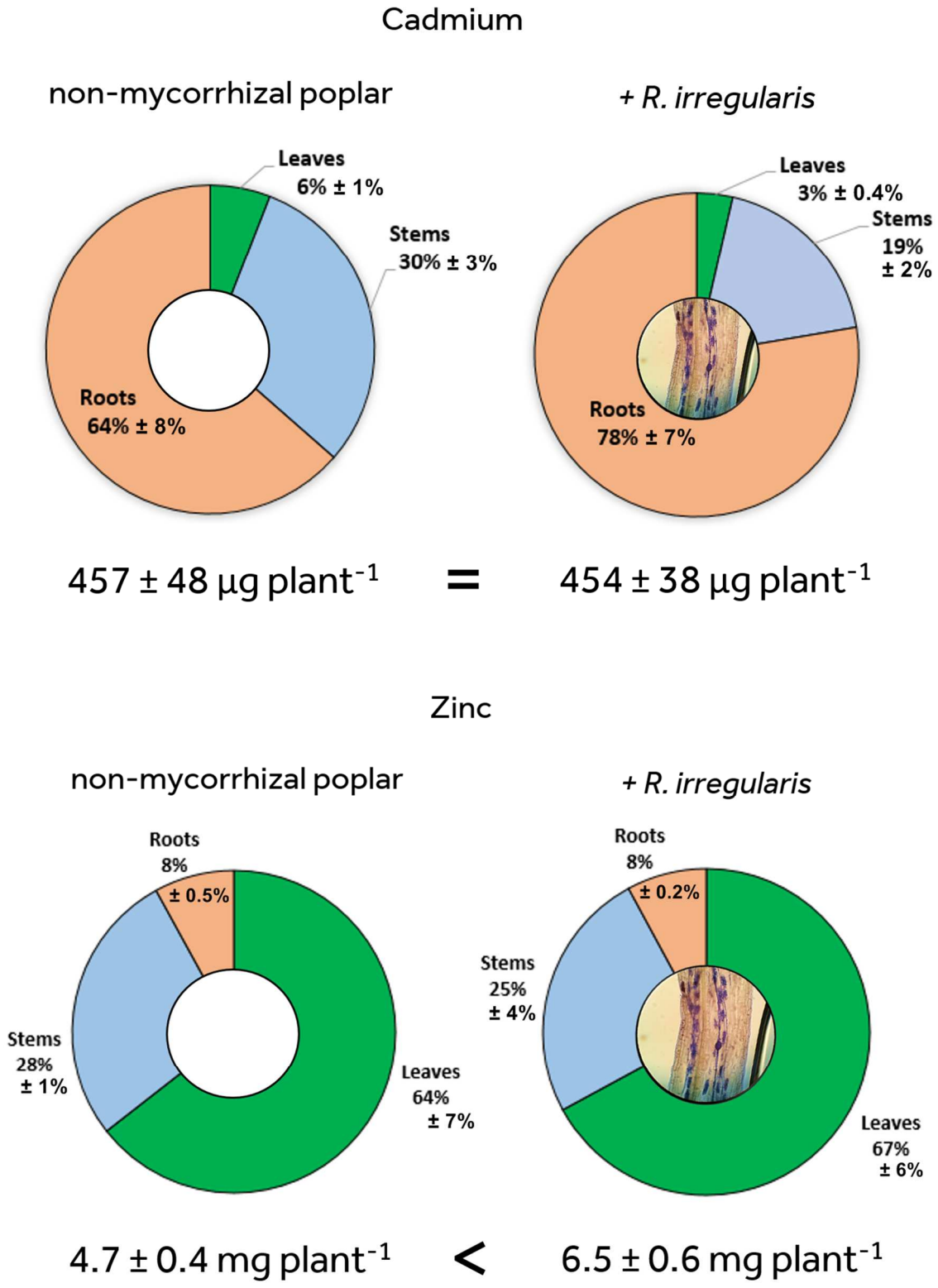


Figure 4.2. Average total content (and standard error) and distribution of Cd and Zn in *Populus trichocarpa* plants under metal stress, with and without inoculation of *Rhizophagus irregularis*. A) under 81 mg kg^{-1} Cd, $p = 0.95$ (t-test); B) under 300 mg kg^{-1} Zn, $p = 0.02$ (t-test).

4.3.3 *Effects of Cd and AMF on gene expression*

Gene expression varied greatly depending on the treatment applied (Cd or inoculation) and the tissue assessed (roots or leaves). The membrane metal transporter *PtHMA4* was down-regulated in poplar roots subjected to Cd contamination, however in leaves, mycorrhizal symbiosis lead to a slightly lower expression regardless of metal addition (Figure 4.3A). *PtMTP1* expression was not significantly different across treatments in both leaf and root tissues (Figure 4.3B), 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* (Figure 4.3C), which had lower expression in root tissues (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 (Figure 4.3E), 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 (Figure 4.3F). *PtPCS1* expression was similar across all treatments (Figure 4.3D) and tissues 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 (Figure 4.3G).

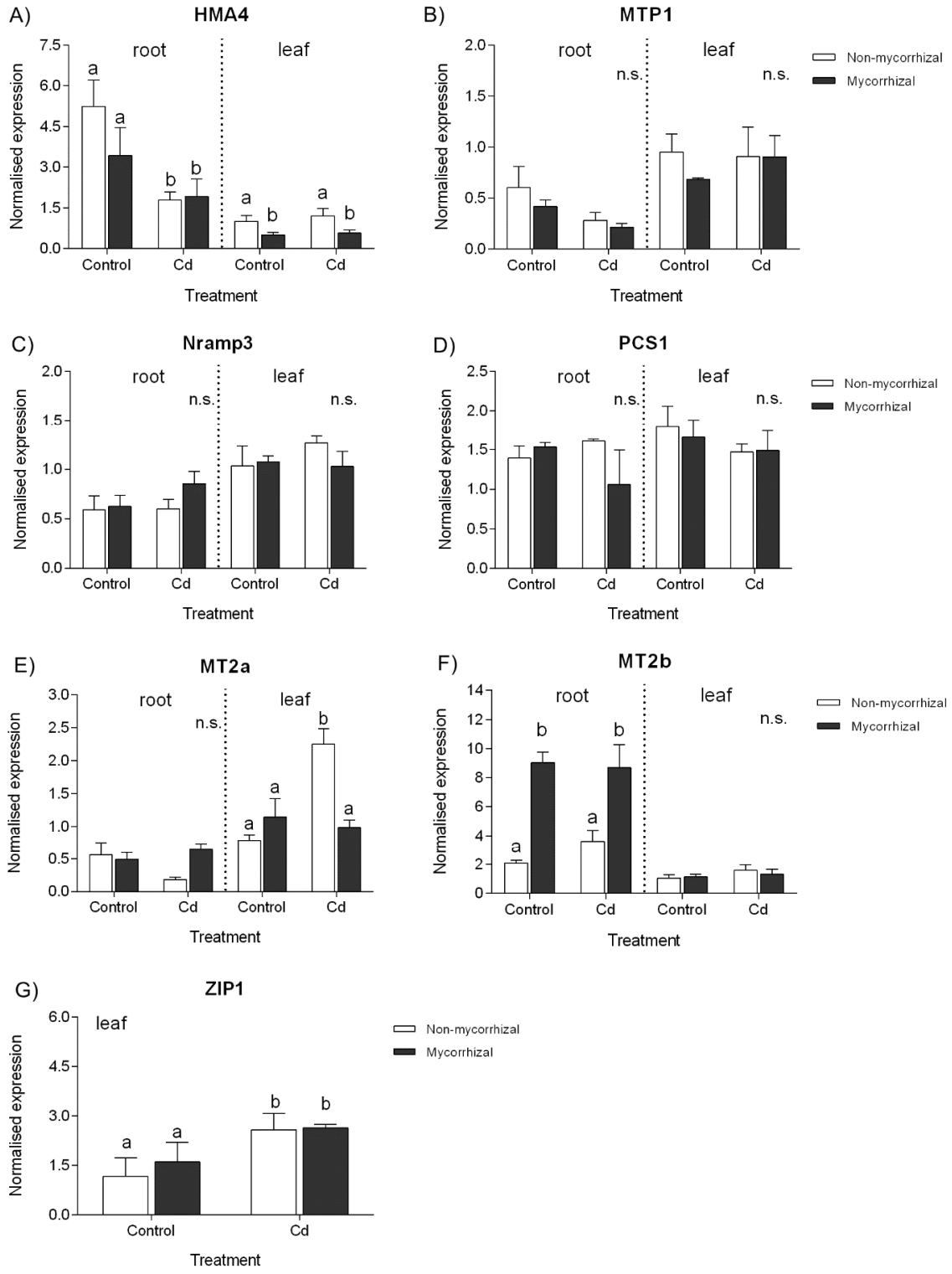


Figure 4.3. Relative gene expression of *PtHMA4* (A), *PtMTP1*(B), *PtNRAMP3* (C), *PtPCS1* (D), *PtMT2a* (E), *PtMT2b* (F), and *PtZIP1*(G) in *P. trichocarpa* cv. Trichobel grown under 81 mg kg^{-1} Cd for eight weeks, with or without mycorrhizal symbiosis (*Rhizophagus irregularis)*. Values are means ± standard error (n = 3) of expression normalised by UBQ. Different letters represent significant differences by ANOVA, Tukey test ($p < 0.05$) for each plant tissue. n.s. = not significant.

4.3.4 *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 (Figure 4.4A), which also had higher expression than in foliar tissues (t-test, $p = 0.006$), although no effects were found in leaves. *PtMTP1* was not differentially expressed (Figure 4.4B) 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 (Figure 4.4C).

As with Cd, expression of the *PtZIP1* transporter was only affected by metal treatment, however Zn exposure quadrupled its expression in poplar leaves (Figure 4.4G) while only a twofold increase was observed under Cd (Figure 4.3G). In roots, *PtMT2a* expression was down-regulated under both high Zn and AMF symbiosis (Figure 4.4E), 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 (Figure 4.4F). Similar to Cd treatments, no changes were verified in *PtPCS1* expression between treatments (Figure 4.4D) or tissues (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 S4.1). Relationships among the genes assessed were mainly negative, with the only positive correlations observed between *PtHMA4* and *PtMTP1* in both roots and leaves (Figure 4.5). *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 (Figure 4.5).

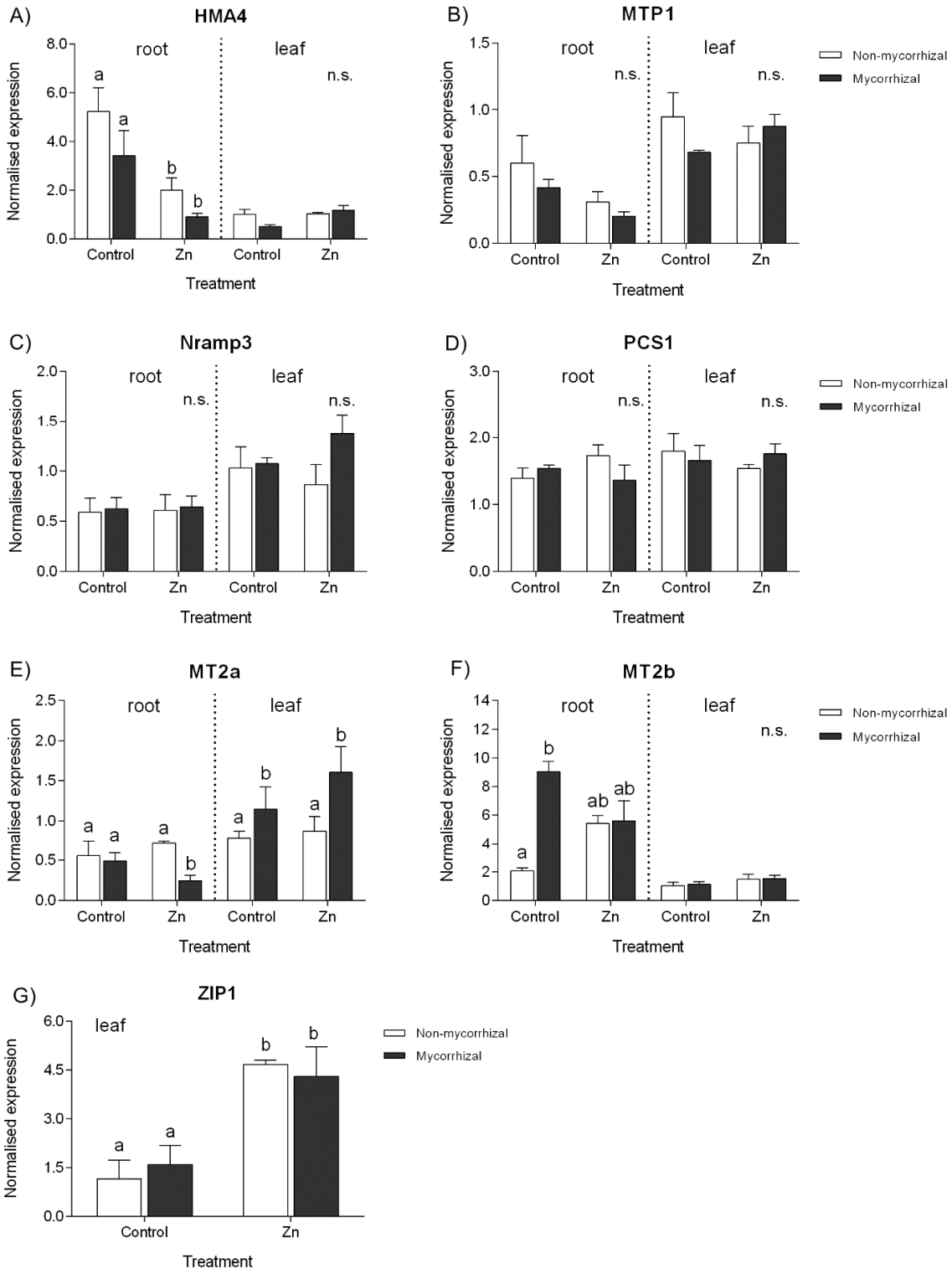


Figure 4.4. Relative gene expression of *PtHMA4* (A), *PtMTP1*(B), *PtNRAMP3* (C), *PtPCS1* (D), *PtMT2a* (E), *PtMT2b* (F), and *PtZIP1*(G) in *P. trichocarpa* cv. Trichobel grown under 300 mg kg^{-1} Zn for eight weeks, with or without mycorrhizal symbiosis (*Rhizophagus irregularis)*. Values are means ± standard error (n = 3) of expression normalised by UBQ. Different letters represent significant differences by ANOVA + Tukey test ($p < 0.05$) for each plant tissue. n.s. = not significant.

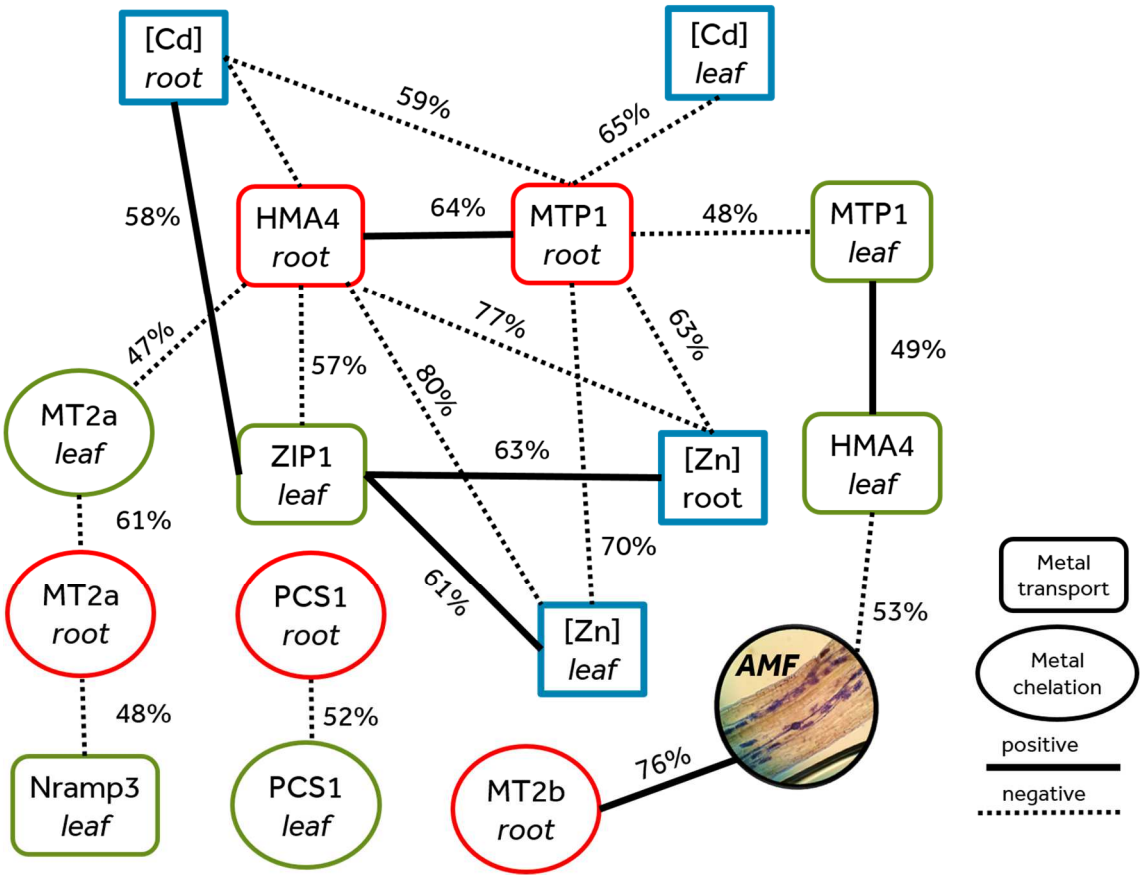


Figure 4.5. Diagram representing significant correlations between the expression of different genes in roots and leaves of *Populus trichocarpa* under Cd and Zn stress, with or without mycorrhizal inoculation (*Rhizophagus irregularis*). Circles: genes associated with metal chelation; Rectangles: genes 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; $p < 0.05$.

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 (Figure 4.6); 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.

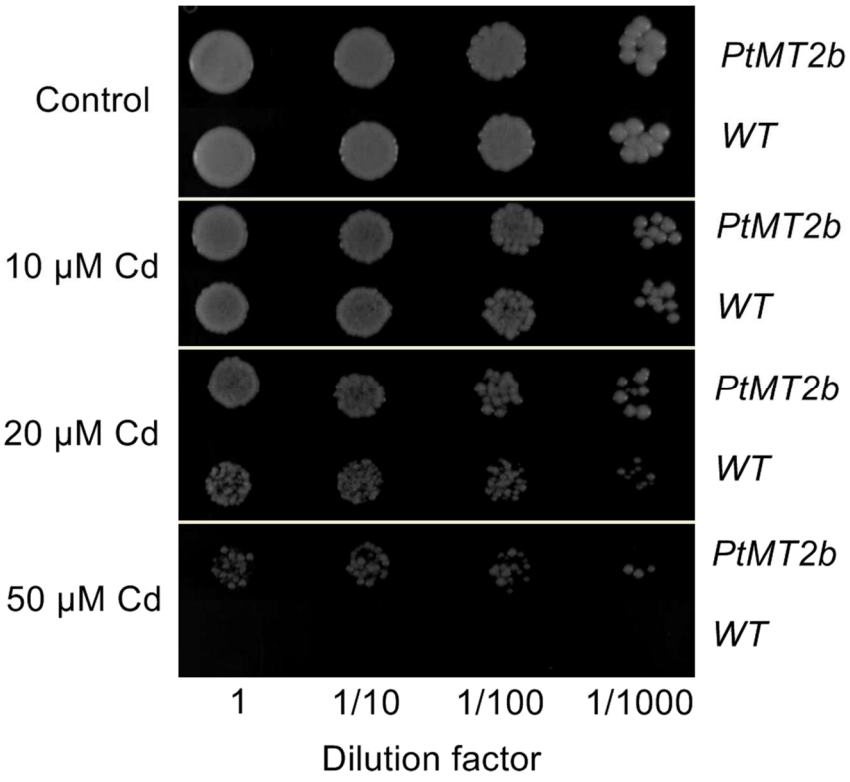


Figure 4.6. Growth of *Saccharomyces cerevisiae* (DY1457) expressing the metallothionein gene *PtMT2b* under increasing Cd concentrations. The wild-type (WT) strain transformed with an empty vector was included as a control. Plates were grown in SC agar medium at 30°C for 72 hours in the dark.

4.4 Discussion

4.4.1 Mycorrhizal effects in Cd and Zn tolerance

Populus trichocarpa cv. Trichobel showed a very high tolerance to Cd and Zn stress in which dry biomass was barely affected (Table 4.2). 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 be one of the reasons for the small change in biomass production found in mycorrhizal plants in comparison to non-inoculated treatments, contradicting our initial hypothesis.

In other poplar clones (e.g. *P. trichocarpa* x *P. maximowiczii*), however, it has been shown by Ciadamidaro et al. (2017) that inoculation with *R. irregularis* can promote an overall increase in biomass of 40% in soils highly contaminated by Cd and Cu. Although it should be noted that their study was carried out over a four-year period, while in the present work plants were exposed to metals for only eight weeks,

which may have been not enough time to verify significant changes. AMF can usually increase plant tolerance to HMs, yet biomass increment can vary depending on plant hosts and ecotypes, fungal partners, metal concentration, soil type etc. (Hildebrandt et al. 2007; Gaurg and Bhandari 2014; Coninx et al. 2017).

All inoculated treatments presented on average 40% of root colonisation, including metal contaminated treatments (Table 4.2). This is not surprising considering AMF are commonly found in roots of plants growing in soils contaminated by heavy metals (Javaid 2011). In polluted soils with high concentrations of Zn, Bedini et al. (2010), by DNA sequencing, reported a high abundance of *R. irregularis* in roots of the dominant flora. In glasshouse conditions, inoculation of *P. deltoides* cuttings with *R. irregularis* in Cd-contaminated substrate resulted in root colonisation rates ranging from 30 to 50%, similar to what was found in the present work. In *P. alba* exposed to 950 mg kg^{-1} Zn, *R. irregularis* colonisation was also similar to controls and at least 20% (Lingua et al. 2012). Abundant root colonisation by arbuscular mycorrhizal species can also be expected during poplar's initial establishment, often decreasing as the plant ages due to displacement by ectomycorrhizal fungi species (Lodge and Wentworth 1990).

4.4.2 Mycorrhizal symbiosis decreases Cd translocation to shoots in poplar

Despite not showing any toxicity symptoms nor biomass reduction, *P. trichocarpa* exposed to Cd and to Zn accumulated considerable amounts of both metals in its tissues, mainly in roots for Cd (Tables 4.3 and 4.4). Roots are usually reported as the main sink for Cd in plants, for poplars (Zacchini et al. 2009; Di Lonardo et al. 2011; De Oliveira and Tibbett 2018), as well as other species (Obata and Umebayashi 1997; Lux et al. 2011).

Mycorrhizal symbiosis decreased Cd concentration in leaves and stems by around 40%, but there was no effect on roots. A similar response was reported previously for some individuals of *P. deltoides* colonised by *R. irregularis* (Chen et al. 2016), but 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 will vary depending on metal concentration and soil characteristics (Audet and Charest 2007), as well as their plant partners; for instance, in both tobacco

and the macrophyte *Phragmites australis*, symbiosis with *R. irregularis* was able to significantly increase Cd concentration in shoots (Janouskova et al. 2006; Huang et al. 2018).

Cd overall extraction (in µg per plant) was similar for both non- and inoculated treatments (Figure 4.2A), 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 tissues. Mycorrhizal fungi have several defence mechanisms against heavy metal toxicity which may have contributed to Cd immobilisation in root tissues, mainly cell wall binding, chelation in cytoplasm and metal transport into intracellular compartments (Coninx et al. 2017), or spores (Gonzalez-Guerrero et al. 2008). In ectomycorrhizal fungi such as *Paxillus involutus*, binding of Cd to cell walls represent a major mechanism of fungal tolerance to toxicity effects (Bellion et al. 2006). Furthermore, *R. irregularis* forms vesicles: thick-walled ovoid structures abundant in lipids that can act as storage units (Smith and Read 2008) and are also believed to be a sink for heavy metal storage within mycorrhizal roots (Göhre and Paszkowski 2006; Nayuki et al. 2014). Up-regulation of metallothionein gene *PtMT2b* in roots due to the symbiosis was probably involved in the higher sequestration of Cd and will be discussed later.

4.4.3 Mycorrhizal symbiosis increased overall Zn phytoextraction in poplars

Regardless of inoculation treatments, *P. trichocarpa* accumulated high contents of Zn under 300 mg kg^{-1} Zn (Table 4.4), and despite concentrations in leaves and roots being similar, the overall Zn accumulated (in mg per plant) had a very different distribution among tissues (Figure 4.2B), with at least 60% accumulated in leaves against only 8% in roots. It should be noted, however, that Zn concentrations in poplar tissues (in mg kg^{-1}) were not significantly affected by mycorrhization (Table 4.4), only the total metal content (in mg per plant), which takes into account the overall plant biomass produced (Figure 4.2B). These findings confirm our initial hypothesis that AMF symbiosis increases Zn accumulation in *P. trichocarpa* and are in line with other reports on other *Salicaceae* species, such as poplars and willows (Laureysens et al. 2004; Lingua et al. 2008; Castiglione et al. 2009; Cicatelli et al. 2010; Todeschini et al. 2011).

Mycorrhizal symbiosis can lead to nutritional benefits in plants under Zn deficiency, as well as protective effects under toxic Zn concentrations - by restricting its uptake and translocation to aboveground parts (Watts-Williams et al. 2013). Although the role of AMF in increasing Zn uptake is well established, especially under Zn-deficient conditions, their effects in plants under high Zn concentrations vary (Smith and Read 2008; 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 (~200 mg kg^{-1}) and leaves (~400 mg kg^{-1}), 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 4.4), a concentration that despite being considered to be highly toxic for foliar tissues (> 300 mg kg^{-1} Zn) (Marschner 1995) did not impair plant growth. It has been suggested that for host plants with high accumulation capacity and HM translocation towards shoots, AMF would increase this phenomenon and enhance phytoextraction (Affholder et al. 2014), which appears to be the case in the present study, considering that the overall Zn content was higher in this case (6.5 mg per plant; Figure 4.2B). Moreover, the intrinsic potential of the host plant to withstand high HM concentrations in its photosynthetic apparatus is probably a major factor influencing this pattern.

4.4.4 Mycorrhizal symbiosis influence on 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 absorb and transport essential metals as well as heavy metals, 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 mycorrhizal inoculation (Figures 4.3A and 4.4A). 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 can be one of the mechanisms by which *P. trichocarpa* avoids metal toxicity in aboveground tissues, and a similar response was 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). Despite being highly tolerant to Cd and Zn, *P. trichocarpa* presented a response common to non-hyperaccumulator plants, shown by Hammond et al. (2006) studying both *N. (Thlaspi) caerulescens* and *N. (Thlaspi) arvense*. Higher expression of *HMA4* in roots than leaves is also in accordance with other results in *P. trichocarpa* (Li et al. 2015), but in the present study, mycorrhizal symbiosis down-regulated *PtHMA4* expression in leaves even further under Cd stress, possibly explaining the decrease in Cd concentration in mycorrhizal treatment (Table 4.3).

PtMTP1: Proteins from this transporter family are known for being involved in metal efflux from the cytoplasm, either to extracellular or into organelles, such as vacuoles and the Golgi apparatus (Peiter et al. 2007; Ricachenevsky et al. 2013). Metal tolerance proteins such as MTP1 usually act on the transport of Zn, Cd, Fe and Mn and tend to have similar roles and localisation among different species (Blaudez et al. 2003; Kramer et al. 2005; Hammond et al. 2006; Ricachenevsky et al. 2013). However, contrary to our hypothesis that metal stress would down-regulate gene expression of HM transporter, *PtMTP1* expression was not significantly different in *P. trichocarpa* regardless of Cd/Zn addition or AMF inoculation (Figures 4.3B and 4.4B). In *P. deltoides*, MTP1 expression was influenced by Zn, but not Cd (Blaudez et al. 2003). Peiter et al. (2007) verified that sensitive yeast transformed with *PtMTP1* had no increase in tolerance to toxic Zn nor Cd, only responding to Mn stress, suggesting that this gene in *P. trichocarpa* is not as involved in Zn/Cd tolerance as in other poplar species. Also, it is important to consider that qPCR analyses was performed after 8 weeks of metal exposure, at which time the transcript levels may have returned to their original baseline (control).

PtNRAMP3: NRAMPs are membrane metal transporter proteins usually located in tonoplasts, from which NRAMP3 is involved in metal efflux from the vacuole into the cytoplasm as a nutrient remobilisation strategy (Sharma et al. 2016), although Cd sequestration into vacuoles by NRAMPs has also been suggested in poplars (Iori et al. 2016). Nonetheless, there are not many studies of *NRAMP3* in poplars, especially

under mycorrhizal symbiosis, but there is agreement that this gene is usually affected by metal exposure, either in *P. trichocarpa* itself (Le Thi 2015), or its homologues in *A. thaliana* and *Nocceae (Thlaspi) caerulescens* (Oomen et al. 2009). The fact that no treatments affected the expression of this gene in the present study (Figures 4.3C and 4.4C) probably means 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, complexation and sequestration in plant cells, with their synthesis catalysed by the enzyme phytochelatin synthase (PCS) (Cobbett and Goldsbrough 2002). Although a variation due to metal exposure was expected, *PtPCS1* expression was not significantly different across treatments (Figures 4.3D and 4.4D). Similar results were observed in tomato plants inoculated with AMF, in which neither symbiosis nor HM exposure affected the expression of this gene (Ouziad et al. 2005). It has been demonstrated that Zn exposure quickly down-regulates (in 48 h) *PtPCS1* expression in poplar stems (*P. trichocarpa* cv. Nisqually), however, Zn did not affect *PtPCS1* expression in leaves and roots (Adams et al. 2011), which is also the case in the present work. 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). It seems that the correlation between PC production and *PtPCS1* expression may not be as significant as it is with the expression glutathione reductase (GSH), another precursor of phytochelatins (Di Baccio et al. 2005). Measuring the actual content of PCs would have been useful in this case, since colonisation by AMF is able to enhance PC production in plants exposed to Cd (Garg and Chandel 2012).

PtZIP1: Members of the ZIP family are able to transport several cations, such as Zn and Cd into the cytosol (Guerinot 2000 – in Rome 2016; Pottier et al. 2015; Iori et al. 2016). Expression of *PtZIP1* was around three times higher in leaves of poplars exposed to 300 mg kg^{-1} Zn than in non-polluted soil, due to the high influx of Zn to those tissues (Figure 4.4G). Cd also up-regulated *PtZIP1* expression, but to a lesser extent, highlighting the role of this gene in Cd transport in poplars (Figure 4.3G). Moreover, in the present study, the level of *ZIP1* transcripts was too low to be detected via qPCR (data not shown), similar results were seen also in the Salicaceae

family; for example, ZIP1 genes of *Salix integra* were expressed mainly in leaves (Shi et al. 2016).

4.4.5 AMF effects on metallothioneins: gene expression and functional expression of MT2b 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 heavy metal exposure (Chen et al. 2014), but in this study, only *MT2a* was affected by metals (Figures 4.3E and 4.4E, with higher expression in leaves of non-mycorrhizal poplars exposed to Cd, where foliar Cd concentration was at its highest compared to other treatments. *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 leaf tissues, and a yeast complementation assay demonstrated *PtdMT2a* function in increasing Cd tolerance (Kohler et al. 2004).

PtMT2b: This gene had very low expression in leaves when compared to roots (Figures 4.3F and 4.4F), similar to results found in *Solanum lycopersicon* by Ouziad et al. (2005), who only detected *MT2b* expression in the root system. Despite not being influenced much by Cd and Zn stress, *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 poplar trees (Cicatelli et al. 2010; Cicatelli et al. 2012; Pallara et al. 2013), highlighting AMF ability in protecting plants against stress by activating detoxifying defences in plants (Miransari 2017). One of the reasons behind the up-regulation of MTs in mycorrhizal roots regardless of metal stress could be related to their secondary role of 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 establishment of the arbuscular mycorrhizal symbiosis, 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 heavy metal stress. This explanation is supported by results from Hrynkiewicz et al. (2012) and research reported in Haq et al. (2003).

From all the Spearman correlations found involving the seven poplar genes assessed, the most interesting was the 76% correlation between *PtMT2b* in roots and the colonisation rates (%) from *R. irregularis* symbiosis (Figure 4.5). Therefore, immobilisation of Cd in roots is probably a combined effect from both fungal binding and MT up-regulation. This observation led us to investigate the function of *PtMT2b* in terms of Cd tolerance. Indeed, we demonstrate here that *PtMT2b* is able to successfully enhance Cd tolerance when expressed in *S. cerevisiae* to at least 50 µM Cd. To the best of our knowledge, the function of *PtMT2b* in Cd tolerance has not been previously tested in yeast, the closest being the work from Kohler et al. (2004), with a poplar hybrid, although most studies are still from herbaceous species (Zhou and Goldsbrough 1994; Guo et al. 2008; Zhang et al. 2014). Therefore, *PtMT2b* could also be a candidate gene for transgenic purposes, with plant or microorganisms to be used in remediation techniques.

4.5 Conclusions

Inoculation of *P. trichocarpa* with the symbiontic fungus *R. irregularis* is able to increase Zn phytoextraction from soils, while for Cd, the decrease in root-to-shoot transport suggests that this association enhances Cd phytostabilisation, which is ideal in terms of remediation techniques, for it reduces the risk of Cd entering the food chain after accumulation in leaves. Overall, the results from this work advance the knowledge on the effects of arbuscular mycorrhizal symbiosis in poplars under Cd and Zn stress, not only in terms of tolerance and phytoremediation 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.

4.6 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.

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*, 3rd edn. Netherlands: Springer.

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.

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M. 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi, *FEMS Microbiology Letters*. **254**: 173-181.

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, *et al.* 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.

Chen L, Ma L, Bai Q, Zhu X *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 *et al.* 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.

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

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 Baccio D, Kopriva S, Sebastiani L, Rennenberg H. 2005. Does glutathione metabolism have

a role in the defence of poplar against zinc excess? *New Phytologist*. **167**: 73-80.

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.

Garg N, Chandel S. 2012. Role of arbuscular mycorrhizal (AM) fungi on growth, cadmium uptake, osmolyte, and phytochelatin synthesis in *Cajanus cajan* (l.) millsp. under NaCl and Cd stresses, *Journal of Plant Growth Regulation*. **31**: 292-308.

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.

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 *et al.* 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 *et al.* 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 *et al.* 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. *et al*. 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, *et al.* eds., *Biomanagement of Metal-Contaminated Soils*, *Environmental Pollution 20*. Springer Science, p. 125-141.

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

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 *et al.* 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.

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. Agricultural sciences. Université Paris Sud - Paris XI, 2015. 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 *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, Bona E, Todeschini V, Cattaneo C, Marsano F, Berta G, Cavaletto M. 2012. Effects of heavy metals and arbuscular mycorrhiza on the leaf proteome of a selected poplar clone: a time course analysis, *PLoS ONE*. **7**: 1-25.

Lingua G, Franchin C, Todeschini V. *et al*. 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.

Lodge DJ, Wentworth TR. 1990. Negative associations among VA-mycorrhizal fungi and some ectomycorrhizal fungi inhabiting the same root system, *Oikos*. **57**: 347-356.

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.

Lux A, Martinka M, Vaculik M, White PJ. 2011. Root responses to cadmium in the rhizosphere: a review, *Journal of Experimental Botany*. **62**: 21–37.

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*, 1st edn. London: Academic Press.

Migeon A, Blaudez D, Wilkins O, et al. 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*. Springer, Singapore, 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 unsing Synchrotron micro X-ray fluorescence, *Microbes and Environments*. **29**: 60-66.

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

Oomen RJFJ, Wu J, Lelievre F, Blanchet S *et al.* 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.

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.

Sarwar N, Imran M, Shaheen MR, Ishaque W *et al.* 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, Y. *et al*. 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 3rd edition.* Academic Press, London.

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.

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, et al. 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, Patti AF, Cavagnaro TR. 2013. Arbuscular mycorrhizas are beneficial under both deficient and toxic soil zinc conditions, *Plant and Soil*. **371**: 299-312.

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.

Zhou J, Goldsbrough PB. 1994. Functional homologs of fungal metallothionein genes from *Arabidopsis*, *The Plant Cell*. **6**: 875-884.

4.7 Supplementary Information

Table S4.1: Pair-wise Spearman correlations (r_s) between the expression of different genes (in roots and leaves) and several parameters assessed in *Populus trichocarpa* grown under Cd and Zn contamination, with and without AMF inoculation. Only significant correlations are shown ($p < 0.05$).

Gene	Covariables[1]	r_s	p-value
Metal transport			
MTP1 $_{root}$	Cd $_{leaf}$	-0.65	0.022
	Cd $_{root}$	-0.59	0.044
	Zn $_{leaf}$	-0.70	0.011
	Zn $_{stem}$	-0.59	0.044
	Zn $_{root}$	-0.63	0.028
	pH	0.54	0.022
	MTP1 $_{leaf}$	-0.48	0.042
	HMA4 $_{root}$	0.70	0.001
	NRAMP3 $_{root}$	-0.48	0.045
MTP1 $_{leaf}$	HMA4 $_{leaf}$	0.50	0.033
	MTP1 $_{root}$	-0.48	0.042
HMA4 $_{root}$	Cd $_{root}$	-0.72	0.008
	Cd $_{stem}$	-0.64	0.024
	Zn $_{leaf}$	-0.80	0.002
	Zn $_{root}$	-0.77	0.003
	Zn $_{stem}$	-0.66	0.020
	pH	0.54	0.020
	MTP1 $_{root}$	0.70	0.001
	ZIP1 $_{leaf}$	-0.57	0.013
	MT2a $_{leaf}$	-0.47	0.049
HMA4 $_{leaf}$	Zn $_{stem}$	-0.58	0.048
	Colonisation	-0.51	0.030
	MTP1 $_{leaf}$	0.50	0.033
NRAMP3 $_{root}$	MTP1 $_{root}$	-0.48	0.045
NRAMP3 $_{leaf}$	MT2a $_{root}$	-0.48	0.046
ZIP1 $_{leaf}$	Cd $_{root}$	0.58	0.048
	Zn $_{leaf}$	0.61	0.036
	Zn $_{root}$	0.63	0.028
	Zn $_{stem}$	0.71	0.009
	HMA4 $_{root}$	-0.57	0.013
	pH	-0.63	0.005

Table S4.1: Cont.

Gene	Covariables[1]	r_s	p-value
Metal chelation			
MT2a $_{root}$	NRAMP3 $_{leaf}$	-0.48	0.046
	MT2a $_{leaf}$	-0.61	0.008
MT2a $_{leaf}$	HMA4 $_{root}$	-0.47	0.049
	MT2a $_{root}$	-0.61	0.008
MT2b $_{root}$	colonisation	0.76	0.0003
MT2b $_{leaf}$	biomass $_{stem}$	-0.56	0.016
PCS1 $_{root}$	PCS1 $_{leaf}$	-0.50	0.034
PCS1 $_{leaf}$	PCS1 $_{root}$	-0.50	0.034

[1] - pH: substrate pH after plant growth; Cd/Zn $_{leaf/root/stem}$: metal concentration (mg kg^{-1}) in either roots, leaves or stems; colonisation: colonisation rate (%) of roots inoculated with *R. irregularis*; biomass $_{stem}$: dry weight (in g) of stems after harvest.

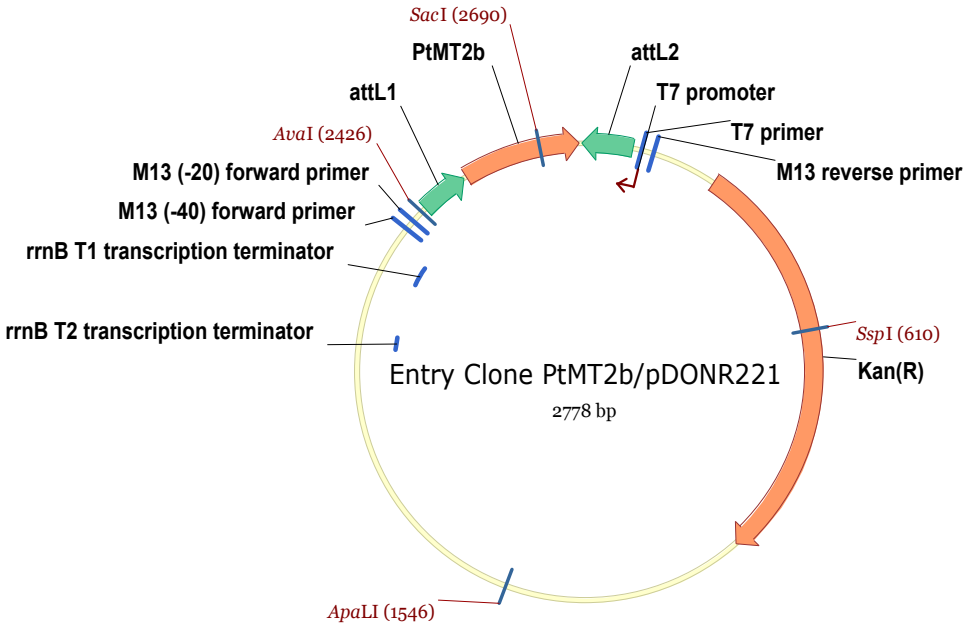


Figure S4.1. Entry clone pDONR221 carrying *PtMT2b* and a gene responsible for Kanamycin resistance, used for transformation and cloning in *E. coli* competent cells.

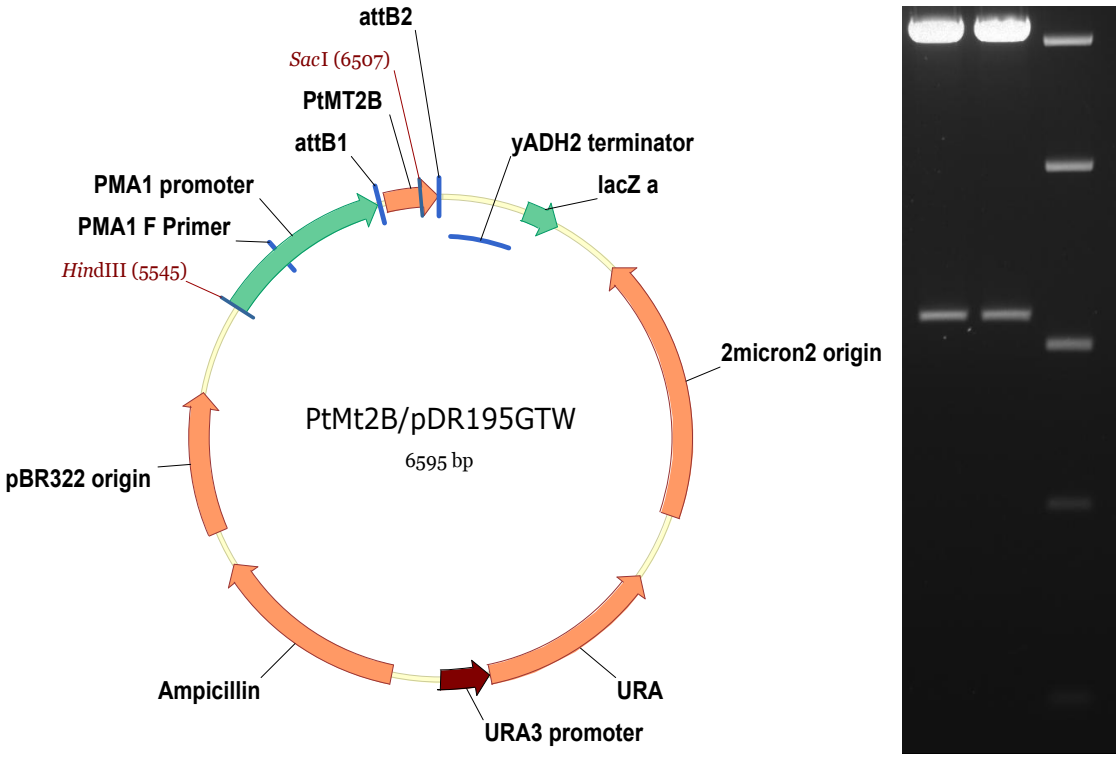
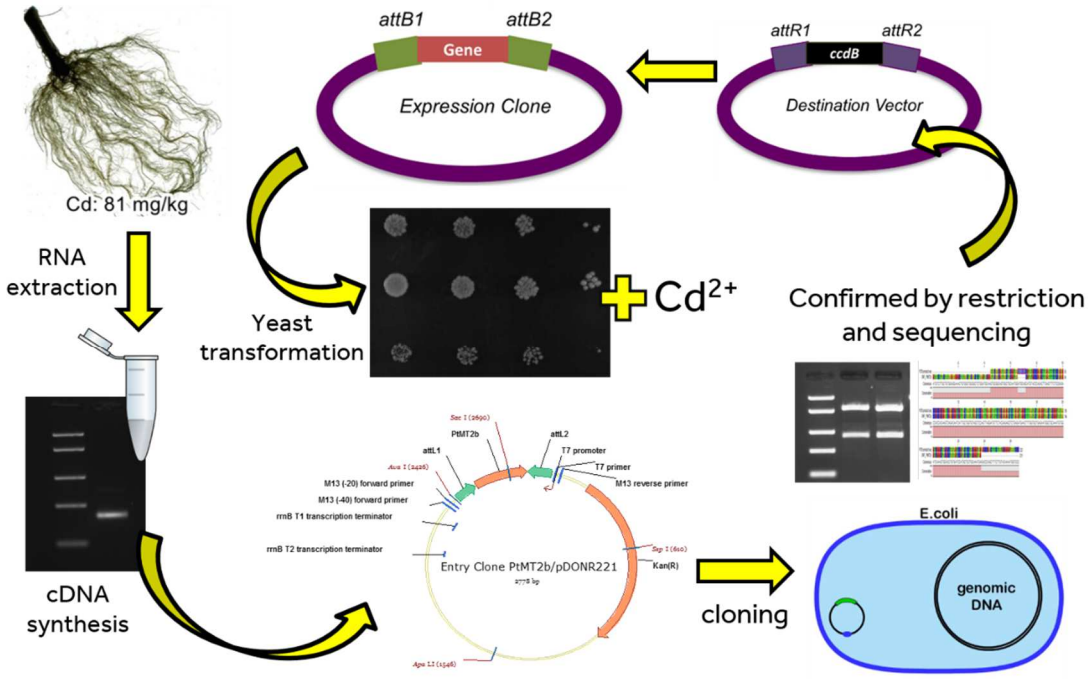


Figure S4.2. Expression vector pDR195GTW carrying *PtMT2b* and a gene responsible for uracyl production (URA) used for the transformation of yeast competent cells. On the right side, the confirmation electrophoresis after a restriction analyses using the enzymes *SacI* and *HindIII* (Expected fragment sizes: 962 and 5633 bp)

Chapter 5

Bioremediation potential of Cd by transgenic yeast expressing a metallothionein gene from *Populus trichocarpa*



Stages for heterologous expression of poplar genes in yeast

Abstract

Cadmium (Cd) is one of the most toxic metals in the environment and soils undergo constant inputs of Cd due to industrial activities, agricultural practices and waste disposal. Due to its high mobility in soils, Cd can also contaminate groundwater increasing its risk of entering the food chain. Biosorption by yeasts (living or not) seem to be a low-cost and effective method for removing Cd from contaminated aqueous solutions.

Here we transformed wild-type *Saccharomyces cerevisiae* strains (WT) with two versions of a *Populus trichocarpa* gene (*PtMT2b*) coding for a metallothionein: one with the original sequence (PtMT2b 'C') and the other with a mutated sequence, with an amino acid substitution in the third position (Cys to Tyr – C3Y, named here: PtMT2b 'Y'). WT and both transformed yeast strains were grown under Cd stress, in agar plates (0; 10; 20; 50 μM Cd) and in liquid media (0; 10; 20 μM Cd) for 72 h. Their growth was assessed visually and by spectrometry OD_{600}, to determine difference in Cd tolerance. The potential of transformed yeasts (*PtMT2b* 'Y') in removing Cd from contaminated media (0; 10; 30 μM Cd), and their intracellular accumulation were also verified by acid digestion and ICP-MS. This gene was also inserted into mutant yeast strains: *fet3fet4*, *zrt1zrt2* and *smf1*, which were grown under Fe, Zn and Mn deficient media, respectively.

Yeast strains had similar growth under 0 μM, but differed under 20 μM Cd, the order of tolerance was: WT < *PtMT2b* 'C' < *PtMT2b* 'Y', in which *PtMT2b* 'Y' had a 37% higher growth than the strain carrying the original gene sequence (*PtMT2b* 'C'). Transgenic yeasts (PtMT2b 'Y') extracted around 80% of the Cd in solution, and had higher intracellular Cd accumulation (around 30 μg g^{-1} cell dry weight) than WT yeasts (0.7 μg g^{-1}). Mutant yeasts carrying *PtMT2b* 'Y' had a slightly higher growth in Mn and Fe deficient media than their non-transgenic counterparts, suggesting that this gene may be involved in chelating these metals as well. *S. cerevisiae* carrying the altered poplar gene (*PtMT2b* 'Y') offers great potential for bioremediation and biosorption of Cd from waste waters. Further studies should be carried in different conditions, and with a mixture of toxic metals, with its efficiency also being tested in bioreactor systems.

5.1 Introduction

Cadmium (Cd) is an element that lacks a known biological function. It is one of the most hazardous metals in the environment, because it can affect animals, plants and microorganisms at relatively low concentrations (Alloway 2013). Several anthropogenic activities are responsible for Cd addition into the environment, such as: atmospheric deposition, industrial and municipal wastes, mining activities, smelting and metal ore processing, battery production, soil fertilisation and sewage sludge application (Mirlean and Roisenberg 2006; Smolders and Mertens 2013; He et al. 2015; Khan et al. 2017). Sewage sludge is an inevitable by-product from industrial or domestic wastewater processing, and is commonly used as an organic amendment in soils, however if wastewater is not pre-treated for metal removal, it can lead to high metal contents being added into agricultural soils and crops (Chen and Wang 2008; Jamali et al. 2009).

Cd is also highly mobile in soils (Lei et al. 2010) with a potential risk of contaminating the groundwater. Estimated leaching of Cd from European soils is between 100 to 5,700 mg Cd ha^{-1} $year^{-1}$ (Smolders and Mertens 2013). Cd is readily taken up by plant roots and poses a risk when entering the food chain, possibly causing biomagnification, in which a low Cd concentration can increase and become even more toxic through different trophic levels (Janssen et al. 1993).

A low-cost and effective method of removing heavy metals from wastewater or aqueous solutions is by using natural materials of biological origin (algae, fungi, bacteria, yeast) in a process known as biosorption (Goksungur et al. 2005; Oliveira et al. 2012). This process has many advantages, such as low operating costs, decreased volume of the sludge generated and high efficiency in detoxifying very dilute effluents (Marques et al. 2000). The yeast *Saccharomyces cerevisiae* has been frequently studied as a biosorbent for several heavy metals, such as Pb, Cr, Zn, Cu and Cd (Oliveira et al. 2012; Vijayaraghavan and Balasubramanian 2015). Although biosorption is a term commonly used for non-living biomaterials that bind and concentrate contaminants, this process occurs in both living and dead organisms (Amirnia et al. 2015).

Employing living microorganisms for metal biosorption has an advantage of simultaneously exploiting their inherent ability of absorbing and accumulating heavy metals intracellularly, a process known as bioaccumulation (Pankiewicz et al. 2015). Recently, a system of continuous growth of *S. cerevisiae* was demonstrated to be an efficient method of removing copper and lead ions from water (Amirnia et al. 2015). *S. cerevisiae* is a great candidate for bioremediation of metal-contaminated waters for several reasons: (1) reproduction by budding (asexual) or spore formation (sexual) (Wang and Chen 2009); (2) it is easy to cultivate and available from various food and beverage industries (Wang and Chen 2006); (3) it has high adsorbent capacity even in dead cells (Goksungur et al. 2005), (4) it can accumulate high intracellular amounts of heavy metals (Brady and Duncan 1994; Joutey et al. 2013), (5) it can flocculate easily in metal solutions and sediment, which facilitates the separation process after remediation (Machado et al. 2008, Soares 2011), and, finally, (6) *S. cerevisiae* is a model system in biology and can be easily manipulated genetically and morphologically to numerous purposes (Karathia et al. 2011; Farcasanu and Ruta 2017).

Genetically engineered microorganisms appear to be the next frontier in terms of bioremediation and biodegradation of contaminants, in which remediation pathways are enhanced by inserting foreign genes of specific interest (Joutey et al. 2013; Kulshreshtha 2013). Genes coding for phytochelatins (PCs) and metallothioneins (MT) are frequently the focus for engineering microorganisms for heavy metal remediation (Sriprang et al. 2003; Singh et al. 2008; Ruiz et al. 2011).

Metallothioneins are low-molecular weight proteins rich in Cys (usually 9-16 Cys residues), which are able to bind metals in metal-thiolate clusters (Cobbett and Goldsbrough, 2002; Sheoran et al. 2011), such as Zn^{2+} and Cu^{2+} (Bulgarelli et al. 2016). Most MT genes belong to the sub-family MT2 of plants, which is known for biding divalent cations, such as Cd^{2+} (Cobbett and Goldsbrough 2002; Bulgarelli et al. 2016), or some nutrients like Fe^{2+}, Zn^{2+} and Cu^{2+} (Jin et al. 2014) and were already demonstrated to increase Cd tolerance through heterologous expression in yeast (Kohler et al. 2004).

Similarly, we have recently demonstrated that the gene *PtMT2b* from tree species *Populus trichocarpa* cv 'Trichobel' was able to reduce Cd toxicity when expressed in *S. cerevisiae* (Chapter 4). This poplar clone is particularly tolerant to elevated Cd concentrations (De Oliveira and Tibbett 2018), whose high expression of MT2b in roots was shown to be correlated to enhanced Cd sequestration (Chapter 4). Moreover, those yeasts expressing poplar's MT2b may effectively remove Cd from contaminated water by preventing the excretion of metals back to the medium through chelation (Ruta et al. 2017).

Therefore, it was hypothesised that i) *PtMT2b* increases yeast tolerance to Cd; ii) a mutated version of the gene *PtMT2b* 'Y' (C3Y substitution) is not as efficient in conferring Cd tolerance in yeast due to the lack of one cysteine in the peptide sequence; and iii) if transformed yeasts are more tolerant to Cd, they can also effectively bioremediate Cd from aqueous solutions (by surface biosorption or intracellular accumulation).

Considering the role of MTs in binding divalent cations, it was also hypothesised that this metallothionein could improve the growth of mutant *S. cerevisiae* strains in nutrient (Fe, Mn or Zn) depleted media, possibly by containing a larger internal metal store than the non-transformed yeast.

5.2 Materials and Methods

5.2.1 RNA extraction, cDNA synthesis and cloning

DNA was extracted, from *Populus trichocarpa* cv. 'Trichobel' roots and leaves with DNeasy Plant Mini Kit (Qiagen, UK), following manufacturer's instructions. Total RNA was extracted from approximately 100 g of fresh weight material (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 65^{o}C (instead of 10 min), LiCl addition was 1/3 of total extract volume (instead of 1/4) and after overnight precipitation at 4^{o}C, extract was centrifuged for 60 min (instead of 20 min); After centrifugation, 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.

The extracted RNA was converted into cDNA using the SensiFAST cDNA synthesis kit (Bioline, UK) following the manufacturer's instructions. The full coding sequence was then amplified with a *PtMT2b* primer set containing attB overhang (annealing temperature: 58^{o}C), with sequences (5' – 3'):

F - GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTTGCTGTGGAGGAAA;

R - GGGGACCACTTTGTACAAGAAAGCTGGGTCTCA TTTGCAGGAGCATGGAT.

5.2.2 Amino acid substitution

During the cloning process, two different *PtMT2b* sequences were obtained due to a probable mishap during DNA amplification (Figure 5.1). This was later confirmed by sequencing the MT2b gene directly from the genomic DNA extracted. One codon had a single nucleotide substitution, from the original 'TGC' to 'TAC', which consequently changed the correspondent amino acid from a cysteine (C) to a tyrosine (Y) at the third position (C3Y). Considering that cysteine is responsible for the divalent cation binding ability in MTs, it was possible that the C3Y substitution would lead to a different Cd tolerance phenotype in yeast. Therefore, these two versions of the same gene were used in yeast transformation, the original (*PtMT2b* 'C') and the mutated (*PtMT2b* 'Y').

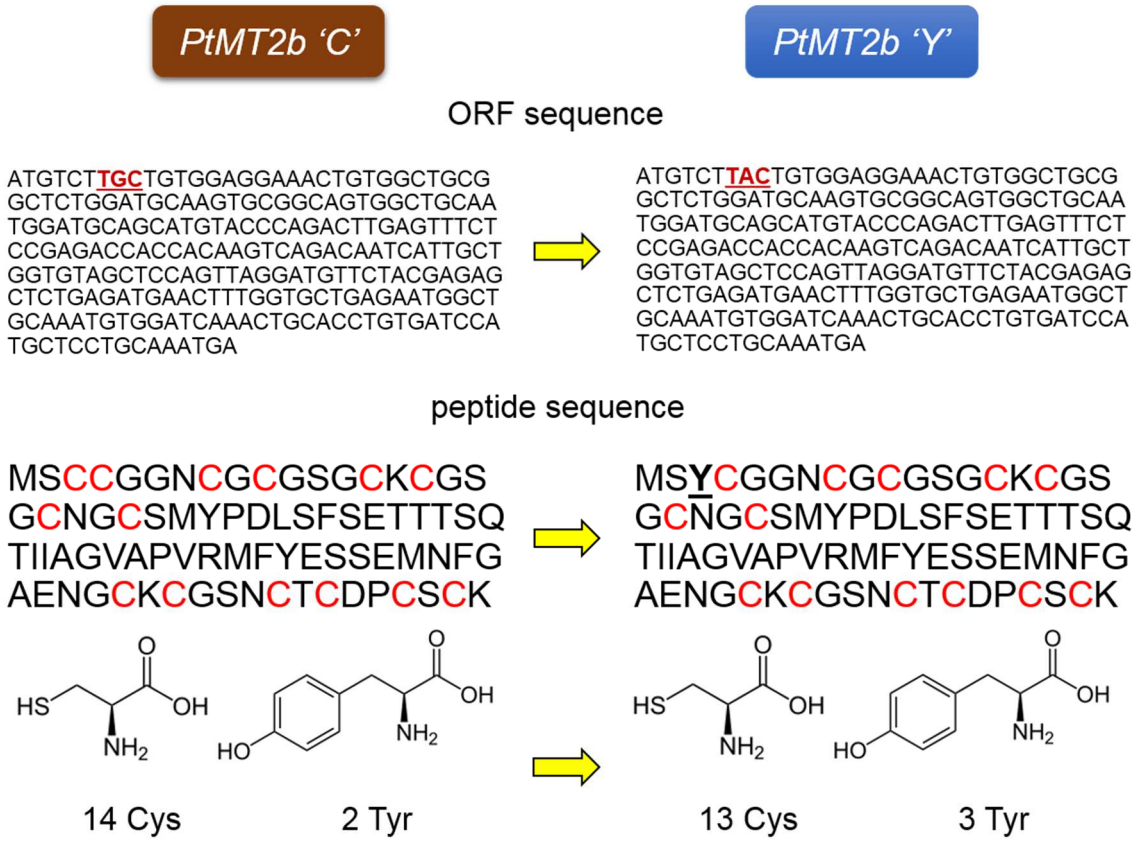


Figure 5.1: Substitution of one single nucleotide in *Populus trichocarpa* MT2b gene during amplification (from TGC to TAC), leading to the cysteine in the third position being replaced by a tyrosine (C3Y).

5.2.3 Yeast transformation

The wild-type *S. cerevisiae* strain DY1457 (WT) was used for transformations. The genes were introduced into a Gateway® donor vector pDONR221 (containing the kanamycin resistance gene – Figure S5.1; Sup. Files) using Gateway® BP Clonase® II enzyme mix. Chemically competent *Escherichia coli* cells (TOP10) were transformed with the entry clones and grown overnight in LB agar + Kanamycin medium at 37ºC. Plasmids were isolated from transformed *E. coli* and introduced into destination vector pDR195 (Figure S5.2; Sup. Files) using the Gateway® LR Clonase® II enzyme mix. *E. coli* cells were transformed with the expression vectors and grown in LB agar + Ampicillin, same parameters as before. WT yeast was transformed with the expression vector containing *PtMT2b* 'C', *PtMT2b* 'Y' and an empty vector (pDR195) 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 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.

5.2.4 Heterologous expression of PtMT2b (C and Y) in yeast under Cd stress

Yeast cells were grown overnight at 30°C (250 rpm) in SC liquid media (5 mL; pH: 5.5). Cells were then pelleted by centrifugation, and re-suspended in 5 mL of sterile water. Optical density at 600 nm of wavelength (OD_{600}) was recorded using SpectraMax i3x (Molecular Devices) microplate reader. Cultures were diluted in sterile water to reach OD_{600} of 0.1, which were used for serial dilutions (1:10 v/v). All dilutions of transformed (*PtMT2b* 'C' and 'Y') and empty vector yeast ('WT') were spotted (5 µL) into SC agar plates at 0; 10; 20; and 50 µM Cd (via $CdCl_2$), then grown at 30°C for 72 hours in the dark (three replicates). In order to quantify yeast growth under Cd stress, all strains were grown in liquid SC media (initial OD_{600}: 0.01), containing either 0; 10 or 20 µM Cd (three replicates) for 48 hours (30°C, 250 rpm; dark), after which the OD_{600} was recorded. The concentration of 50 µM Cd was not used in any liquid media assays due to high toxicity.

5.2.5 Cd tolerance, accumulation and extraction potential of yeast containing PtMT2b 'Y'.

The *PtMT2b* 'Y' and WT (empty vector) yeasts were grown in 5mL of SC liquid media + 2% dextrose, containing 0; 10 and 30 µM Cd at 30°C in the dark with constant shaking (initial OD_{600}: 0.01; four replicates). After 72 hours, OD_{600} was recorded and cells were pelleted by centrifugation (10 min, 4000 rpm). All contaminated media were transferred to new tubes without disturbing the pellet, these were denominated Left Over (LO) and were later analysed by ICP-MS to determine the remaining Cd concentration after yeast growth. Pelleted cells were re-suspended in 10 mL of EDTA (20 mM) and washed for 10 minutes (by inverting tubes) in order to remove adhering Cd ions from yeast surface

(Ullah et al. 2018). Cells were pelleted again and washed twice with 10 mL of deionised water. Yeasts were oven-dried at 80°C for 48 hours. Dried cells were digested in 5 mL of 69% nitric acid (TraceSELECT™ grade) in closed glass vessels for 8h at 110°C (in duplicates). Pure acid was used as blank and 0.05 g of reference material (IAEA-359 cabbage leaves) was digested in the same manner for quality control. Cd accumulation in cells and the remaining Cd in Left Over media were determined via ICP-MS (Thermo Scientific™ iCAP™ Q). Cd extraction potential was calculated by the following equation:

$$(1) \quad Cd\ extracted\ (\%) = 100 - \frac{LO\ Cd \times 100}{Initial\ Cd}$$

In which *"LO Cd"* is the Cd concentration determined in the left over media solution after yeast growth (mg L^{-1}); and "*Initial Cd"* the concentration of Cd added in the growth media before yeast inoculation, also determined via ICP-MS (mg L^{-1}).

5.2.6 Cell Dry Weight vs OD_{600}

In order to estimate Cd concentration in terms of cell dry weight (CDW), transformed (*PtMT2b* 'Y') yeast was grown in conical flasks (three replicates), containing 60 mL of uncontaminated SC media, with OD_{600} starting at 0.01. Every 3h an aliquot of 10 mL from each flask had its OD_{600} determined, cells were pelleted and washed with deionised water and dried in previously weighed glass vials at 80°C. After 72h, dry weight was recorded. The relationship between CDW and OD_{600} was determined by linear regression model ($\alpha = 0.05$; 15 samples).

5.2.7 PtMT2b 'Y' expression in mutant yeast under nutrient-deficient media

In order to verify the specificity of this gene, transgenic mutant yeast were subjected to nutrient deficient conditions (Fe, Mn and Zn). If MT2b 'Y' proteins also bind these nutrients, these yeast strains would be able to grow under deficiency due to a higher nutrient storage capacity in their cells. Strains

used for transformation were the single mutant SMF1 (*smf1*), and the double mutants DEY1453 (*fet3fet4*) and ZHY3 (*zrt1zrt2*), as well as the corresponding parental wild type strain DY1457 (WT). DEY1453 is defective for low and high-affinity Fe^{2+} uptake systems, while ZHY3 lack two Zn^{2+} transporters (ZRT1 and ZRT2); SMF1 strain lacks a high affinity Mn^{2+} uptake gene (SMF1) (Ullah et al. 2018). All strains were transformed either with *PtMT2b* 'Y' or an empty vector (e.v.) as control. Mutant yeasts were also transformed with *TcNramp5,* a metal transporter gene from cocoa trees known to increase Cd^{2+} and Zn^{2+} uptake in yeast (Ullah et al. 2018), and were used as a positive control. Transformations were carried out as described previously.

Primary cultures were stablished from a single colony, and grown in 10 mL SC media supplemented with either 0.4% (v/v) Fe, 0.2% Mn or 0.4% Zn; for DEY1453, SMF1 and ZHY3 strains, respectively (30^{o}C, 72h, 250 rpm, dark). Initial growth in a rich media was carried out to promote a nutrient stock in yeast cells before being transferred to deficient media. Cultures were serial diluted and spotted (5 µL) into SC + agar plates, with or without chelating agents to decrease nutrient availability: 10 µM BPS for creating iron deficient plates (- Fe); 12.5 mM EGTA for Mn deficiency (- Mn); and 100 µM EDTA for Zn deficiency (- Zn).

5.2.8 Statistical analyses

ANOVA and Tukey test was performed for all datasets that met ANOVA's assumptions: (i) OD_{600} between WT, *PtMT2b* 'C' and *PtMT2b* 'Y' under 10 and 20 µM Cd; (ii) OD_{600} between WT and *PtMT2b* 'Y' under 0, 10 and 30 µM Cd; (iii) Cd extraction from liquid media (%); and (iv) growth of *SMF1* strains with and without MT2b under Mn deficiency (transformed by x^2). After being unable to transform the Cd content (µg g^{-1}) data to attain normality, the non-parametric Kruskal-Wallis test was employed. Linear regression analysis was used for obtaining the CDW (mg mL^{-1}) and OD_{600} relationship, in which the Min/Max accuracy and MAPE (mean absolute percent error) were used for calculating the model accuracy. All statistical analyses were performed using R software.

5.3 Results

5.3.1 Amino acid substitution in MT2b increased Cd tolerance in yeast

The spot assay clearly showed that the strains transformed with both versions of the *PtMT2b* gene were able to cope with higher Cd concentrations than the strain transformed with the empty vector only, especially at 50 µM, in which its growth was completely suppressed (Figure 5.2).

In liquid media contaminated by Cd, yeast strains had similar growth under 0 µM, but differed under 10 and 20 µM Cd (ANOVA: $p < 0.001$). Under the highest Cd concentration the order of tolerance was WT < *PtMT2b* 'C' < *PtMT2b* 'Y'; determined after Tukey test (variation coefficient = 6.5%), in which the growth of yeasts carrying the tyrosine-replaced MT2b was around 37% higher than strain expressing the original gene sequence (*PtMT2b* 'C') (Table 5.1).

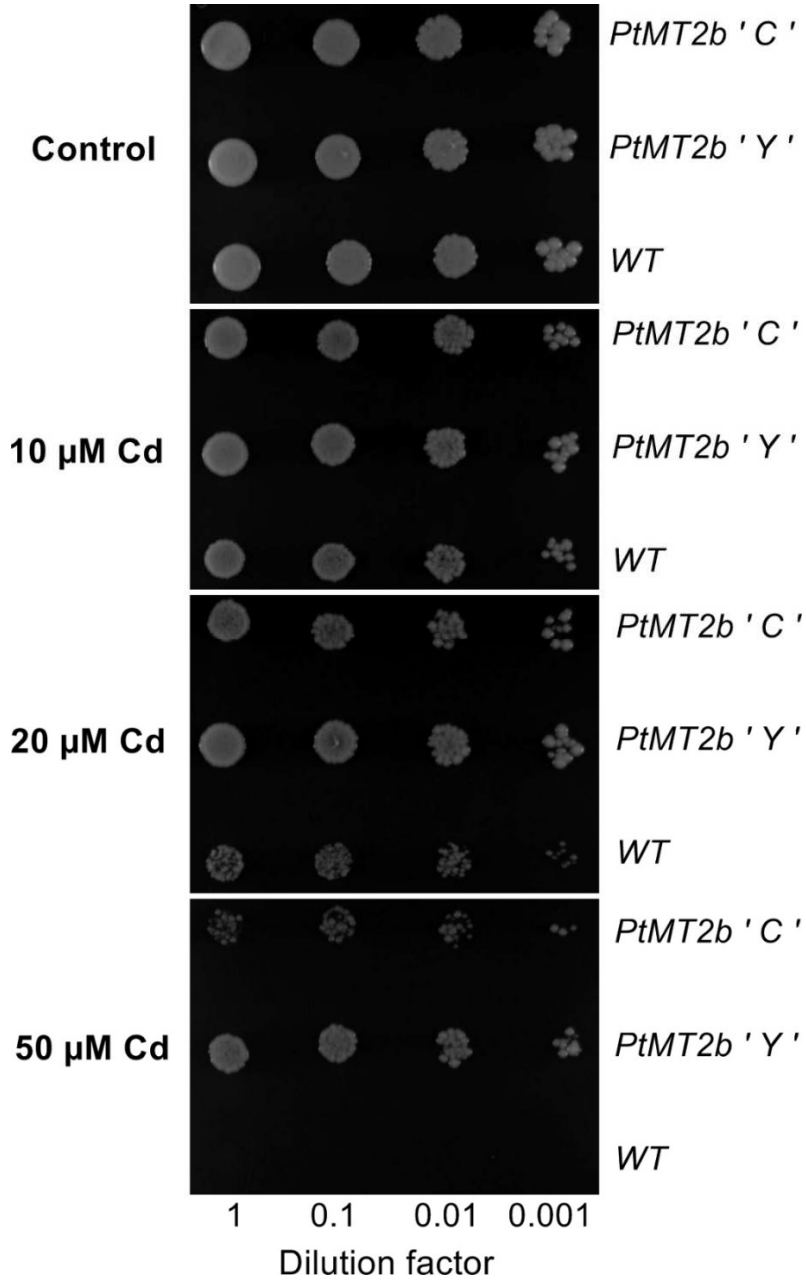


Figure 5.2 Heterologous expression of *PtMT2b* in *S. cerevisiae* growing under increasing Cd concentrations. WT: Wild type (DY1457) strain; MT2b 'C': wild type yeast transformed with the original *PtMT2b*; MT2b 'Y': transformed yeast expressing the modified *PtMT2b* gene, with cysteine to tyrosine replacement (C3Y).

Table 5.1 – Growth of transformed *Saccharomyces cerevisiae* strains under Cd stress, determined by OD_{600} after 48 hours (mean ± st. error). WT: wild type; e.v.: empty vector; *PtMT2b* 'C': gene with original sequence; and *PtMT2b* 'Y': gene with cysteine to tyrosine replacement (C3Y).

Strain	Cd concentration	
	10 µM	20 µM
WT + e.v.	0.20 ± 0.009 a	0.15 ± 0.009 a
WT + *PtMT2b* 'C'	0.31 ± 0.005 b	0.25 ± 0.003 b
WT + *PtMT2b* 'Y'	0.29 ± 0.005 b	0.35 ± 0.012 c

Different letters correspond to significant differences among strains within columns (same Cd concentrations), as determined by Tukey test after ANOVA ($p < 0.001$).

5.3.2 Mutated PtMT2b gene increased Cd accumulation and removal by yeast

Since yeast carrying the mutated gene sequence (*PtMT2b* 'Y') were more tolerant than the strains expressing the original gene, they were ultimately selected for Cd bioremediation trials. Results showed that WT strain (empty vector) was significantly affected by Cd toxicity, while growth of transformed strain was unaffected by Cd additions (Figure 5.3).

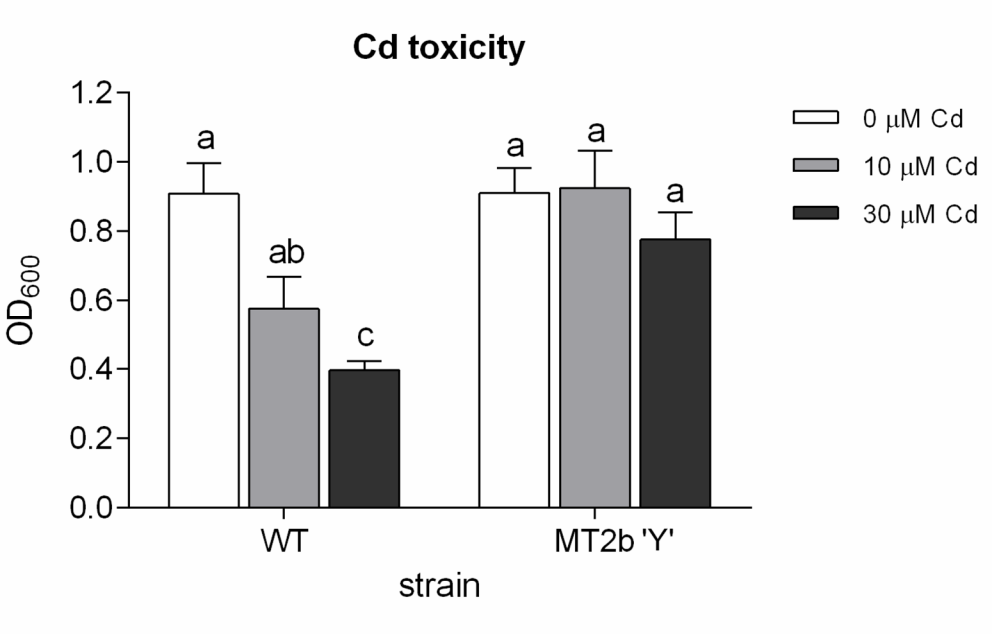


Figure 5.3 *S. cerevisiae* growth under three Cd concentrations, as determined by OD_{600} in liquid SC media after 72h. WT: Wild type (DY1457); MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). Different letters represent significant differences among treatments by Tukey test (ANOVA; $p = 0.00085$).

Transformed yeast accumulated high contents of Cd within cells, with concentrations at least 30 times higher than the strains carrying empty vectors only (Figure 5.4 A). In the WT yeast, internal Cd uptake was similar regardless of media concentration, but in transformed yeast accumulation significantly increased under the higher Cd dose (30 µM). In order to convert the OD_{600} values into CDW (cell dry weight) and express the results in µg of Cd per g CDW, the following equation was used:

$$CDW_{(mg/ml)} = 2.496 \times OD_{600} + 0.0303$$

This equation was obtained by a linear regression analysis between CDW (mg mL^{-1}) and OD_{600} values of 15 samples at different growth stages ($R^2 = 0.974$; $p < 0.001$); with 94.8% of Min/Max accuracy and 5.6% of MAPE (mean absolute percent error) (Figure S5.3).

In terms of Cd removal from the media (%), which includes internal Cd accumulation, cell wall binding and sorption processes; the transformed yeast removed around 84% and 77% of the total Cd concentration initially added (10 µM and 30 µM, respectively) in a 72 h period, while in WT strain those values were on average under 30% (Figure 5.4 B).

5.3.3 Mutated PtMT2b gene slightly increases yeast growth under Fe and Mn deficiency

Spot assay of transformed mutant yeasts under deficient conditions showed that *PtMT2b* 'Y' could not recover the growth double mutant *zrt1zrt2* under Zn deficiency, but slightly promoted growth in *SMF1* and *fet3fet4* strains in Mn and Fe deficient plates, respectively (Figure 5.5). From those strains, *PtMT2b* 'Y' effect appeared to be more pronounced only in *SMF1* (Figure 5.5 B). For quantification purposes, this mutant strain was cultivated in liquid media under Mn deficiency, which then *SMF1* + *PtMT2b* 'Y' had on average 71% higher growth (OD_{600}: 0.90) than when carrying an empty vector (OD_{600}: 0.52) (ANOVA; p = 0.008).

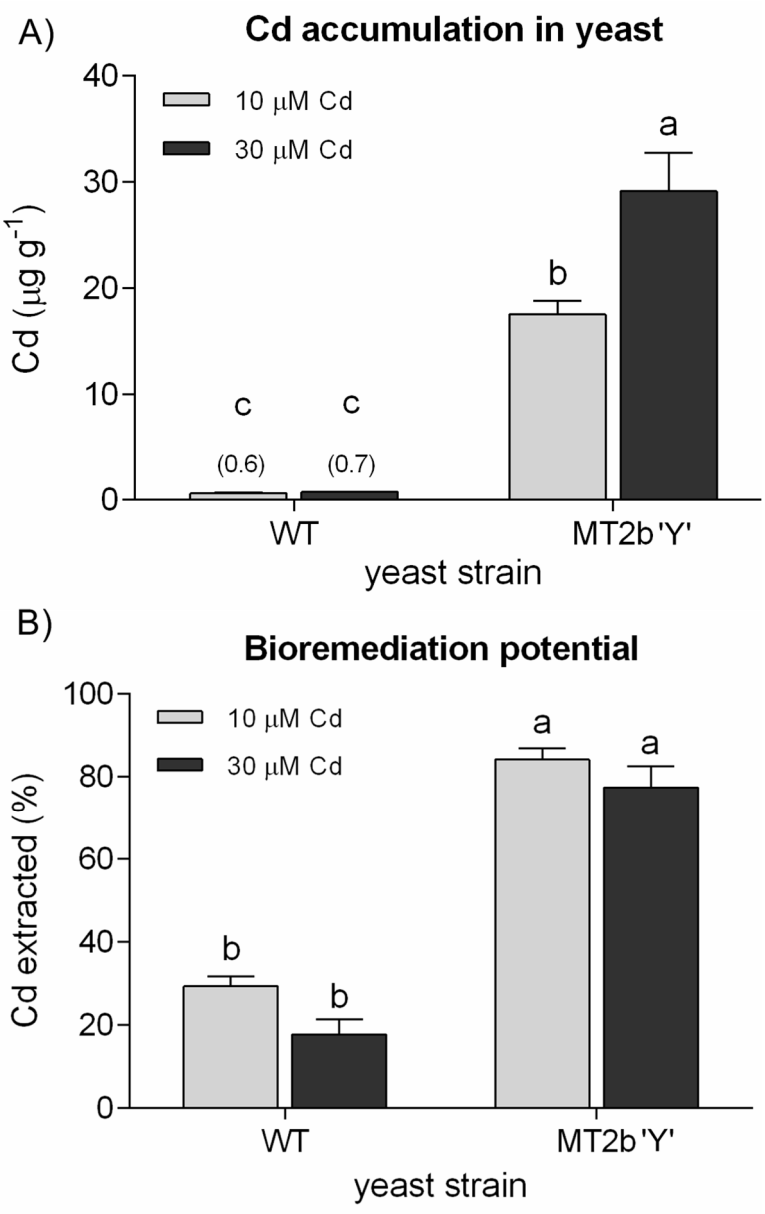


Figure 5.4 Cd accumulation in *S. cerevisiae* strains after 72 hours of growth. WT: Wild type + empty vector (DY1457); MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). A) Amount of Cd in dried yeast cells (μg g^{-1}) after EDTA washing and acid digestion. B) Percentage of Cd removal from liquid media after yeast growth (72 h). Different letters represent significant differences among treatments by Kruskal-Wallis and Dunn test (p = 0.004) in A); and by Tukey test (ANOVA; p < 0.001) in B).

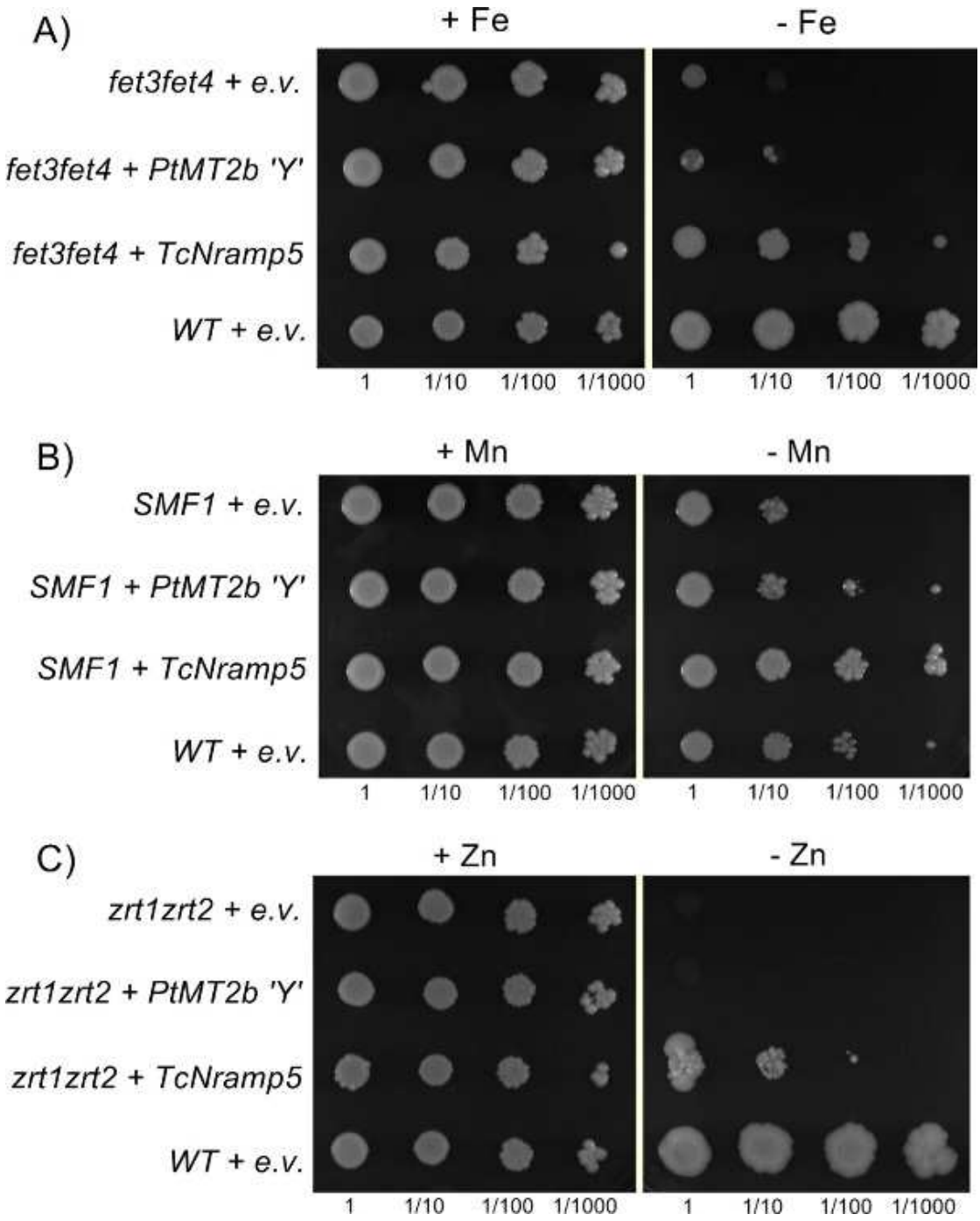


Figure 5.5 Growth of mutant *S. cerevisiae* strains in nutrient sufficient (+ X) and nutrient deficient (- X) plates for 72 hours. Yeast strains were: *fet3fet4*, with double mutation for Fe uptake (A); *SMF1*, with single mutation for Mn uptake (B); and *zrt1zrt2*, with double mutation for Zn uptake (C). WT: wild type; e. v.: empty vector (DY1457); *PtMT2b* 'Y': poplar metallothionein with cysteine to tyrosine replacement (C3Y); and *TcNramp5*: cocoa tree metal transporter Nramp5. Dilution 1 = 0.1 OD_{600}.

5.4 Discussion

5.4.1 Poplar's mutated metallothionein and Cd tolerance

As demonstrated previously, *P. trichocarpa*'s metallothionein MT2b is indeed able to increase Cd tolerance in transformed *S. cerevisiae* (Chapter 4). Besides chelating and inactivating metals in their toxic forms, such as Cd^{2+}, MTs have a role in scavenging reactive oxygen species (ROS) from cells under stress (Wong et al. 2004; Ruttkay-Nedecky et al. 2013). Genes for ROS tolerance are highly expressed in WT *S. cerevisiae* exposed to Cd (Thorsen et al. 2009), therefore it is clear that the addition of *PtMT2b* would enhance Cd tolerance by producing even more ROS-scavenging proteins than a WT strain.

Heterologous expression of other plant metallothionein genes in yeast have been assessed under heavy metal stress, with similar results, but mostly from herbaceous plant species (Zhou and Goldsbrough 1994; Guo et al. 2008; Zhang et al. 2014; Zhang et al. 2014b). For Cd, metallothioneins from sunflower, rice, *Arabidopsis*, *Noccaea caerulescens*, and even mycorrhizal fungi *Rhizophagus irregularis* and *Hebeloma cylindrosporum* were shown to complement Cd sensitivity in mutant yeast (Farcasanu and Ruta 2017). In the present study, the non-transgenic strain (WT + empty vector) had a decrease in biomass of around 50% under 10 µM Cd in liquid media (Figure 5.3), which is in accordance with the results from Hosiner et al. (2014), who reported an EC50 (half maximal effective concentration) of 10 µM $CdCl_2$ for *S. cerevisiae*. In the transgenic strain, however, growth was barely affected even at 30 µM Cd, confirming our initial hypothesis that *PtMT2b* increases Cd tolerance.

Metallothioneins are characterised by their high content of Cys residues - generally 10 to 17 in plants – which are able to bind divalent metal cations in their sulfhydryl (R–SH) group, thus forming thiolate bonds (Hassinen et al. 2011; Nguyen et al. 2017) and, in the case of the type II sub-family, their amino-terminal portion has a highly conserved domain, starting with Cys-Cys arrangement (Bulgarelli et al. 2016). Because of this obvious role of the cysteine content in providing metal binding sites in these proteins, it was interesting to observe that *PtMT2b* 'Y', a gene encoding a MT with one fewer Cys residue (replaced by one tyrosine - Tyr), not only did not lose its function as we

hypothesised, but in fact enhanced Cd tolerance in transformed yeast. We could speculate two main reasons for this: 1) tyrosine's aromatic ring; 2) the position in which the substitution took place (C3Y).

Despite lacking the characteristic sulfhydryl group from Cys, Tyr has a phenolic aromatic ring, that can also effectively bind divalent cations such as Cd^{2+} in their aromatic structure forming tyrosine-metal complexes (Hu et al. 1995), from which different conformations have been proposed (Figure 5.6). In this sense, Vandenbossche et al. (2014) developed a synthetic material enriched with tyrosine molecules that was able to efficiently remove copper from contaminated waters. Another reason for increased Cd tolerance is also related to the aromatic group in Tyr, which can form a non-covalent bond with cationic metals, known as cation-π interactions. This interaction is essentially electrostatic, in which a cation is attracted to the negatively charged cloud of electrons from aromatic groups (π systems), and is considered one of the strongest noncovalent interactions (Ma and Dougherty 1997; Mahadevi and Sastry 2013). Although mostly reported for monovalent cations, cation-π can also happen with divalent metal ions, such as seen with Mg^{2+} (Stewart et al. 2013).

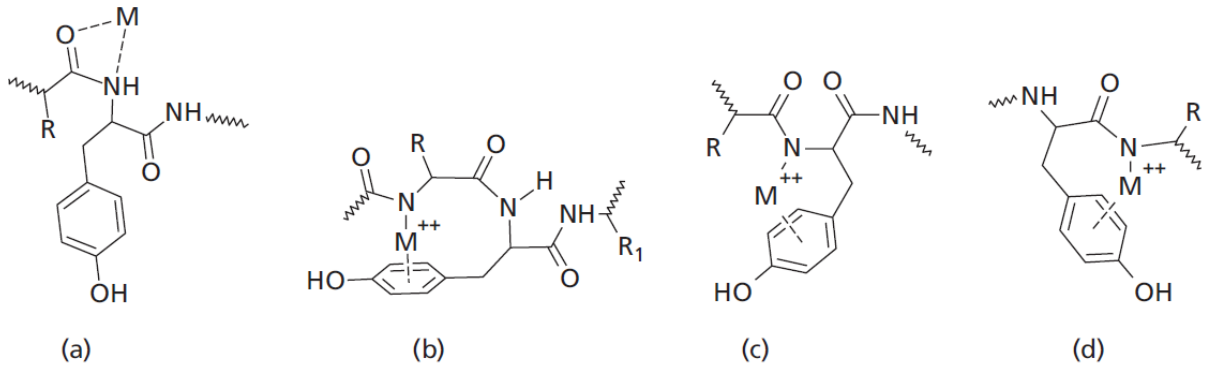


Figure 5.6 - Different conformations of tyrosine-based peptides for metal chelation. M^{++} represents a divalent metal cation (Source: Vandenbossche et al. 2014)

The position in which the substitution took place may possibly have influenced the results observed. Plant MTs have two short cystein-rich terminal domains linked by a long spacer, devoid of Cys, and of around 40 amino acids (Domenech et al. 2006). These Cys domains in opposite ends can interact with

each other and bind metals, forming a cluster, conferring the *hairpin* structure model typical of MT2 proteins (Hassinen et al. 2011). In the present work, *PtMT2b* 'Y' had only the third amino acid of the peptide chain (Cys) replaced by a Tyr (C3Y; Figure 5.1), which means that it is unlikely for it to have affected the overall protein folding, considering that this domain had another seven Cys residues to interact with the six Cys from the opposite domain. Moreover, the domain in which this substitution occurred may also explain why there was no loss of protein function. For instance, Cismowski et al. (1991) observed that yeast carrying a mutated MT gene (Cys to Tyr substitution) had a markedly lower resistance to Cd when it occurred in one domain (C50Y), but no effects when this mutation was present in another domain (C13Y).

5.4.1 Bioaccumulation and removal of Cd by transgenic yeast

Yeast can bioremediate metals from solutions by mainly two mechanisms, one is passive and requires no energy expenditure (e.g. cell wall binding and metal diffusion) and the other active, metabolism-dependent and being carried out only by living cells, involving compartmentalisation in subcellular organelles such as vacuole or mitochondria (Vijver et al. 2004; Wang and Chen 2009). Metal binding by metallothioneins is one of the most important strategies for metal accumulation (or toxicity avoidance) in living cells, a process seen in almost all eukaryotic organisms, such as animals, plants, yeast and ectomycorrhizal fungi (Vijver et al. 2004; Nguyen et al. 2017). Although in *S. cerevisiae* the induction of MT production seems to occur mainly through exposure to Cu (Wang and Chen 2006) or Ag (Hosiner et al. 2014).

Linear regression resulted in a good prediction model for converting OD_{600} measurements into cell dry weight (CDW) and allowed converting Cd concentrations in yeast to µg of Cd per gram of biomass. It should be noted, however, that those predictions should be applied only under the experimental conditions of the present work (strain type, growth period, temperature etc.), as well as the equipment use for OD_{600} determination, since it can vary according to the device used (Ude et al. 2014).

Transgenic strains carrying the mutated *PtMT2b* gene were not only highly tolerant but also effectively accumulated more Cd (in µg g^{-1}) than wild type yeast, with Cd contents at least 10 times higher, which supports our hypothesis that Cd tolerance can lead to enhanced Cd accumulation. Ruta et al. (2017) recently showed that *S. cerevisiae* transformed with *NctMT2a* and *NcMT2b* (from *Noccaea caerulescens*) had a 5-fold and a 4-fold increase in Cd accumulation, respectively, compared to the non-transformed strain. Yeast expressing *SaMT2* from hyperaccumulator *Sedum alfredii* also had a 50% increase in Cd accumulation in relation to the control (Zhang et al. 2014b). However enhanced Cd accumulation is not always observed, such as the case of the *S. cerevisiae* strains transformed with a range of MTs from *Arabidopsis thaliana* (Guo et al. 2008). Bacteria may also display similar effects, such as the *E. coli* expressing a metallothionein from mice (*mt-1*), in which the gene promoted higher tolerance and accumulation of mercury from contaminated media (Ruiz et al. 2011), and the *CeMT2b* gene from tolerant weed species *Colocasia esculenta*, that doubled Cd accumulation in *E. coli* (Kim et al. 2011).

Due to their biosorption characteristics, yeast cell walls can remove heavy metals from aqueous wastes even if the cells are no longer alive. Machado et al. (2008) verified that after applying dead *S. cerevisiae* biomass (12 mg mL^{-1}) in nickel contaminated water, almost 80% of the Ni^{2+} in solution was removed after only 30 minutes. By using the OD_{600} to CDW (mg mL^{-1}) conversion equation previously determined, we were able to estimate that despite removing around 80% of Cd^{2+} from the growth media, this amount would represent a biosorption capacity of 1.5 mg g^{-1} of dried yeast. Even though this assay ran for only 72 hours and did not reach saturation, the result is quite low compared to other biosorbent materials, such as dried chestnut burr, which is able to remove 16.2 mg of Cd per gram, pinecones (4.3 mg g^{-1}) or the breakthrough biosorbent known as MMBB (a mix of tea wastes, mandarin peels and maple leaves), which can absorb 31.7 mg g^{-1} of Cd from solution (Kim et al. 2015; Abdolali et al. 2016). However, those are dead materials, and are not susceptible to metal toxicity effects. Living yeasts provide a constant source of biosorbent material, which is also able to actively accumulate metals within cells, removing metals

continuously through internal detoxification mechanisms (Wang and Chen, 2006). In this sense, Amirnia et al. (2015) developed a continuous bioreactor-biosorption system, which is efficient for simultaneous production of *S. cerevisiae* and removal of Cu^{2+} and Pb^{2+} from liquid waste without requiring much nutritional input for yeast growth. The authors also suggested that this process is facilitated by using flocculant strains that are easily able to decant and separate from the growth solution (Soares 2011), a feature that was observed in the WT strains in the present work.

5.4.3 Transgenic yeast under nutrient deficiency

Considering the evidence that metals such as Zn and Cu can affect the expression of MT2b in white poplar (Cicatelli et al. 2010), and both Cd and Zn concentrations were verified to be highly correlated to *MT2b* expression in leaves of *P. tremula* x *P. tremuloides* (Hassinen et al. 2009), we hypothesised that the double mutant strain *zrt1zrt2*, lacking two Zn transporters, would have increased growth if carrying the *PtMT2b* gene. This was based on the concept that prior to yeast inoculation into the Zn-depleted media, during pre-growth stage, transgenic yeast would have built up a larger nutrient storage capacity within their cells by forming MT-Metal chelates, which could then be accessed under nutrient deficiency. The same was tested for Fe and Mn, using their respective mutant strains, for it is known that MTs are also able to bind these metals (Farcasanu and Ruta 2017), for example, yeast expressing plant gene *PutMT2* under toxic metal concentrations had enhanced accumulation of Fe, Man Zn and Ag, usually promoting tolerance as well, expect for Mn, Cu and Ni (Zhang et al. 2014).

In our work, the spot assay showed that mutant strain *zrt1zrt2* had no effects from *PtMT2b* ‘Y’ transformation under Zn deficiency, showing virtually no growth. One reason could be the double mutation did not allow enough Zn to penetrate the yeast cells during pre-growth. *S. cerevisiae* acquires Zn via mainly three transporters: Zrt1 (high affinity), Zrt2 (low affinity) and Fet4 (non-specific), therefore this mutation severely hinders Zn acquisition pathways (Zhao and Eide 1996; Schothorst et al. 2017). The *PtMT2b* gene was also shown to have slightly

lower expression in poplars under high Zn concentrations (Chapter 4), so it is probably involved in Zn binding, however it is also possible that the amino acid substitution (C3Y) in this gene could have lead to an MT with lower Zn affinity, resulting in poor Zn storage.

Transgenic yeasts were able to grow, to some extent, in Mn- and Fe- agar deficient media, confirming in part the initial hypothesis, although only by verifying metal contents intracellularly would allow a more empirical conclusion. In liquid media, transgenic *SMF1* strains had a 71% increase in growth under Mn deficiency, suggesting that this gene is involved in Mn binding. The involvement of MTs in Mn homeostasis has not been thoroughly explored in plants thus far, and usually between them is not commonly observed (Chyan et al. 2005), except for a few studies with MTs from animals or plants (Kobayashi et al. 2007; Benatti et al. 2014).

5.5 Conclusions

Heterologous expression of the metallothionein gene (MT2b) from *Populus trichocarpa* is able to confer tolerance to *S. cerevisiae* under Cd concentrations up to 50 µM. Replacement of Cys by Tyr (C3Y) in the amino acid sequence did not affect protein function, and, in fact, increased yeast growth under Cd. The transgenic strains carrying the mutated gene were able to extract up to 80% of Cd from contaminated media solution, mostly due to continuous growth and constant metal biosorption. This specific strain offers great potential for bioremediation Cd from waters or effluents, and further studies should be carried out to assess its potential use in a mixture of cationic metals, such as Zn, Mn or Cu, as well as tested on different bioreactor systems.

5.6 References

Abdolali A, Ngo HH, Guo W, et al. **2016**. A breakthrough biosorbent in removing heavy metals: Equilibrium, kinetic, thermodynamic and mechanism analyses in a lab-scale study, *Science of the Total Environment*. **542**: 603-611.

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

Amirnia S, Ray MB, Margaritis A. 2015. Heavy metals removal from aqueous solutions using *Saccharomyces cerevisiae* in a novel continuous bioreactor–biosorption system, *Chemical Engineering Journal*. **264**: 863-872.

Benatti MR, Yookongkaew N, Meetam M et al. **2014**. Metallothionein deficiency impacts copper accumulation and redistribution in leaves and seeds of Arabidopsis, *New Phytologist*. **202**: 940-951.

Brady D, Duncan JR. 1994. Bioaccumulation of metal cations by *Saccharomyces cerevisiae*, *Applied Microbiology and Biotechnology*. **41**: 149-154.

Bulgarelli RG, Araujo P, Tezotto T, Mazzafera P, Andrade SAL. 2016. Expression of metallothionein genes in coffee leaves in response to the absence or excess of Cu and Zn, *Theoretical and Experimental Plant Physiology*. **28**: 371-383.

Chen C, Wang J. 2008. Removal of Pb^{2+}, Ag^{+}, Cs^{+} and Sr^{2+} from aqueous solution by brewery's waste biomass, *Journal of Hazardous Materials*. **151**: 65-70.

Chyan CL, Lee TTT, Liu CP, Yang YC, Tzen TC. **2005**. Cloning and expression of a seed-specific metallothionein-like protein from Sesame, *Bioscience, Biotechnology, and Biochemistry*. **69**: 2319-2325.

Cismowski MJ, Narula SS, Armitage IM, Chernaik ML, Huang PC. 1991. Mutation of invariant cysteines in mammalian metallothionein alters metal binding capacity, cadmium resistance and ^{113}Cd NMR spectrum, *The Journal of Biological Chemistry*. **266**: 24390-24397.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis, *Annual Review in Plant Biology*. **53**: 159-182.

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

Domenech J, Mir G, Huguet G, Capdevila M, Molinas M, Atrian S. 2006. Plant metallothionein domains: functional insight into physiological metal binding and protein folding, *Biochimie*. **88**: 583-593.

Farcasanu IC, Ruta LL. 2017. Metallothioneins, *Saccharomyces cerevisiae*, and heavy metals: a biotechnology triad? In: Lucas C, Pais C, eds. *Old Yeasts - New Questions*, InTech Open, 21-39.

Goksungur Y, Uren S, Guvenc U. 2005. Biosorption of cadmium and lead ions by ethanol treated waste baker's yeast biomass, *Bioresource Technology*. **96**: 103-109.

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.

Hassinen V, Vallinkoski VM, Issakainen S et al. **2009**. Correlation of foliar MT2b expression with Cd and Zn concentrations in hybrid aspen (*Populus tremula* x *tremuloides*) grown in contaminated soil, *Environmental Pollution*. **157**: 922-930.

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.

Hosiner D, Gerber S, Lichtenberg-Frate H, et al. 2014. Impact of acute metal stress in *Saccharomyces cerevisiae*, *PLoS ONE*. **9**: e83330.

Hu P, Sorensen C, Gross ML. 1995. Influences of peptide side chains on the metal ion binding site in metal ion-cationized peptides: participation of aromatic rings in metal chelation, *Journal of The American Society for Mass Spectrometry*. **6**: 1079-1085.

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.

Jamali M, Kazi TG, Arain MB, Afridi HI et al. 2009. Heavy metal accumulation in different varieties of wheat (*Triticum aestivum* L.) grown in soil amended with domestic sewage sludge, *Journal of Hazardous Materials*. **164**: 1386-1391.

Janssen MPM, Ma WC, Van Straalen NM. 1993. Biomagnification of metals in terrestrial ecosystems, *Science of the Total Environment*. **134**: 511-524.

Jin S, Sun D, Wang J, Li Y, Wang X, Liu S. **2014**. Expression of *rgMT* gene, enconding for a rice metallothionein-like protein in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, *Journal of Genetics*. **93:** 709-718.

Joutey NT, Bahafid W, Sayel H, Ghachtouli NE. 2013. Biodegradation: involved microorganisms and genetically engineered microorganisms. In: Chamy R, ed. *Biodegradation - Life of Science*, InTech, Rijeka, 289-320.

Karathia H, Vilaprinyo E, Sorribas A, Alves R. 2011. *Saccharomyces cerevisiae* as a model organism: a comparative study, *PLoS ONE*. **6**: e16015.

Khan MA, Khan S, Khan A, Alam M. 2017. Soil contamination with cadmium,

consequences and remediation using organic amendments, *Science of the Total Environment*. **601-602**: 1591-1605.

Kim N, Park M, Park D. **2015**. A new efficient forest biowaste as biosorbent for removal of cationic heavy metals, *Bioresource Technology*. **175**: 629-632.

Kim YO, Patel DH, Lee DS, Song Y, Bae HJ. **2011**. High cadmium-binding ability of a novel *Colocasia esculenta* metallothionein increases cadmium tolerance in *Escherichia coli* and tobacco, *Bioscience, Biotechnology, and Biochemistry*. **75**: 1912-1920.

Kobayashi K, Kuroda J, Shibata N et al. **2007**. Induction of metallothionein by manganese is completely dependent on interleukin-6 production, *The Journal of Pharmacology and Experimental Therapeutics*. **320**: 721-727.

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

Kulshreshtha S. 2013. Genetically engineered microorganisms: a problem solving approach for bioremediation, *Journal of Bioremediation and Biodegradation*. **4**: e133.7

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.

Ma JC, Dougherty D. 1997. The Cation−π Interaction, *Chemical Reviews*. **97**: 1303-1324.

Machado MD, Santos MSF, Gouveia C, Soares HMVM, Soares EV. 2008. Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: the flocculation as a separation process, *Bioresource Technology.* **99**: 2107-2115.

Mahadevi AS, Sastry GN. 2013. Cation−π interaction: its role and relevance in chemistry, biology, and material science, *Chemical Reviews*. **113**: 2100-2138.

Marques PASS, Rosa MF, Pinheiro HM. 2000. pH effects on the removal of Cu^{2+}, Cd^{2+} and Pb^{2+} from aqueous solution by waste brewery biomass, *Bioprocess Engineering*. **23**: 135-141.

Mirlean N, Roisenberg A. 2006. The effect of emissions of fertilizer production on the environment contamination by cadmium and arsenic in southern Brazil, *Environmental Pollution*. **143**: 335-340.

Nguyen H, Rineau F, Vangronsveld J, Cuypers A, Colpaert JV, Ruytinx J. **2017**. A novel, highly conserved metallothionein family in basidiomycete fungi and characterization of two representative *SlMTa* and *SlMTb* genes in the

ectomycorrhizal fungus *Suillus luteus*, *Environmental Microbiology*. **19**: 2577-2587.

Oliveira RPS, Basso LC, Junior AB et al. 2012. Response of *Saccharomyces cerevisiae* to cadmium and nickel stress: the use of the sugar cane vinasse as a potential mitigator, *Biological Trace Element Research*. **145**: 71-80.

Pankiewicz U, Sujka M, Jamroz J. 2015. Bioaccumulation of the selected metal ions in *Saccharomyces cerevisiae* cells under treatment of the culture with Pulsed Electric Field (PEF), *The Journal of Membrane Biology*. **248**: 943-949.

Ruiz ON, Alvarez D, Gonzalez-Ruiz G, Torres C. 2011. Characterization of mercury bioremediation by transgenic bacteria expressing metallothionein and polyphosphate kinase, *BMC Biotechnology*. **11**: 1-8.

Ruta LL, Lin YF, Kissen R, Nicolau I, et al. 2017. Anchoring plant metallothioneins to the inner face of the plasma membrane of *Saccharomyces cerevisiae* cells leads to heavy metal accumulation, *PLoS ONE*. **12:** e0178393.

Ruttkay-Nedecky B, Nejdl L, Gumulec J, et al. 2013. The role of metallothionein in oxidative stress, *International Journal of Molecular Sciences*. **14**: 6044-6066.

Schothorst J, Van Zeebroeck G, Thevelein JM. 2017. Identification of Ftr1 and Zrt1 as iron and zinc micronutrient transceptors for activation of the PKA pathway in *Saccharomyces cerevisiae*, *Microbial Cell*. **4**: 74-89.

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.

Singh S, Kang SH, Mulchandani A, Chen W. 2008. Bioremediation: environmental clean-up through pathway engineering, *Current Opinion in Biotechnology*. **19**: 437–444.

Smolders E, Mertens J. 2013. Cadmium. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, 283–311.

Soares EV. 2011. Flocculation in *Saccharomyces cerevisiae*: a review, *Journal of Applied Microbiology*. **110**: 1-18.

Sriprang R, Hayashi M, Ono H et al. 2003. Enhanced accumulation of Cd^{2+} by a *Mesorhizobium* sp. transformed with a gene from *Arabidopsis thaliana* coding for phytochelatin synthase, *Applied And Environmental Microbiology*. **69**: 1791-1796.

Stewart M, Dunlap T, Dourlain E, Grant B, McFail-Isom L. 2013. Cations form sequence

selective motifs within DNA grooves via a combination of cation-pi and ion-dipole/hydrogen bond interactions, *PLoS ONE*. **8**: e71420.

Thorsen M, Perrone GG, Kristiansson E, et al. 2009. Genetic basis of arsenite and cadmium tolerance in *Saccharomyces cerevisiae*, *BCM Genomics*. **10**: 105.

Ude C, Schmidt-Hager J, Findeis M, John GT, Scheper T, Beutel S. 2014. Application of an online-biomass sensor in an optical multisensory platform prototype for growth monitoring of biotechnical relevant microorganism and cell lines in single-use shake flasks, *Sensors*. **14**: 17390-17405.

Ullah I, Wang Y, Eide DJ, Dunwell JM. 2018. Evolution, and functional analysis of Natural Resistance-Associated Macrophage Proteins (NRAMPs) from *Theobroma cacao* and their role in cadmium accumulation, *Scientific Reports*. **8**: 14412.

Vandenbossche M, Dehaese A, Casetta M, Jimenez M, Traisnel M. 2014. Tyrosine: an efficient natural molecule for copper remediation, *Green Materials*. **3**: 1-9.

Vijayaraghavan K, Balasubramanian R. 2015. Is biosorption suitable for decontamination of metal-bearing wastewaters? A critical review on the state-of-the-art of biosorption processes and future directions, *Journal of Environmental Management*. **160**: 283-296.

Vijver MG, Gestel CAM, Lanno RP, Van Straalen NM, Peijnenburg WJGM. **2004**. Internal metal sequestration and its ecotoxicological relevance: a review, *Environmental Science and Technology*. **38**: 4705-4712.

Wang J, Chen C. 2006. Biosorption of heavy metals by *Saccharomyces cerevisiae*: a review, *Biotechnology Advances*. **24**: 427-451.

Wang J, Chen C. 2009. Biosorbents for heavy metals removal and their future, *Biotechnology Advances*. **27**: 195-226.

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.

Zhang J, Zhang M, Tian S, Lu L, Shohag MJI, Yang X. 2014b. Metallothionein 2 (*SaMT2*) from *Sedum alfredii* Hance confers increased Cd tolerance and accumulation in yeast and tobacco, *PLoS ONE*. **9**: e102750.

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.

Zhao H, Eide D. 1996. The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*, *The Journal of Biological Chemistry*. **271**: 23203-23210.

Zhou J, Goldsbrough PB. 1994. Functional homologs of fungal metallothionein genes from *Arabidopsis*, *The Plant Cell*. **6**: 875-884.

5.7 Supplementary Files

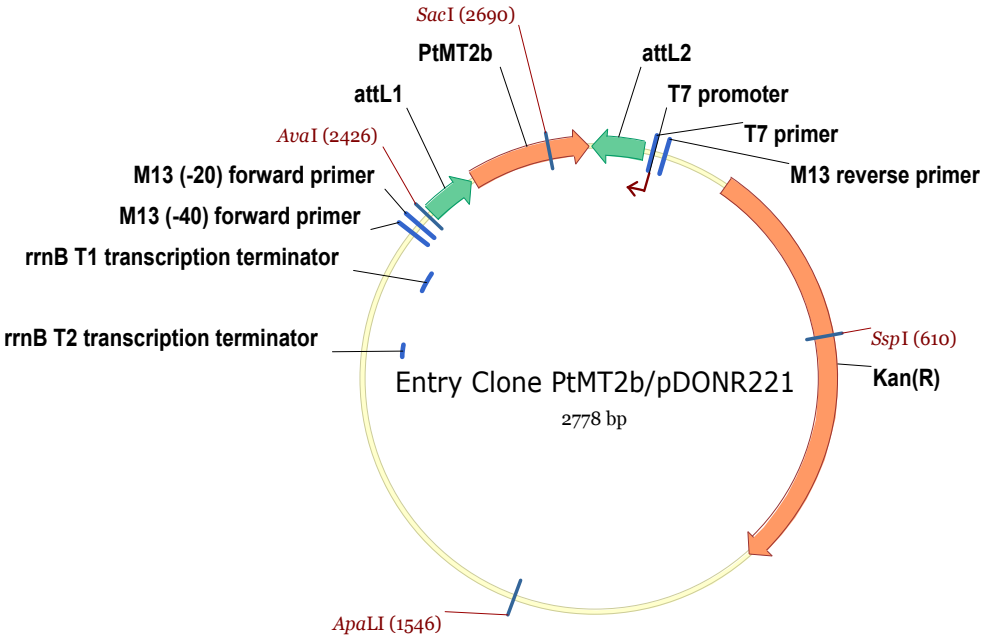


Figure S5.1. Entry clone pDONR221 carrying *PtMT2b* and a gene responsible for Kanamycin resistance, used for transformation and cloning in *E. coli* competent cells.

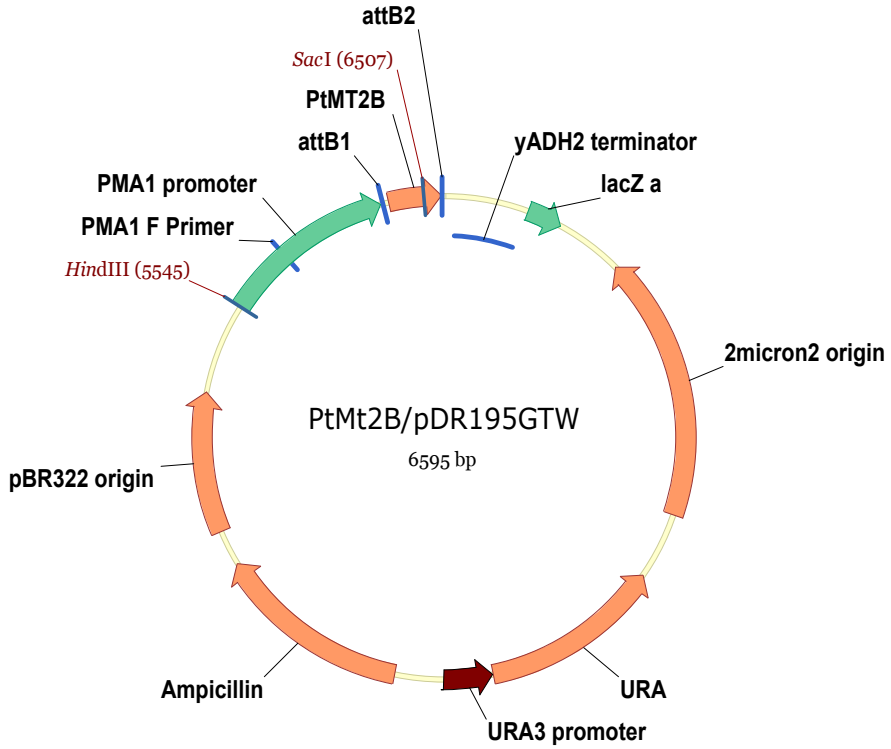


Figure S5.2. Expression vector pDR195GTW carrying *PtMT2b* and a gene responsible for uracyl production (URA) used for the transformation of yeast competent cells.

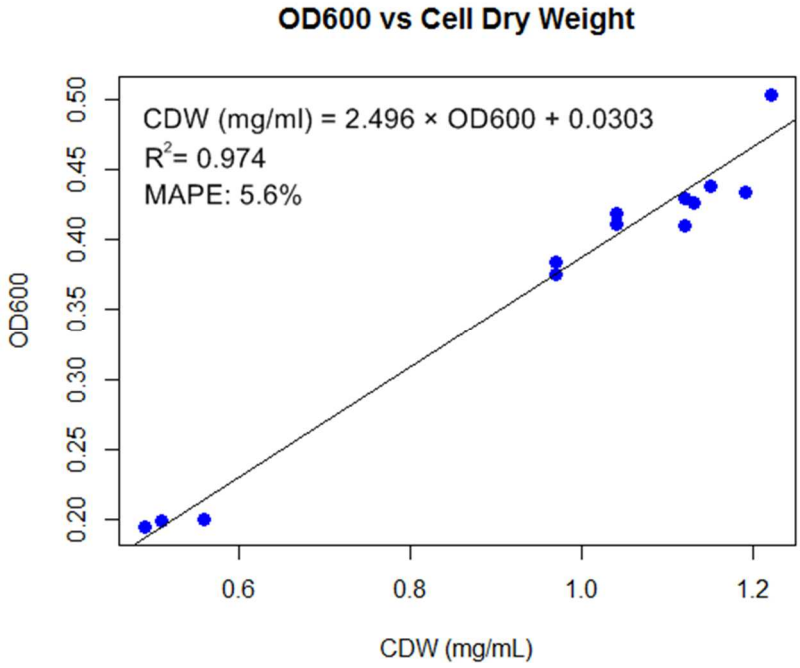


Figure S5.3. Linear regression between OD_{600} values and yeast cell dry weight (CDW) (n = 15). Equation, R^2 and MAPE (mean absolute percent error) presented were all significant ($p < 0.05$).

Chapter 6

General discussion

Miscellaneous photos taken during this PhD

The initial goal of this PhD project was to evaluate the potential of *Populus trichocarpa* for phytoremediation of soils contaminated by Cd and Zn. However, by following the results at each step (i.e. experimental assays and preliminary tests), along with the information inferred and drawn from the literature, more questions emerged, which guided new developments in my research hypotheses.

This chapter will cover the overall findings in this thesis and what they mean in terms of practical application, as well as some of the constraints and limitations encountered and the possibilities for future research arising from the data generated in this work.

6.1 Fundamental scientific findings

Although this thesis adds to the practical knowledge necessary for investigating and enhancing the potential of poplar trees in Cd and Zn phytoremediation, it also provides some fundamental knowledge on metal ecotoxicity, plant uptake and their associated mechanisms.

Chapter 2 shows that Cd and Zn toxicity thresholds in ectomycorrhizal fungi (*in vitro*) are dependent on the type of media used, in which solid medium allows not only a more realistic threshold determination, but also provides the assessment of mycelial density as possible mechanism for Cd tolerance (Table 2.2). It was shown that Zn is not necessarily able to alleviate Cd toxicity under these conditions, sometimes causing a synergistic toxicity (Figure 2.4).

In chapter 3, I was able to show that the restriction of root-to-shoot translocation in poplars is a major mechanism for avoiding Cd and Zn toxicity (Table 3.2; Figure 3.4), and that it involves the down-regulation of *PtHMA4* in roots (Figure 3.5). This chapter also confronts the common assumption that Zn amendment will decrease Cd uptake by plants, wherein I have demonstrated that, under those experimental conditions, the opposite occurs in *P. trichocarpa*.

In chapter 4, I demonstrated that Cd and Zn uptake and accumulation differ in mycorrhizal poplars as hypothesised, in which the former is mostly immobilised in roots and the latter is highly accumulated in terms of mg of Zn per plant. By assessing some gene expression responses to metals as well as mycorrhization, I could demonstrate that *PtHMA4* and *PtZIP1* are affected by both Cd and Zn,

although not to the same extent (Figures 4.3 and 4.4). I was also able to show that inoculation of poplars with *R. irregularis* up-regulated the expression of *PtMT2b* in roots (Figure 4.3F) and that this gene was highly correlated to the AM fungi colonisation rates and the sequestration of Cd in mycorrhizal roots (Figure 4.5). To the best of my knowledge, the function *PtMT2b* has not been tested in yeast until now, and here I could clearly demonstrate its involvement in enhancing Cd tolerance, therefore confirming our hypothesis (Figure 4.6).

In chapter 5, I showed that not only *PtMT2b* increases Cd tolerance in yeast, but also increases its intracellular accumulation (Figures 5.2 and 5.3). In this chapter, I also discovered, rather accidentally, that a substitution in the peptide sequence of *PtMT2b* (a tyrosine instead of a cysteine on the third position; C3Y) has an even greater effect in conferring Cd tolerance in yeast (Figure 5.4), despite this substitution leading to one fewer Cys in the protein, a crucial amino acid in metallothioneins. This was an unexpected result and, to the best of my knowledge, such occurrence has not been verified elsewhere in plant MTs. Finally, within this chapter I showed that a mutant yeast (*SMF1*) was able to grow slightly better in Mn-deficient medium when carrying the mutated poplar gene (*PtMT2b* 'Y'), suggesting its involvement in Mn chelation (Figure 5.5).

6.2 Is Populus trichocarpa useful for phytoremediation?

Phytoremediation is a solar-driven, *in situ*, and natural technology that can clean up soils from heavy metals or other contaminants (Sas-Nowosielska 2011). In addition it has a lower installation and maintenance cost (almost 5% of other remediation technologies), without detrimental impacts to the topsoil (Ali et al. 2013). For an effective Cd and Zn phytoremediation process, ideal plants need to have rapid growth, high biomass production, deep root systems, high tolerance to toxicity and metal accumulation capacity, and not be a direct source of food for humans or herbivores (Yadav et al. 2018). A plant that perfectly fulfils all these criteria is yet to be discovered (Mahar et al. 2016). Moreover, the ability to attract and develop relationships with mutualistic microorganisms is definitely desirable, for they can not only favour plant growth and the remediation process itself, but also increase metal availability along with microbial diversity in the rhizosphere,

thus promoting better conditions in contaminated soils for other species to flourish (Audet 2014).

Populus trichocarpa is a plant species that possesses most of those characteristics. In the present work, I demonstrated that when exposed to elevated Cd and Zn simultaneously (Figure 3.6), which is often the case in contaminated soils, leaves and stems concentrations reached hyperaccumulator levels of Cd (> 100 mg kg^{-1} dry biomass), without apparent phytotoxicity, which is also a requirement for considering a plant as hyperaccumulator (Baker et al. 2000; Ali et al. 2013). It should be clear, however, that the fact high metal concentrations were found in shoots of a given plant does not necessarily mean that they are hyperaccumulators, especially if plants were not grown under natural conditions. Van der Ent et al. (2013) argue that experiments under artificial contamination ('spiked' soils), either in hydroponics or other substrates are not sufficient to define a species as a hyperaccumulator, and understandably so. For instance, environmental factors and soil properties will largely affect these accumulation values, mostly the soil pH, texture, organic matter content, microbiota composition and fertility status, which will all have a direct effect on metal availability for plant uptake. Likewise humidity and temperature can alter water balance and leaf transpiration process, with an obvious consequence to the accumulation potential of plants (Yadav et al. 2018).

This variation can be exemplified by the contrasting Cd accumulation values found between the third and fourth chapters in this thesis. The experiment described in chapter 3 was carried out in glasshouse conditions and consequently plants were exposed to varying temperatures, from 22.1 to 26.9°C. In addition, all substrates were spiked by metal solutions in one single pulse. As for the following experiment, poplars were put into growth chambers with temperature control set to 23°C, while this time metal additions were applied gradually, over a three-day period. Results diverged as a consequence and, despite similar Cd concentrations in poplar roots and stems for both cases (Cd treatment of 81 mg kg^{-1}), the leaves accumulated around five times more Cd (~ 45 mg kg^{-1}) under glasshouse conditions (at higher temperatures) than when in growth chambers (~ 8.5 mg kg^{-1}). The effect of temperature in phytoextraction was studied in willows by Yu et al.

(2010), whose results showed higher and faster chromium accumulation with increasing temperatures.

Nonetheless, the metal extraction capacity of *P. trichocarpa* stems are on average of at least 40 mg kg^{-1} for Cd and around 500 mg kg^{-1} for Zn, considering all experiments in this thesis. Those numbers allow the estimation of how much metal could be extracted from contaminated soils by poplar trunks (harvestable wood), not considering of course the obvious variation arising from extrapolating pot experiments into large scale field applications, longer timeframes and genotypic differences among poplar ecotypes.

For this purpose, we can utilise the equation (eq. 4) suggested by Antoniadis et al. (2017), which allows the estimation of total metal uptake (kg metal ha^{-1} $year^{-1}$) and the timescale (in years) necessary to remediate soils to below critical levels:

$$t_R = \Delta[M]soil \times \frac{SWH}{AMU} \qquad (4)$$

In which: **Δ $[M]_{soil}$** (mg of metal per kg of soil) is the difference between the initial soil concentration of the contaminated soil and the final concentration desired after remediation. **SWH** is the soil weight in a hectare (kg soil ha^{-1}), and equals: $10^7 \times \rho_b \times D$. In which ρ_b is the dry bulk density of soil (g cm^{-3}) and D is the depth of soil to be remediated (in meters); 10^7 is a conversion factor calculated by the authors, involving soil volume, weight and density (Antoniadis et al. 2017; see *Supplementary Information*). **AMU** is the annual metal uptake and equals: $[M]_{plant}$ × Y, in which $[M]_{plant}$ is the metal concentration in the plant (mg kg^{-1}) and Y is the annual plant yield (as aboveground harvestable dry biomass).

Considering the average yield of poplar trees to be around 10 to 30 t ha^{-1} $year^{-1}$ poplar (Dillen et al. 2013; Searle and Malins 2014; Verlinden et al. 2015) and the Cd accumulation potential of 40 mg kg^{-1} or 100 mg kg^{-1} (when exposed to Zn simultaneously); it could be estimated that, in order to decrease Cd concentrations of a contaminated soil from 7 mg kg^{-1} Cd (He et al. 2015), for instance, to 3 mg kg^{-1} – which is the limit according to the Council of the European Communities Directive, 86/278/EEC (CEC 1986) - it would take around 20 years if

Cd accumulation is of 40 mg kg^{-1}, or eight years if accumulation is stable at 100 mg kg^{-1}. This time scale could be further reduced considering that older poplar trees usually have a higher accumulation of Cd in barks (Zarubova et al. 2015).

For Zn however, even if we consider the highest values obtained for Zn accumulation in stems in this thesis, on average 700 mg kg^{-1} Zn, it would require at least 771 years to decrease soil Zn concentrations from 2900 mg kg^{-1} Zn (average in contaminated European soils – Salminen et al. 2005) to 200 mg kg^{-1}, which is a common target value for Zn concentration in affected soils (Alloway 2008).

These figures are only estimations based on a single equation and does not account for several factors influencing yield production, metal extraction such as annual weather cycles, innate soil fertility and extreme climatic events. However, it does suggest that this particular poplar ecotype (i.e. 'Trichobel') is not very a promising candidate for Zn phytoextraction. Yet, for Cd, it seems to be a good alternative, especially if the technique is combined with other available methods, for example: biochar application, use of chelating agents to increase uptake, manipulation of soil pH as well as the association between the plant and the soil microbiota (Yadav et al. 2018). I have shown that mycorrhizal symbiosis with *R. irregularis* in fact decreases Cd extraction by immobilising the metal in poplar roots (phytostabilisation) (Figure 4.2A). However, this is useful if the goal is to immobilise the metal and prevent toxicity to other organisms, leaching and/or groundwater contamination (Ali et al. 2013; Montpetit and Lachapelle 2017).

It must be stressed here, that such estimation is just an exercise, since it is based mostly on our results from pot experiments, but it seems that in the context presented, *P. trichocarpa* has indeed potential to be used in phytoremediation schemes, albeit not as effective yet, it has all the other requirements of an ideal plant for such purpose. Moreover, poplars in general have more traits and uses other than accumulating metals, which would make them even more attractive for soil remediation.

6.2.1 Poplar as a bioenergy source

In order to reduce the human dependency on non-renewable fossil fuels, decrease CO_2 emissions to the atmosphere and cater to an increasing population,

bioenergy is gaining more interest worldwide. Bioenergy is the name given to energy derived from biomass, usually heat, electricity and transport fuels (Creutzig et al. 2015). The use of energy crops for bioenergy generation, such as biofuel, is considered a renewable and sustainable energy source with a closed carbon-cycle system that does not contribute to greenhouse effects (Pandey et al. 2016).

Poplars are high-yielding perennial trees that have been commonly used for bioenergy, within a short-rotation coppice (SRC) system, and are useful for thermal energy, electricity and bioethanol production (Sannigrahi et al. 2010; Manzone et al. 2014; Sabatti et al. 2014). SRC is an old concept in which a tree is cut at the base in order to mimic a natural disturbance, which results in new shoots regenerating and sprouting from the stumps and, not only does this avoid the need for replanting, but can also lead to higher yields in the following growing seasons (Blake 1983; Sabatti et al. 2014; Verlinden et al. 2015). In addition, poplars grown in a sustainable SRC system for energy production may improve carbon sequestration as well as decrease greenhouse gas emissions and mitigate climate change, especially compared to energy derived from fossil fuels (Whitaker et al. 2018).

6.2.2 Phytoremediation wood as source of biomass

A common obstacle regarding the use of land for bioenergy production is the concern that it may displace existing productive lands used for food crops (Whitaker et al. 2018). Thus the use of contaminated lands - which are often unsafe for food production and cannot sustain a rich biodiversity - to implement a bioenergy production system might be a solution (Pandey et al. 2016). In this sense, poplar species have another advantage in comparison to other energy crops, which is their ability to grow and remediate polluted soils, either with organic or inorganic contaminants (Gullner et al. 2001; Brentner et al. 2010; Guerra et al. 2011). *Populus* species can also rapidly invade disturbed sites, reproduce asexually and are not a source of food for farm animals, consequently reducing the risk of heavy metals entering the human food chain (Sebastiani et al. 2004; Hamberg et al. 2011; Shim et al. 2013). In this thesis, I have demonstrated the potential of *P. trichocarpa* cv 'Trichobel' in growing under extreme Cd

concentrations (up to 81 mg kg^{-1}), and to stabilise high amounts of Cd in roots, especially under mycorrhizal symbiosis, which makes this poplar variety a good candidate for further studies in terms of energy production (biomass) in contaminated environments under SRC systems

Another concern for bioenergy production using woody species in SCR systems is that it may not be as profitable as other energy crops (Schweier and Becker 2013). A study from Manzone et al. (2014) concluded that a poplar plantation for bioenergy production in Italy was not able to achieve full sustainability without increasing the price of the biomass, or without economic support for the production. In this sense, combining bioenergy production with phytoremediation, could be an alternative to obtain financial incentive or subsidy from governments interested in land reclamation, bioenergy production and climate change mitigation.

Using contaminated biomass for energy production also appears to be the best strategy for the common conundrum of how to deal with the plants after phytoremediation. According to Witters et al. (2012), willow biomass from phytoremediation sites could be used, for instance, in co-combustion with coal, for heating purposes to replace cokes in zinc smelters and to produce electricity via combustion. Resulting ashes will have high metal concentrations and will need to be landfilled afterwards, as it appears to be the case with Cd, Zn and Pb, which can highly accumulate in the filters used during the combustion of contaminated wood, requiring proper and safe disposal (Chalot et al. 2012). Nevertheless, there are prospects of using this remaining ash or contaminated filters for further processing and metal recovery before the disposal, in a process known as phytomining (Chalot et al. 2012; Ali et al. 2013; Yadav et al. 2018). The amount of metals recovered during combustion, gasification or pyrolysis will depend of several technical parameters, such as temperature, as well as the nature of the metal being targeted (Bert et al. 2017). Overall, it seems that without valorisation of the generated biomass, phytoremediation cannot be a sustainable process (Vigil et al. 2015).

6.3 Beyond the plant: mycorrhizal interaction

Although great part of the literature surrounding phytoremediation rather *phytocentric*, that is, focusing mainly on the plant aspect of the process, we know nowadays that multi- and interdisciplinarity are key in studying this technique. As neatly put by Ali et al. (2013), phytoremediation requires background knowledge in soil chemistry and microbiology, plant biology, ecology and environmental engineering; to that I would add that ecotoxicology, agronomy, biotechnology and economics are also integral aspects of this process (Figure 6.1).

In order to expand the knowledge slightly beyond this *phytocentric* view, I have included a fundamental relationship for most plant species: mycorrhizal symbiosis. This interspecies interaction is an important aspect in any phytoremediation study, since it can greatly affect the outcomes in terms of plant nutrition, metal tolerance, accumulation and/or extraction (Audet 2014).

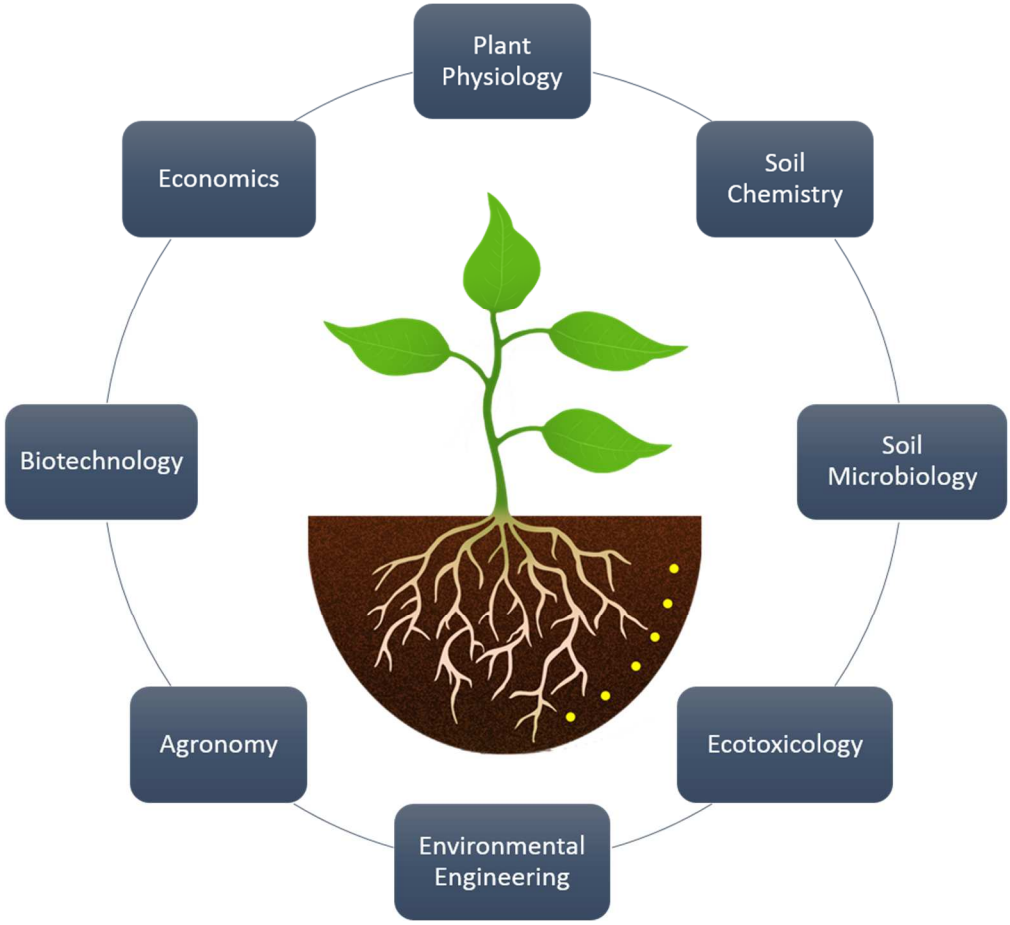


Figure 6.1 - Multi and interdisciplinary knowledge as integral aspects in phytoremediation.

6.3.1 Ectomycorrhizal symbiosis

Chapter 2 was the starting point in addressing the main question of using *P. trichocarpa* in Cd and Zn remediation. The main facts that lead us to include ECM fungi in this project were, in summary: (i) poplars can form symbiosis with both endo- and ectomycorrhizal fungi (Bissonnette et al. 2010; Marmiroli et al. 2011); (ii) woody species are known to be colonised predominantly by ECM fungi (Smith and Read 2008), (iii) inoculation of trees with ECM fungi usually leads to higher tolerance to metal toxicity (Jentschke and Godbold 2000; Baum et al. 2006; Colpaert 2008); (iv) Basidiomycota fungi are commonly found in trees of contaminated area (Krpata et al. 2008; Colpaert et al. 2011) and have an important role in facilitating the re-forestation of HM-contaminated areas (Gherghel and Krause 2012); (v) metal tolerant ECM strains may promote higher tolerance in host plants (Krznaric et al. 2010); and (vi) metal-chelating agents (e.g. siderophores, organic acids) produced by ECM fungi may enhance metal availability and therefore enhance phytoremediation (Machuca 2011).

This experiment assessed Cd and Zn tolerance in five ECM species, and eventually lead to the selection of two strains tolerant to Cd and Zn: *Hebeloma subsaponaceum* and *Scleroderma* sp. (Figure 2.1). This particular work also lead to other questions, such as if Zn is able to alleviate Cd toxicity in the selected species, and the more methodological question concerning the type of media (liquid vs solid) generally used in toxicity assays in ECM fungi. Conclusions were that Zn and Cd cause a synergistic toxicity in mostly sensitive species (Figure 2.3), and that solid media leads to higher metal tolerance (i.e. different toxicity thresholds) and allows assessment of mycelial density (Table 2.2), which may be crucial in withstanding toxicity.

Unfortunately, after several attempts to inoculate *P. trichocarpa* (by different methods) with those selected strains, as well other strains freshly isolated from the field, no ECM colonisation was verified in plant roots. Therefore I decided to not follow through with ECM symbiosis, and work only with arbuscular mycorrhizal fungi (*R. irregularis*), which successfully colonised *P. trichocarpa* roots at high rates (Table 4.2).

6.3.2 Arbuscular mycorrhizal symbiosis

Our results described in chapter 4 clearly showed that the symbiosis between *P. trichocarpa* and the arbuscular mycorrhizal fungus *R. irregularis* increased significantly the phytoextraction potential of the host plant exposed to Zn contamination (Figure 4.2B), while under Cd stress, symbiosis did not affect metal uptake, but altered its distribution within plant tissues, increasing poplars Cd phytostabilisation potential (Figure 4.2A). Therefore, inoculation of plantation of poplars in contaminated sites might be necessary, not only to modulate metal uptake and sequestration, but also to improve colonisation of poplar roots, since other microorganisms and symbionts may be lacking in contaminated sites (Phanthavongsa et al. 2017).

Although results such as these are very important when screening plants and fungal partners for remediation application, understanding some of the mechanisms by which plant-symbiont interactions can consequently influence the phytoremediation process does not only increase the overall scientific knowledge in this field, but also offers new tools and alternative avenues for exploring or improving this technique. In this thesis I focused mainly on some poplar genes involved in heavy metal transport and chelation, and the effects of metals and/or symbiosis on their expression.

6.4 Biotechnology in Phytoremediation

6.4.1 Molecular mechanisms

Understanding the genetic mechanisms underlying metal acquisition, tolerance and accumulation in plants allows the application of molecular techniques, such as genetic engineering, to manipulate other organisms (e.g. plants, bacteria, yeasts) in order to enhance their tolerance, accumulation, sequestration and/or extraction of pollutants (Hassinen et al. 2007; Poonam et al. 2014). For poplars specifically, deeper molecular information is important to identify the key mechanisms regarding their tolerance to heavy metals. This may assist in strategies for breeding and selecting different hybrids/varieties or genetically modify poplars themselves to be more effective in phytoremediation techniques (Sebastiani et al. 2014) and biomass yield. In addition, vascular plants

are not purely autonomous individuals and rely greatly on symbiotic microorganisms, which are able to induce systemic responses in the plants, increasing tolerance to both biotic and abiotic stresses (Vaishnav et al. 2014); and investigating molecular mechanisms in these processes are equally important.

By quantifying the gene expression of some heavy-metal related genes in *P. trichocarpa*, I have mainly established that (i) *PtHMA4* is probably involved in the xylem loading of both Cd and Zn (Figure 3.5), (ii) *PtZIP1* is related involved in the influx of both Cd and Zn in poplar leaves (Figures 4.3G and 4.4G), and that (iii) *PtMT2b* is overexpressed under mycorrhizal symbiosis (Figure 4.3F) and possibly responsible for high Cd sequestration into poplar roots. Therefore, it is possible that poplars overexpressing those genes would lead to a genotype even more suitable for Cd and Zn remediation. *P. trichocarpa* overexpressing their own transporter genes (*PtNramp3.1* and *PtNramp3.2*) were shown to accumulate twice as much Zn in their leaves compared to wild type poplars (Le Thi 2015), while a similar technique was applied to *P. angustifolia* to overexpress glutamyl cysteine synthetase and increase heavy metal accumulation (Fulekar et al. 2009).

6.4.2 Transgenic Poplars

Biotechnology has the potential to overcome phytoremediation limitations by allowing the direct gene transfer between organisms (Yang et al. 2005), and is believed to be a realistic possibility in combining important traits from a hyperaccumulator species into a high-biomass producing plant. Main approaches include: increasing the number of metal transporters and enhancing intracellular ligand production and metal sequestration in order to not disturb cellular processes (Kotrba et al. 2009).

How many genes and proteins to turn a tree into a *Nocceae caerulescens* is still not known, but inserting/manipulating genes to increase root-to-shoot metal transport and chelation/sequestration seems to be a strategic approach (Chaney et al. 2010). The metal transporters *PtHMA4* and *PtZIP1* as well as the metallothionein *PtMT2b* highlighted in this thesis, are therefore classic examples of target genes for genetic manipulations (Kotrba et al. 2011).

Examples of transgenic poplar trees for possible phytoremediation applications include: *P. tremula* × *P. alba* overexpressing γ-glutamylcysteine synthetase (from *E. coli*), which promoted Cd tolerance and uptake (He et al. 2015b); *P. alba* carrying the metallothionein MT2a1 (from *Pisum sativum*), that resulted in higher Cu and Zn tolerance (Balestrazzi et al. 2009; Turchi et al. 2012). And *P. alba* × *P. tremula* with vacuolar transporter YCF1 (from *S. cerevisiae*), enhancing Cd accumulation and tolerance (Shim et al. 2013). In the United States, transgenic poplars grown in sites contaminated by the organic compound trichloroethylene (TCE) have already displayed promising results in terms of pollutant removal (Legault et al. 2017). For more examples for heavy metal remediation, please refer to Song et al. (2007) and Fasani et al. (2017), or for cases involving organic pollutants, refer to Van Aken (2008).

Despite recent advances in this field, there are several challenges to overcome before the effective application of transgenic poplars in phytoremediation and SCR schemes, mainly the lack of field trials, the need for risk assessments to prevent unwanted breeding and spread of transgenes among other poplar species, contingency plants to avoid these modified plants from becoming extremely invasive due to their better fitness and tolerance, as well as addressing possible societal concerns (Yadav et al. 2018).

Moreover, instead of producing genetically engineered plants carrying genes from different or highly distant species (such as yeasts and mammals), the exchange of genes within the same genus (e.g. among *Populus* sp.) could facilitate the generation of a good phytoextractor phenotype and avoid some of the challenges described previously. For instance, by inserting the genes flagged in this thesis (i.e. *PtHMA4*, *PtZIP1* or *PtMT2b*) into other poplar varieties that are already known to accumulate high amounts of metals, such as the *Populus alba* clone (AL35), frequently used for Cu and Zn phytoextraction from contaminated soils, (Cicatelli et al. 2010; Cicatelli et al. 2012; Pallara et al. 2013), may enhance Cd tolerance and accumulation as well.

6.4.3 Populus trichocarpa as source of transgenes

While poplar is often considered as a recipient of transgenes for phytoremediation enhancement, as a donor species that is barely the case. From 70 studies involving transgenic plants with enhanced heavy metal tolerance and extraction potential, reported by Fasani et al. (2017), there was not a single work in which a *Populus* species was the source of the transgene, those mostly coming from *E. coli* (24%), *Arabidopsis* (21%), *S. cerevisiae* (7%) and *Noccaea* sp. (4%). It is a missed opportunity not considering poplar species as possible sources of genetic material for heavy metal remediation, especially when there are highly tolerant varieties available, such as the one studied in this thesis. In this sense, the high concentration of Cd sequestered in mycorrhizal roots instigated us to explore the function of the PtMT2b gene, which was highly up-regulated in this case.

Expressing PtMT2b in *S. cerevisiae*, both original and modified sequences, demonstrated that this poplar's metallothionein effectively enhances Cd tolerance and is also able to increase its accumulation and removal from liquid solutions (Figures 5.2); as seen with the modified version of the gene, by an amino acid substitution (C3Y) in the protein sequence (Figure 5.4). Studies like the one described and discussed in Chapter 5 can open up new avenues for exploring heavy metal remediation which, in this case, involved using yeasts as living biosorption material for Cd bioremediation in contaminated waste water. The overall findings in this work are represented in Figure 6.2

6.5 Research limitations

Because this thesis was designed to answer hypotheses regarding genetic mechanisms and mycorrhizal symbiosis in poplar trees under Cd and Zn stress, I opted for eliminating as many variables as I could. Thus I used a fairly inert substrate (mostly sand and TerraGreen® clay), which had to be autoclaved in order for us to evaluate the effects of a single mycorrhizal species. In the end, this work was limited to potted plants, artificially spiked substrates, glasshouse conditions and growth chambers, which are commonly the central criticisms regarding phytoremediation studies (Robinson et al. 2009; Van der Ent et al. 2013).

Therefore, in terms of practical application, those results need further complementation.

Several aspects were not studied in this thesis, due to time constraints or methodological complications, such as the unsuccessful ectomycorrhizal symbiosis formation, as discussed previously. Another experiment that ultimately failed, was supposed to address if the gene expression patterns differed from colonised roots to non-colonised roots for the same individual, the main hypothesis was that mycorrhizal symbiosis could alter gene expression patterns systemically, instead of a local effect in colonised roots. Plants died a few days after being transplanted to a double-pot system, which separated mycorrhizal from non-mycorrhizal roots, as well as contaminated from non-contaminated substrates.

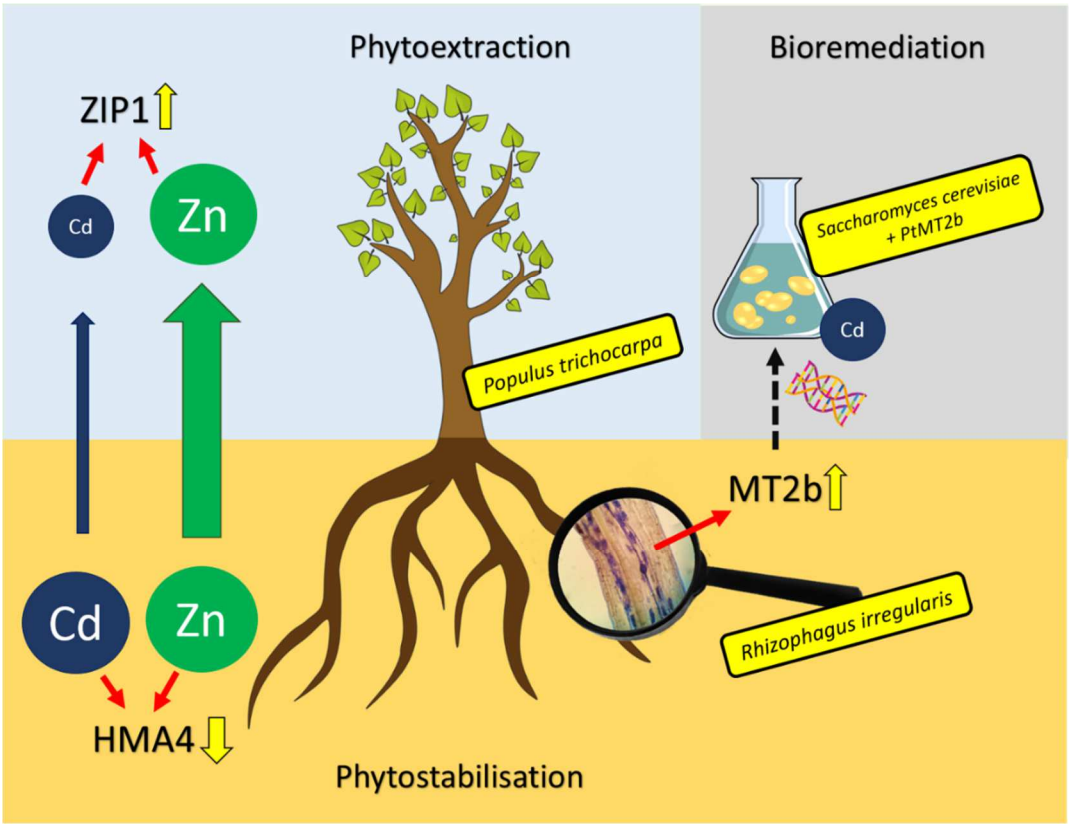


Figure 6.2 – Schematic overview of the main findings in this thesis. Mycorrhizal symbiosis increased Zn uptake and extraction, while increased Cd sequestration in roots. Metal exposure down-regulated the expression of HMA4 in roots, while up-regulated ZIP1 in leaves. *R. irregularis* increased *PtMT2b* transcripts in roots, which when inserted in yeasts, displayed great potential for Cd bioremediation from contaminated water.

6.6 Future research

Considering all the findings from this thesis and the unexplored ideas arising from the data, knowledge gaps, as well as all the limitations from our methodological approach, there are several opportunities for future research.

Poplar studies in the field or in pots using non-spiked contaminated soils (with varying soil properties) are very important in terms of practical remediation experiments, such as the ones reported by Cicatelli et al. (2014). This would allow a better prediction of the potential of *P. trichocarpa* to extract or stabilise Cd and Zn. The manipulation of soil pH, soil nutritional status and microbiota will also provide a better understanding of the process, since they greatly affect metal availability (Alloway 2008; Smolders and Mertens 2013).

Glomalin production by arbuscular mycorrhizal fungi are also an important factor influencing metal uptake and availability to plant roots. Glomalins are glycoproteins with high retention capacity for heavy metals (Khan et al. 2014; Meyer et al. 2017). Thus it would be interesting to determine glomalin concentrations in mycorrhizal poplars under Cd and Zn stress and possible correlation to their stabilisation potential in soils. In addition, because mycorrhizal effects are very dependent on plant and fungal species, symbiosis with different AM and/or ECM partners might better elucidate and enhance the phytoremediation process. For instance, *Funneliformis mossae* is a species widely considered in soil remediation and was also able to colonise *P. trichocarpa* cv 'Trichobel' roots in a previous test (data not shown).

In chapter 4 I was not able to distinguish the Cd bound to plant cell walls, to the fraction bound to fungal walls, vesicles and hyphae of *R. irregularis* and although this is not an easy task, scanning electron microscopy (SEM) with X-ray detection can allow not only the visualisation of morphological effects of HMs in root tissues, but also detect metal concentrations and localisation within root (or mycorrhizal root) tissues (Marzilli et al. 2018).

Other means of enhancing Cd and Zn phytoremediation potential of *P. trichocarpa* (other than mycorrhizal symbiosis) could also be added to this work, such as the root inoculation with synergistic bacteria (Cocozza et al. 2015); soil application of chelating agents such as EDTA (ethylenediaminetetraacetic acid),

EDDS (ethylenediamine-N,N'-disuccinic acid) and NTA (nitrilotriacetic acid) (Guo et al. 2014; Khalid et al. 2017) or soil amendment using biochar (Beesley et al. 2010; Bian et al. 2014; Qiao et al. 2015); to name a few.

In summary, other main topics for future research arising directly from this thesis could include:

- The potential of *P. trichocarpa* in extracting/stabilising other metals such as Pb, Cu and Ni within the same framework of this thesis.
- The effects of multi-contaminated soils in *P. trichocarpa* uptake and tolerance.
- The effects of dual symbiosis (ECM + AM) in modulating heavy metal uptake.
- Effect and interactions between mycorrhizal poplars and other soil microorganisms as well as other organisms, such as earthworms, that can increase Cd availability (Aghababaei et al. 2014).
- Phytoremediation potential of mycorrhizal *P. trichocarpa* in soils contaminated by organic pollutants.
- Transcriptome analyses of mycorrhizal poplars under Cd and Zn stress, for the selection of a range of target genes for remediation.
- Engineering of *P. trichocarpa* overexpressing heavy metal-related genes, such as the ones described in this thesis, or from other organisms, for enhancing Cd/Zn extraction and tolerance.
- More studies with *S. cerevisiae* carrying poplar genes for Cd remediation (e.g. *PtMT2b*), such as in different experimental conditions or in conjunction with other genes that may increase Cd extraction (*PtHMA4* or *PtZIP1*).
- The use of the transgenic yeast strains developed in chapter 5, for different applications, such as in the cocoa bean fermentation process, potentially reducing their Cd contents.

6.7 Concluding remarks

Soil pollution is a prevalent problem that has only been increasing with industrialisation, population growth and continuing inputs of wastes into the environment. The widespread contamination of soils with heavy metals, which are

highly persistent and toxic represents one of the most severe environmental problems that can seriously affect environmental quality and human health (Khalid et al. 2017). Although still very challenging, a possible solution to mitigate this problem is the use of plants and their associated microbiota to remediate contaminated soils, in a process commonly known as phytoremediation (Mahar et al. 2016). It is therefore of major importance for soil scientists, plant biologists, plant breeders and biotechnologists to understand the mechanisms by which plant cope and handle heavy metals in soils (Gallego et al. 2012).

My thesis offers some new opportunities for this non-costly, eco-friendly and possibly sustainable soil remediation. By evaluating some of the factors underlying Cd and Zn tolerance and accumulation in *P. trichocarpa*, a species with several traits crucial for an effective phytoremediation technique. The overall results are useful for better explaining the mechanisms by which mycorrhizal symbiosis can affect Cd and Zn uptake in woody plants and highlights the importance of mycorrhizal symbiosis in phytoremediation. The knowledge gathered and generated in this thesis may serve as basis for the genetic engineering of poplars or other organisms for heavy metal remediation, or further research in refining and enhancing this technique.

6.8 References

Aghababaei F, Raiesi F, Hosseinpur A. 2014. The significant contribution of mycorrhizal fungi and earthworms to maize protection and phytoremediation in Cd-polluted soils, *Pedobiologia*. **57:** 223–233.

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

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

Antoniadis V, Levizou E, Shaheen SM, Ok YS, *et al.* 2017. Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation – A review, *Earth-Science Reviews*. **171**: 621-645.

Audet P. 2014. Arbuscular mycorrhizal fungi and metal phytoremediation: ecophysiological complementarity in relation to environmental stress. In:

Ahmad P, ed., *Emerging Technologies and Management of Crop Stress Tolerance, Volume 2.* Elsevier Inc.

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.m *Phytoremediation of Contaminated Soil And Water*. London: LewisPublisher, p. 85–107.

Balestrazzi A, Botti S, Zelasco S, Biondi S, *et al.* 2009. Expression of the PsMTA1 gene in white poplar engineered with the MAT system is associated with heavy metal tolerance and protection against 8-hydroxy-20-deoxyguanosine mediated-DNA damage, *Plant Cell Reports*. **28**: 1179-1192.

Baum C, Hrynkiewicz K, Leinweber P, Meißner R. 2006. Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (*Salix* × *dasyclados*), *Journal of Plant Nutrition and Soil Science*. **169**: 516–522.

Beesley L, Moreno-Jimenez E, Gomez-Eyles JL. 2010. Effects of biochar and greenwaste compost amendments on mobility, bioavailability and toxicity of inorganic and organic contaminants in a multi-element polluted soil, *Environmental Pollution*. **158:** 2282-2287.

Bert V, Allemon J, Sajet P, Dieu S, *et al.* 2017. Torrefaction and pyrolysis of metal-enriched poplars from phytotechnologies: Effect of temperature and biomass chlorine content on metal distribution in end-products and valorization options, *Biomass and Bioenergy*. **96**: 1-11.

Bian R, Joseph S, Cui L, Pan G, *et al.* 2014. A three-year experiment confirms continuous immobilization ofcadmium and lead in contaminated paddy field with biocharamendment, *Journal of Hazardous Materials*. **272**: 121-128.

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.

Blake TJ. 1983. Coppice systems for short-rotation intensive forestry: The influence of cultural, seasonal and plant factors, *Australian Forest Research*. **13:** 279-291.

Brentner LB, Mukherji ST, Walsh SA, Schnoor JL. 2010. Localization of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT) in poplar and switchgrass plants using phosphor imager autoradiography, *Environmental Pollution*. **158:** 470–475.

Chalot M, Blaudez D, Rogaume Y, Provent AS, Pascual C. 2012. Fate of trace elements during the combustion of phytoremediation wood, *Environmental Science and Technology*. **46**: 13361−13369.

Chaney R, Broadhurst CL, Centofanti T. 2010. Cadmium and Zinc. In: Hooda PS, ed., *Trace Elements in Soils*. Blackwell Publishing Ltd, UK, p. 311-352.

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, 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, 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.

Cocozza C, Trupiano D, Lustrato G, Alfano G, *et al.* 2015. Challenging synergistic activity of poplar–bacteria association for the Cd phytostabilization, *Environmental Science and Pollution Research*. **22**: 19546-19561.

Colpaert JV, Wevers JHL, Krznaric E, Adriaensen K. 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution, *Annals of Forest Science* **68**: 17-24.

Colpaert JV. 2008. Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi. In: Avery SV, Stratford M, Van West P, eds, *Stress in yeasts and filamentous fungi*. Academic, Amsterdam, p. 157–174.

Council of the European Communities (CEC). 1986. *The protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture*. Council Directive of 12 June 1986 (86/278/EEC). In: Official Journal of the European Communities No L 181/6.

Creutzig F, Ravindranath NH, Berndes G, Bolwig S, *et al*. 2015. Bioenergy and climate change mitigation: an assessment, *GCB Bioenergy*. **7**: 916–944.

Dillen SY, Djomo SN, Afas NA, Vanbeveren S, Ceulemans R. 2013. Biomass yield and energy balance of a shortrotation poplar coppice with multiple clones on

degraded land during 16 years, *Biomass and Bioenergy*. **56**: 157-165.

Fasani E, Manara A, Martini F, Furini A, DalCorso G. 2017. The potential of genetic engineering of plants for the remediation of soils contaminated with heavy metals, *Plant, Cell and Environment*. **41:** 1201-1232**.**

Fulekar MH, Singh A, Bhaduri AM. 2009. Genetic engineering strategies for enhancing phytoremediation of heavy metals, *African Journal of Biotechnology*. **8**: 529-535.

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, *et al.* 2012. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms, *Environmental and Experimental Botany*. **83**: 33-46.

Gherghel F, Krause K. 2012. Role of mycorrhiza in re-forestation at heavy metal-contaminated sites. In: Kothe E, Varma A, eds., *Bio-Geo Interactions in Metal-Contaminated Soils*, Springer, Berlin, p. 183-199.

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, p. 387–413.

Gullner G, Komives T, Rennenberg H. 2001. Enhanced tolerance of transgenic poplar plants overexpressing γ-glutamylcysteine synthetase towards chloroacetilide herbicides, *Journal of Experimental Botany*. **52**: 971-979.

Guo J, Feng R, Ding Y, Wang R. 2014. Applying carbon dioxide, plant growth-promoting rhizobacterium and EDTA can enhance the phytoremediation efficiency of ryegrass in a soil polluted with zinc, arsenic, cadmium and lead, *Journal of Environmental Management.* **141:** 1-8.

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.

Hassinen VH, Tervahauta AI, Karenlampi SO. 2007. Searching for genes involved in metal tolerance, uptake and transport. In: Willey N, ed., *Phytoremediation: Methods and Reviews*. Humana Press Inc., New Jersey, p. 265-290.

He J, Li H, Ma C, Zhang Y, Polle A, *et al.* 2015b. Overexpression of bacterial γ-glutamylcysteine synthetase mediates changes in cadmium influx, allocation and detoxification in poplar, *New Phytologist*. **205**: 240-254.

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, p. 135–225.

Jentschke G, Godbold DL. **2000.** Metal toxicity and ectomycorrhizas, *Physiologia Plantarum*. **109**: 107–116.

Khalid S, Shahid M, Niazi NK, Murtaza B, *et al.* 2017. A comparison of technologies for remediation of heavy metal contaminated soils, *Journal of Geochemical Exploration*. **182**: 247–268.

Khan A, Sharif M, Ali A, Shah SNM, *et al.* 2014. Potential of AM fungi in phytoremediation of heavy metals and effect on yield of wheat crop, *American Journal of Plant Sciences*. **5**: 1578-1586.

Kotrba P, Mackova M, Macek T. 2011. Transgenic Approaches to Improve Phytoremediation of Heavy Metal Polluted Soils. In: Khan MS, Zaidi A, Goel R, Musarrat J, eds., *Biomanagement of Metal-Contaminated Soils*, Springer Science, p. 409-430.

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.

Krpata D, Peintner U, Langer I, Fitz WJ, Schweiger P. 2008. Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site, *Mycological Research*. **112**: 1069–1079.

Krznaric E, Wevers JHL, Cloquet C, Vangronsveld J, Vanhaecke F, Colpaert JV. 2010. Zn pollution counteracts Cd toxicity in metal-tolerant ectomycorrhizal fungi and their host plant, *Pinus sylvestris*, *Environmental Microbiology*. **12**: 2133-2141.

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

Legault EK, James A, Stewart K, Muiznieks I, *et al.* 2017. A field trial of TCE phytoremediation by genetically modified poplars expressing cytochrome P450 2E1, *Environmental Science and Technology*. **51**: 60906099.

Machuca A. 2011. Metal-chelating agents from ectomycorrhizal fungi and their biotechnological potential. In: Rai M, Varma A, eds., *Diversity and Biotechnology in Ectomycorrhizae*, Springer, Berlin, p. 347-370.

Mahar A, Wang P, Ali A, Awasthi MK, *et al.* 2016. Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review, *Ecotoxicology and Environmental Safety*. **126:** 111–121.

Manzone M, Bergante S, Facciotto G. **2014.** Energy and economic evaluation of a poplar plantation for woodchips production in Italy, *Biomass and Bioenergy*. **60**: 164-170.

Marmiroli M, Pietrini F, Maestri E, Zacchini M, Marmiroli N, Massacci A. 2011. Growth, physiological and molecular traits in Salicaceae trees investigated for phytoremediation of heavy metals and organics, *Tree Physiology*. **31:** 1319–1334.

Marzili M, Di Santo P, Palumbo G, Maiuro L, *et al.* 2018. Cd and Cu accumulation, translocation and tolerance in *Populus alba* clone (Villafranca) in autotrophic in vitro screening, *Environmental Science and Pollution Research*. **25**: 10058-10068.

Meyer E, Londono DMM, Armas RD, Giachini AJ, *et al.* 2017. Arbuscular mycorrhizal fungi in the growth and extraction of trace elements by *Chrysopogon zizanioides* (vetiver) in a substrate containing coal mine wastes, *International Journal of Phytoremediation*. **19**: 113-120.

Montpetit E, Lachapelle E. 2017. New environmental technology uptake and bias toward the status quo: The case of phytoremediation, *Environmental Technology and Innovation*. **7:** 102-109.

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.

Pandey VC, Bajpai O, Singh N. **2016.** Energy crops in sustainable phytoremediation, *Renewable and Sustainable Energy Reviews*, **54:** 58–73.

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.

Poonam RB, Sharma R, Handa N, *et al*. 2014. Prospects of field crops for phytoremediation of contaminants. In: Ahmad P, ed., *Emerging Technologies and Management of Crop Stress Tolerance*, *Volume 2*, Elsevier Inc., p. 449-470.

Qiao Y, Crowley D, Wang K, Zhang H, Li H. 2015. Effects of biochar and arbuscular mycorrhizae on bioavailability of potentially toxic elements in an aged contaminated soil, *Environmental Pollution*. **206**: 636-643.

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.

Sabatti M, Fabbrini F, Harfouche A, Beritognolo I, *et al.* 2014. Evaluation of biomass production potential and heating value of hybrid poplar genotypes in a short-rotation culture in Italy, *Industrial Crops and Products*. **61**: 62–73.

Salminen R, Batista MJ, Bidovec M, Demetriades A, De Vivo B *et al*. 2005. Geochemical atlas of Europe. Part 1: Background Information, Methodology and Maps. Espoo, Geological Survey of Finland, p. 526.

Sannigrahi P, Ragauskas AJ. **2010.** Poplar as a feedstock for biofuels: A review of compositional characteristics, *Biofuels, Bioproducts & Biorefining*. **4**: 209–226.

Sas-Nowosielska A. 2011. Phytotechnologies: Importance in Remediation of Heavy Metal–Contaminated Soils. In: Khan MS, Zaidi A, Goel R, Musarrat J, eds., *Biomanagement of Metal-Contaminated Soils*, Springer Science, p. 277-296.

Schweier J, Becker G. 2013. Economics of poplar short rotation coppice plantations on marginal land in Germany, *Biomass and* Bioenergy. **59:** 494-502.

Searler SY, Malins CJ. 2014. Will energy crop yields meet expectations? *Biomass and Bioenergy*. **65**: 3-12.

Sebastiani L, Francini A, Romeo S, Ariani A, Minnocci A. 2014. Heavy metals stress on poplar: molecular and anatomical modifications. In: Gaur RK, Sharma P, eds., *Approaches to Plant Stress and their Management*. Springer, New Delhi, p. 267-280.

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.

Shim D, Kim S, Choi, Y. *et al*. 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 3rd edition.* Academic Press, London.

Smolders E, Mertens J. 2013. Cadmium. In: Alloway BJ, ed. *Heavy Metals in Soils*, 3rd edn. Netherlands: Springer, p. 283–311.

Song WY, Choi YI, Shim D, Kim DY, *et al.* 2007. Transgenic poplar for phytoremediation. In: Xu Z, Li J, Xue Y, Yang W, eds., *Biotechnology and Sustainable Agriculture 2006 and Beyond*. Springer, Dordrecht, p. 265-271.

Turchi A, Tamantini I, Camussi AM, Recchi L. 2012. Expression of a metallothionein A1 gene of *Pisum sativum* in white poplar enhances tolerance and accumulation of zinc and copper, *Plant Science*. **183**: 50-56.

Vaishnav A, Jain S, Kasotia A, Kumari S, *et al.* 2014. Molecular mechanism of benign microbe-elicited alleviation of biotic and abiotic stresses for plants. In: Gaur RK, Sharma P, eds., *Approaches to Plant Stress and their Management*. Springer, New Delhi, p. 281-296.

Van Aken B. 2008. Transgenic plants for phytoremediation: helping nature to clean up environmental pollution, *Trends in Biotechnology*. **26**: 225-227.

Van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction, *Plant and Soil*. **362**: 319-334.

Verlinden MS, Broeckx LS, Ceulemans R. 2015. First vs. second rotation of a poplar short rotation coppice: Above-ground biomass productivity and shoot dynamics, *Biomass and Bioenergy*. **73**: 174-185.

Vigil M, Marey-Perez MF, Huerta GM, Cabal VA. 2015. Is phytoremediation without biomass valorization sustainable? — Comparative LCA of landfilling vs. anaerobic co-digestion, *Science of the Total Environment*. **505**: 844-850.

Whitaker J, Field JL, Bernacchi CJ, Cerri CEP, *et al.* 2018. Consensus, uncertainties and challenges for perennial bioenergy crops and land use, *GCB Bioenergy*. **10**: 150-164.

Witters N, Mendelsohn RO, Van Slycken S, Weyens N, *et al.* 2012. Phytoremediation, a sustainable remediation technology? Conclusions from a case study. I: Energy production and carbon dioxide abatement, *Biomass and Bioenergy*. **39:** 454-469.

Yadav KK, Gupta N, Kumar A, Reece LM, *et al.* 2018. Mechanistic understanding and holistic approach of phytoremediation: A review on application and future prospects, *Ecological Engineering*. **120:** 274–298.

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.

Yu XZ, Peng XY, Xing LQ. **2010.** Effect of temperature on phytoextraction of hexavalent and trivalent chromium by hybrid willows, *Ecotoxicology*. **19**: 61-68.

Zarubova P, Hejcman M, Vondrackova S, Mrnka L, *et al.* 2015. Distribution of P, K, Ca, Mg, Cd, Cu, Fe, Mn, Pb and Zn in wood and bark age classes of willows and

poplars used for phytoextraction on soils contaminated by risk elements, *Environmental Science and Pollution Research*. **22**: 18801-18813.

6.9 Supplementary Information

Calculations concerning the conversion factor 10^7 for the time parameter t_R in section 6.2 (source: Antoniadis et al. 2017):

Step 1: Area in a hectare is 10^4 m^2 ha^{-1}.

Step 2: Volume of soil in depth D (m) in the area of a hectare is 10^4 m^2 x D m ha^{-1} = 10^4 x D m^3 ha^{-1}.

Step 3: Weight of this volume is: Weight = Density x Volume = Dry bulk density x Volume = ρ_b (g cm^{-3}) x 10^4 x D (m^3 ha^{-1}) [1].

Step 4: The units of g cm^{-3} are converted to kg m^{-3} multiplying by a factor of 10^3 (i.e., g = 10^{-3} kg, and cm^3 = 10^{-6} m^3; thus g cm^{-3} = 10^3 kg m^{-3}) [2]

Step 5: Combining [1] and [2] we have soil weight in a hectare in depth D: 10^3 x ρ_b x 10^4 x D = **10^7 x ρ_b x D**.