

**Cadmium and its effect on the physiology of
mycorrhizal and saprotrophic fungi**

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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Publications related to this thesis

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Thesis Abstract

Cadmium (Cd) is one of the most harmful metals in the environment, because even in a low concentration they affect human and other organisms. Cd when in the soil impose severe threats on crop production and food safety. One of the major remediation techniques is phytoremediation, which is a use of plants to immobilise or extract heavy metal from the soil. This thesis studies the effect of Cadmium (Cd) in the mycorrhizal system and in saprotrophic fungi. The main hypothesis tested was common mycorrhizal network increases the amount of cadmium extracted from the soil and the cadmium uptake dynamics. In order to understand that system, ectomycorrhizal fungi were tested under Cd along with its effect on carbon cycling enzymes. The experiments conducted in this project aimed to investigate the potential of poplars trees (*Populus trichocarpa*), colonised by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, to improve metal uptake in the host plant as well as in combination with different species under contaminated soil with Cd. Additional aims were to investigate common mycorrhizal networks and their potential to transfer Cd between plants, and to understand the physiological processes by which Cd can affect ectomycorrhizal and saprotrophic fungi. Results showed that mycorrhizal *P. trichocarpa* have increased Cd accumulation in their roots, which supports their use for Cd phytoextraction. Experiments with common mycorrhizal networks showed that Cd can be transported between *P. trichocarpa* trees. This result opens many other opportunities for exploring this complex system and the likely importance of having neighbouring plants to establish new vegetation in contaminated soil. Cd contamination increased the production of GSRP (glomalin) in all arbuscular mycorrhizal plants, but mostly in leeks. Glomalin as known as soil glycoprotein, which apart from being responsible for stabilization of soil aggregation also act as a soil protector by binding the metal under Cd contamination. It was also found that Cd can affect mycorrhizal and soil microorganism producing of carbon enzymes, however there was a wide variation within different species. Cd was able to reduce soil enzyme activity of xylanase by 50%, demonstrating how Cd can affect contaminated areas and its dynamics. Some species of saprotrophic fungi showed a potential to be used for Cd remediation, but there are a wide range of species to be explored in this field. This thesis offers new opportunities to explore properties of common mycorrhizal networks and to enhance our knowledge on Cd contamination. The knowledge gathered in this project may help further research in Cd remediation technique.

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Chapter 1

1. General Introduction and literature review

1.1 Heavy metals in soils

Heavy metals are defined as a group of elements that have weights greater than 5g/cm (Adriano, 2001). The term heavy metal is strictly ascribed to transition metals with an atomic mass over 20 (Koller & Saleh, 2018). They are a series of metals and metalloids that can be toxic to both plants and animals even at very low concentrations. Unlike organic contaminants, metals (metalloids) do not undergo microbial or chemical degradation and persist for a long time after their introduction (Adriano, Wenzel & Bolan, 2004). This series of metals can be one of the most problematic and persistent environmental contaminants.

Metal can travel long distances, either in watersheds or through the atmosphere. Their ecological half-life is long, and although their structure can be modified in the environment or in the animals by speciation and processes such as ionization, methylation, and binding to organic ligands. For instance, the half-life of cadmium in soils is estimated to be from 13 to 1,100 years (Kabata-Pendias, 2001), and it has a significantly high half-life of approximately 30 years in the human body (Rahimzadeh et al., 2017). Heavy metal content can be affected by the interaction of soil physicochemical properties such as soil pH (Kicinska, Pomykala and Izquierdo, 2022), soil granularity (Błonska, Lasota & Gruba, 2016; Gasiorek et al., 2017), organic matter content (Zeng et al., 2011) and heavy metal storage capacity (Bourg & Loch, 1995), which play an important role in the retention, mobilization, and migration of heavy metals in soil (Weber et al., 2018).

Heavy metal contamination is a worldwide problem, as many anthropogenic activities release increasingly high quantities of these compounds into the environment with mine tailing, leaded gasoline and paints, atmospheric deposition, fertilizer application, sewage sludge, pesticides, coal combustion residues, spillage of petrochemicals, batteries, electroplating, etc (Khan et al., 2008; Zhang, Liu & Wang, 2010). Heavy metals also occur naturally in soil environment under low concentrations due to pedogenetic processes through time (Alloway, 2013). However, anthropogenic activities are still leading the higher inputs of those elements.

Mining and extensive chemical use in agriculture are major causes of heavy metal accumulation in soil. For instance, the soils of approximately three million sites in the European Union (EU) are suspected of being contaminated and 250,000 contaminated sites are known to

require cleanup. (European Environment Agency, 2007) In Western Europe, 1.4 million sites are contaminated with potentially toxic metals such as zinc (Zn), cadmium (Cd), lead (Pb), and copper (Cu) (ETCS, 1998; McGrath, Zhao & Lombi., 2001). Trace metals are chemically reactive in the environment leading to their increased mobility and bioavailability to living organisms. Consequently, heavy metals can get into the food chain, depending on the amount and the source of metal/metalloids input, the properties of the soil, the rate and magnitude of uptake by plants, and the extent of absorption by animals (Tibbett et al., 2021).

Food security and safety are of great concern throughout the world due to heavy metal contamination and their associated health risks (Khan et al., 2014; Shaheen et al., 2016; Chen et al., 2016; Yousaf et al., 2016) Historically, heavy metals have often been reported in several countries, which shows significant adverse impact on human health. For example, in Bangladesh, India, and China it is claimed that millions of people are potentially at risk from poisoning (Bhattacharya et al., 2012). Cd accumulation in the offal of grazing animals in New Zealand and Australia made it unsuitable for human consumption (Loganathan, Hedley & Grace, 2008).

1.2 Heavy metals in plants

According to roles in biological systems, heavy metals can be classified as essential and nonessential. Essential heavy metals are important for living organisms and may be required in the body in low concentrations. Cd, Pb, and Hg are toxic and regarded as biologically nonessential, however, Mn, Fe, Cu, Co, Ni, Mo and Zn are essential elements for plants (Marschner, 2012). These latter elements can easily lead to poisoning when their concentration rises to supra-optimal values.

Heavy metal phytotoxicity may result from alterations of numerous physiological processes caused at the cellular/ molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting for essential elements, and disrupting membrane integrity (Hossain et al., 2012). All those processes started in the root system because they are the primary form of contact between plants and the metal ions in soil solution, whereby toxic metal ions as HMs enter plant cells by the same uptake processes that move essential micronutrient metal ions. HM interacting with cell DNA and nuclear proteins, cause DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis, or apoptosis (Chang et al., 1996; Beyersmann & Hartwig, 2008).

Therefore, to survive plants need to adapt to this stressed environment. Heavy metal resistance can be achieved by avoidance and tolerance. Plants that can avoid this are those able to protect themselves by preventing metal ions from entering their cellular cytoplasm, while tolerant plants can detoxify metal ions that have crossed the plasma membrane or internal organelle bio membranes (Millaleo et al., 2010). Baker and Walker (1990) classified them into 3 categories based on their skills used in growing on metal-contaminated soils. This first group includes the majority of plant species, the excluder, limits the translocation of heavy metals and maintains low levels of contaminants in their aerial tissues. The metal indicators accumulate metal in their harvestable biomass, and metal accumulators are plants that increase internal sequestration, translocation, and accumulation of metals in their harvestable biomass to level that far exceed those in the soil (Baker & Walker 1990; Mganga et al., 2011;). Plants have different approaches to dealing with heavy metal concentrations. Some of those approaches include the extracellular level which can increase plant metal tolerance as mycorrhizal symbioses (Schutzendubel & Pole, 2002) and exudation of organic compounds like phytochelatins (Schat et al., 2002).

1.3 Cadmium in soil and plants

Cadmium in soil is derived from both natural and anthropogenic sources. Natural sources include underlying bedrock or transported parent material such as glacial till and alluvium. Generally, it occurs in the lithosphere (0.2 mg kg^{-1}), sedimentary rocks (0.3 mg kg^{-1}), and soil (0.53 mg kg^{-1}) (Greenwood & Earnshaw, 1997). Kabata-Pendias and Pendias (1992) reviewed the world literature and reported that average Cd concentrations in soils not exposed to obvious sources of pollution lie in the range $0.06\text{-}1.1 \text{ mg kg}^{-1}$ with a minimum of 0.01 mg kg^{-1} and a maximum of 2.7 mg kg^{-1} , although values can vary between continents, countries, and soil types. (Greenwood and Earnshaw, 1997, Kabata-Pendias & Pendias, 2001). This element is mobile in the soil (Alloway and Jackson, 1991; Nies, 1999; Nies 2003) and their availability increased under lower soil pH (Lux et al., 2011).

Compared with natural sources, anthropogenic sources are commonly considered as the major cause of the increasing cadmium pollution in the environment. Activities like the combustion of fossil fuels, compost or fertiliser application, metallurgical activities, wastes from the cement industry, industrial and municipal wastes, mining, smelting, and metal ore processing contribute to a significant amount of Cd in soils. The worldwide main Cd use, and thus the primary source of Cd directed to landfills with municipal solid waste, are nickel-cadmium batteries (UNEP, 2010; Khan et al., 2017). Another reason for elevated Cd

concentrations in soil and groundwater is the use of phosphate fertilizers, which contain Cd as an impurity (Bigalke et al., 2017). Phosphate fertilizers contain an average of 77 mg Cd per kg P₂O₅ in the Eastern Mediterranean countries (Azzi et al., 2017), 36 mg Cd per kg P₂O₅, and 60 mg Cd per kg P₂O₅ in Europe (Schuetze et al, 2003; Six & Smolder, 2014).

Cadmium can replace calcium in minerals due to its similar ionic radius, identical charge, and similar chemical behaviour (Thornton, 1986). Cd can be also frequently found in zinc (Zn) bearing minerals (Alloway, 2013) and due to their similar geochemical characteristics, Cd and Zn are often associated in soils (Kabata-Pendias & Pendias, 2001). Zinc ores (ZNS) generally contain 0.1-5% and sometimes even higher cadmium (Adriano, 1986). This association of Cd and Zn in the environment can lead to interaction during plant uptake, transport from roots to aboveground parts, or accumulation in edible parts (Das et al., 1997).

High Cd concentration can have toxic effects on soil organisms and can easily transfer into vegetative cover and ultimately enter the food chain (Li et al., 2016, Wahid et al., 2009). A good quantity of Cd taken up by plants is retained in the roots, but a portion is translocated to the aerial part of the plant and into the seeds. The amount of Cd accumulated and translocated in plants varies with species and with cultivars within species. There is no known metabolic function of Cd in plants, however, it also accumulates in edible parts of the plant along with essential elements (Zarcinas et al., 2004; Rascio & Navari-Izzo, 2011). The uptake of Cd by vegetables from the soil is a major exposure pathway for humans (Franz et al., 2008, Kobayashi et al., 2008) and this exposure through consumption represents approximately 70-80% of total intake in humans (Olsson et al., 2002; Wang et al., 2016). If the content of Cd in the human body is high it is associated with glucose metabolism disorders, breast and lung cancer, various types of cancer (Pan et al., 2010) cerebral infarction, and cardiac failure (Khan et al., 2017). According to WHO (2011), the tolerable monthly Cd intake is 25 µg/kg body weight. Having in mind the above adverse health impacts of Cd, it is necessary to ensure that Cd concentrations in food crops meet the regulatory standards set by different agencies such as the World Health Organization (FAO/WHO, 2001) and the United States Environmental Protection Agency (USEPA, 2005).

Cadmium can interfere with numerous biochemical and physiological processes of plant cells, such as plant growth, photosynthesis, nutrient uptake, enzyme activities (Muradoglu et al., 2015). Even at low Cd levels, a negative impact on vegetative and reproductive plant organs can be induced by Cd uptake and transport of Cd from roots to shoots (DalCorso et al., 2010). Seed germination and seedling growth are also greatly affected by Cd (De Oliveira et al., 2016).

1.4 Heavy metals and mycorrhizas

When plants have high heavy metal concentrations, this can interfere with essential enzymatic activities by modifying protein structure or by replacing a vital element resulting in deficiency symptoms. The plasma membrane is particularly vulnerable to heavy metal toxicity since membrane permeability and thus functionality can be affected by alterations of important membrane intrinsic proteins such as H⁺ ATPases (Hall, 2002). Toxicity symptoms include chlorosis, growth retardation, browning of roots, and effects on both photosystems, but the plant can become more tolerant to HM toxicity through symbioses with soil microorganisms, such as plant growth-promoting bacteria (Khan, 2006) or mycorrhizal fungi (Vamerali et al., 2010; Saraswat & Rai 2011).

Most of the plants in the land 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). Depending on the plant and fungal species involved as well as distinct morphological characteristics, at least six different types of mycorrhizal associations have been described: (1) arbuscular mycorrhizas (AM), (2) arbutoid mycorrhizas, (3) ectomycorrhizas (EM), (4) ericoid mycorrhizas, (5) monotropoid mycorrhizas, and (6) orchid mycorrhizas (Harley & Smith, 1983; Brundrett et al., 1996; Smith & Read, 1997). However, the main types are AM (also known as “endomycorrhizas”), where the fungus colonises the interior of a host plant root cells, and ectomycorrhizas (ECM) where the fungus is located outside plant root cells (Smith & Read, 2008; Gadd, 2010). The establishment of mycorrhizal symbiosis generally benefits their host plant by: - increasing the absorbing surface area of the root system, - increasing the ability of plants to capture water and nutrients such as nitrogen (N), phosphorus (P), or other essential elements from the soil; - increasing plant tolerance to heavy metal, droughts, high soil temperatures; - providing protection from certain plant pathogenics; amongst other benefits. In return for these activities, the fungus receives carbohydrates and nutrients supplied by the plant (Smith & Read, 1997).

AM and ECM symbioses have a crucial role in alleviating metal stress, generally by influencing HM availability in the rhizosphere and providing the host plant with water and nutrients in a poor environment (Gherghel & Krause, 2012). The metal alleviation stress can happen by the resistance, avoidance, or tolerance of these organisms. Metal tolerance as a mechanism due to intrinsic properties and/or modification of the environment to reduce toxicity, including binding or precipitation via, for example, extracellular polysaccharide secretion. Metal avoidance, where the organism is able to restrict metal uptake (reduce uptake

or increased efflux, formation of complexes outside cells, organic acid release, etc.), and finally for last tolerance where the organism survives in the presence of high internal metal concentrations (Gobran & Wenzel, 2000).

AM fungi affect metal uptake by plants from soil and translocation from root to shoot by forming extensive hyphal networks. The external mycelium can produce glycoprotein, glomalin, with heavy metal binding properties (Li & Feng 2001; Vivas et al., 2003; Agely et al, 2005) In addition, AM fungi have been shown to induce plants to increase antioxidants levels to protect their cells from oxidative damage (Gopi et al., 2007; Zhang et al., 2007).

1.5 Common mycorrhizal network and Cadmium

One or more mycorrhizal fungi can colonize two or more plants, forming mycelial links in a common mycorrhizal network. Common mycorrhizal networks (CMN) add a level of complexity to the analysis of benefits in mycorrhizal interactions (Jakobsen & Hammmmer, 2015). Both partners in the symbiosis can choose among multiple trading partners and do not depend on a single partner for their carbon or nutrient resources. CMN can connect plants of the same or of different plant species and different developmental stages and are involved in the long-distance transport of nutrients, water, stress chemicals, and allelochemicals in soil ecosystems (Voets at al, 2008; Babikova et al., 2013, Teste et al., 2015, Gorzalak et al., 2015).

There are two major common mycorrhizal networks, the asexual and obligate mycorrhizal which form arbuscular endomycorrhizas (AM) and ectomycorrhizas with basidiomycete and ascomycete fungi. CMN can link multiple plant species in a community (Simard & Durall 2004, Giovannetti et al., 2004). Water and possibly nutrients move between plants via the CMN (Querejeta, 2003; Allen, 2007; Gyuricza et al., 2010; Whiteside et al., 2019) and, likely, signals inducing plant defenses are also transported (Song et al., 2010). Many studies have focused mainly on seedling establishment in the neighbourhood of larger trees sharing the same fungal species (Kytoviita, Vestberg & Tuomi, 2003; Simard and Durall 2004; Selosse et al., 2006; Whitfield, 2007; Bennett & Groten, 2022; Varga & Kytoviita, 2016). Another very well studied area is already established plants which may also be affected to different degrees by the CMN, potentially altering intra and inter-specific competitive interactions among plants within a plant community (Simard & Durall, 2004; Booth & Hoeksema, 2010).

It has also been recently proven that CMNs help a plant cope with heavy metal toxicity (Cd), and they can transfer Cd from Cd-treated maize plants to untreated soybean plants as Chaohui Ding et al. (2022) show in their experiment. This work demonstrated that mycorrhizal

colonization significantly increased the Cd tolerance of maize plants. One of the fundamental roles of the mycorrhizal fungal network has been demonstrated in the distribution of resources in plant communities (He et al., 2003; Fellbaum et al., 2014). The increasing evidence shows that mycorrhizal networks facilitate inter-plant communication by biochemical signal transfer (Barto et al., 2011; Babikova et al., 2013; Weremijewicz and Janos, 2013; Gorzelak et al., 2015) and consequently influence the behavior of their neighboring plants (Gorzelak et al., 2015; Simard, 2018; Weremijewicz et al., 2018). This finding has a great effect on the understanding of the potential of CMN to distribute heavy metals in soils and minimize the detrimental effect of heavy metals on plants.

Arbuscular mycorrhizal and ectomycorrhizal mycelial systems can immobilize and reduce plant uptake of nonessential and toxic metals such as Cd and Pb and thereby decrease the passage of these elements through the food chain. The ability of mycorrhizal mycelium to selectively take up and pass on nutrients to their hosts whilst excluding toxic metals enables mycorrhizas to play an important role in the revegetation of metal-contaminated sites (Leake et al., 2004).

1.6 Carbon cycle and the mycorrhizal role

Mycorrhizal fungi are present in almost all terrestrial ecosystems forming a symbiotic association with land plants, from deserts to tropical forests to arable lands (van der Heijden & Sanders, 2002). These below-ground fungi play a substantial role in terrestrial ecosystems as they regulate nutrient and carbon cycle as well as influencing soil structure and multifunctionality (van der Heijden & Martin 2015; Soudzilovskaia et al 2015).

This symbiosis is driven by carbon, which the host plant provides to the mycorrhizal fungus in exchange for the acquisition of soil nutrients. As heterotrophs, mycorrhizal fungi are completely dependent on C as their energy resource for growth and their metabolism. Their mycelia through intimate contact can absorb and access organic molecules, mostly sugars, which afterwards are distributed in the rhizosphere. The majority of C redistributed from the host plant to the mycorrhizal fungus is approximately 10-50% of the total plant-fixed C (Simard et al., 2003) and the majority of C is released by the fungi via respiration and exudation (Van Hees et al., 2005). Changes in the plant community can modify the colonization of roots by mycorrhizal fungi and may affect the sequestration of carbon in ecosystems, and it is suggested that ecosystem C cycling and storage might be determined by the type of mycorrhizal on the ecosystem (Averill et al., 2014). It has been reported that areas where ECM fungi are abundant

are also characterized as having high soil C levels (Read et al., 2005), one of the reasons may be that plants colonized by ECM tend to have low-litter quality, which might promote soil C storage.

There is some evidence that mycorrhizal fungi contribute to the C cycle through the redistribution of new fixed C through the soil, by feeding organic matter mineralisation pathways. Mycorrhizal fungi obtain their C in a highly labile form directly from their partner (photosymbiont) and they are not C-limited to the same extent as other saprotrophs that rely on C from decomposing soil organic matter (SOM) (Orwin et al., 2011). Despite the fact of their symbioses' dependency on C, there is accumulating evidence that mycorrhizal fungi also contribute to the direct loss of soil C in all types of ecosystems by performing as a decomposer (Moorhead & Sinsabaugh, 2006; Lindahl & Tunlid 2015).

These organisms might be responsible for promoting a unique contribution to carbon storage in the soil, however, the direct contribution is not well understood yet. It is still unknown how much mycorrhizae can add to the C pools. Some authors believe that their contribution to the C pool is not of a magnitude immediately relevant to ecosystem level considerations (Rillig et al., 2001). Despite this statement, a growing body of work suggests that ecosystem models should incorporate the traits of mycorrhizal fungi, as they fundamentally alter ecosystem C cycling is rising (McGuire & Treseder, 2010; Ostle et al., 2009).

Mycorrhizal symbioses may contribute to soil C storage and they are capable of expressing a wide range of attributes on nutrition and protection, such as mobilising a range of nutrients from complex substances (Rich et al., 2017), resistance to disease (Pozo et al., 2010), climatic stress, as well as to the impact of pollutants (Ferrol et al., 2016; Meier et al., 2015). Mycorrhizal fungi also influence other ecosystem processes, e.g. soil aggregation, seedling survival, plant growth and performance, tolerance to biotic and abiotic stress, such as, water deficit, (van der Heijden & Sanders, 2002).

AMF can have different effects on C cycling. Studies show that AMF promotes carbon stocks and has a positive correlation with AMF extraradical hyphae in long-term field experiments (17 and 6 years) (Wilson et al., 2009). Another way by which AMF affects soil C cycling is through its effects on soil structure, for example, the distribution and size of soil aggregations. As Leifheit et al., (2015) confirm in their experiment, one of the few studies that show that in the presence of AMF, there is a reduced decomposition of woody plant litter while promoting an increase in the soil aggregation, which means AMF has yet another influence on soil carbon storage (Leifheit, Verbruggen, & Rillig 2015).

As the hyphae and spores from AM decompose, glomalin is released and deposited in the soil, which can constitute a non-trivial portion of the terrestrial carbon pool. Glomalin is a type of glycoprotein that is produced by AMF hyphae and not by another group of fungi, it was discovered by using specialized extraction protocols for soil, and there is still limited information. Glomalin carbon concentration can be 2-25 times that of humic acid (HA). This compound can enhance soil aggregation which can contribute to the reduction of organic matter degradation and enhance carbon sequestration (Wu et al., 2014).

Recent studies have identified the use of ^{14}C labeling indicated that photosynthate is transferred from host plants to AMF hyphae within hours of labelling. It is widely accepted that AMF receive all their carbohydrate from their host plant(s) (Zhu & Miller, 2003). These symbioses could create a sink demand for carbohydrates, which potentially results in a 4-20% drain of carbon from the host plant and could indirectly influence carbon storage in soils (Johnson, Leake, & Read, 2002). AMF hyphae are responsible for the production of glycoprotein-like substance, glomalin (Wright & Upadhyaya, 1998), which is fairly stable in soils. Radiocarbon dating of the operationally defined glomalin extracts indicates a residence time in soils of 6-42 years, much longer than the residence time reported for AMF hyphae (Miller, Jastrow & Reinhardt, 1995). The close correlation of the amount of glomalin in soil with hyphae and the stability of soil aggregates, suggests that glomalin could influence soil carbon storage indirectly by stabilizing soil aggregates. One of the modes of action of glomalin could be facilitating the formation of a sticky string bag of hyphae, the primary mode by which AMF contributes to soil aggregation. However, with glomalin still only operationally defined, the challenges ahead are to isolate and characterize the substance, identify the mechanism of its stabilizing action in soils, and define the relationship between hyphal turnover and glomalin inputs to the soil.

1.7 Ectomycorrhizal fungi

Ectomycorrhizal has a wide range of different species, according to Molina et al., (1992) it is approximately around 5000 and 6000, within a diverse structural and functional recognition. These fungi appear to produce more extensive mycelial networks (Van Der Heijden & Horton, 2009) as well as a more vast and powerful range of extracellular enzymes to mine nutrients from organic matter than AMs (Kohler et al. 2015). Trees that are colonised by ectomycorrhizas serve as the primary nutrient gathering interface, scavenging nitrogen, phosphorus, and elements from organic and inorganic pools in the soil (Smith & Read, 2008).

There is evidence that EM ecosystems store 1.7 times more carbon per unit of soil N than AM ecosystems. According to Soudzilovskaia et al., (2015), EM fungi have a higher capability to accumulate more C than AM fungi from their host plants and, correspondingly release more C into the soil (Van der Heijden and Martin, 2015; Soudzilovskaia et al. 2015).

It has been suggested that ECM fungi immobilize both C and N through active secretion of proteins that may persist in soil, stabilizing soil organic matter (Aber et al. 1998). However, the amount of C belowground is different between soil locations consequently EMF will respond differently depending on its conditions. Some field experiment estimates that 40-73% of assimilated C (Persson 1978), in laboratory studies, 3-36% more C belowground than plants without ectomycorrhizal (Churchland and Grayston 2014; Durall M., Jones, and Tinker 1994). C allocation to ECM fungi can be beneficial to plants if plants get a net gain in terms of acquired N. When the C is tracked on the ECM fungi, it may identify an optimal level of C investment in ECM fungi that maximizes plant growth (Pringle, 2016).

Ectomycorrhizal fungi also have the potential to produce a range of hydrolytic and oxidative enzymes that have the potential to break down C-containing compounds and mobilize nutrients from SOM (Courty et al, 2010; Plassard and Fransson, 2009; Floudas et al., 2012). Clemmensen et al. (2015) demonstrated that a higher abundance of ECM fungi in early successional-stage forests was linked to high N mobilization to trees and low C sequestration, and proposed that certain ECM fungi may contribute directly to N release from recalcitrant organic stores and alleviate N limitation, playing a role as “decomposers”.

This process of carbon allocation from trees to ectomycorrhizal fungi appears to be sensitive to anthropogenic influences such as elevated nitrogen deposition, ozone, and carbon dioxide (Rillig et al. 2002).

1.8 Phytoremediation

More than 1.4 million sites in Western Europe, not less than 160,000 sites in Europe (Montpetit and Lachapelle 2017), and 20% of China’s agricultural land (Zhang et al. 2015) are contaminated with heavy metals. These metals are steadily accumulated in the environment because of the expansion of industrial activities and their non-degradability (Clemens et al. 2013). Regarding their toxicity, the enrichment of heavy metal ions leads to decreases in biodiversity, and productivity and consequently changes in the structure and function of ecosystems (Mayor et al. 2013).

Cadmium is one of the most harmful metals in the environment because has been highly toxic to humans and other organisms at a relatively small concentration. In humans, if under long-term exposure, it leads to mutagenic effects acting as carcinogenic (Templeton and Liu 2010). The toxicity is so severe that can contribute to problems in the DNA protein structures that function to protect the ends of eukaryotic chromosomes (Zota et al. 2015).

Most heavy metals are not biologically important, some elements as Zn, Cu, and Mn are micronutrients, but higher concentrations of them can be toxic (Dučić and Polle 2007). Other HMs such as Cd, As, Pb, Hg, and Ni have no biological function and are toxic elements for most organisms (Jourand et al. 2010). When these elements are taken up by plants and placed in edible parts, they consequently enter the human body through the food chain (Kaplan, Ince, and Yaman 2011). The toxicity of HMs to human health depends on the element, some are carcinogenic, and others affect the central nervous system, kidneys, and bones (Bertin and Averbeck 2006).

These metals are toxic not only for human health but also for all living organisms. They inhibit photosynthesis and affect soil biogeochemical progress including microbial and enzyme activities, microbial community structure, and the contents of organic compounds. Heavy metal contamination can affect plant development and ecosystem functions, inhibiting several species of plants to grow. However, plants are also major accumulators of inorganic nutrients, such as metal (Ross and Kaye, 1994), in order to survive, plants developed some specific mechanisms to tolerate HM uptake from soils (Zenk 1996).

Heavy metals can be absorbed by plants, but are dependent on their bioavailability and solubility (Shah et al. 2010). Plants adopt different strategies to cope with metal toxicity which provide several tolerance mechanisms such as metal sequestration, compartmentalization in certain cell organelles, exclusion, and inactivation by exudation of organic ligands (Choppala et al. 2014). The use of plants and their associated microorganisms for environmental cleanup, by removing, destroying, or sequestering hazardous contaminants (Prasad 2003), is called phytoremediation. It is a technique that can be useful for several contaminants, eco-friendly, and cost-effective, making it an alternative to conventional methods (Guerra et al. 2011; Ali et al. 2013).

Many techniques and applications are included in the term phytoremediation. The difference is in the process by which plants can remove, immobilize, or degrade contaminants. Phytostabilisation is one of the most important for heavy metals (Prasad 2003). Metal ions can

become less available in soil due to absorption, complexation, reduction, or precipitation within the roots or the rhizosphere (Ali et al. 2003, Thakur et al. 2016), consequently, the plant will restrict the transfer of metals to its shoots (Qasim et al. 2016). This prevents off-site contamination through their migration via wind and water erosion, leaching, and soil dispersion (Bolan et al. 2011).

Phytoremediation is recognised as cost cost-effective method for mitigating soil contaminated by heavy metals. Since the 1980s, there has been increasing demand for fast-growing, stress-tolerant trees that can be used for the reforestation of post-agricultural lands, for environmental cleanup or reclamation (Willey 2007). Comparing agricultural species with trees, the latter has more advantages in remediating soil contaminated by heavy metals. The root system is potentially deeper reaching a bigger area and site stabilization which will even help to reduce leaching in the groundwater, (Dos Santos Utmazian and Wenzel 2007) erosion control, increase of organic matter in the soil, perennial and long-lived plant (Brunner et al. 2008; Domínguez et al. 2008). The soil condition can even be improved by mycorrhizal which can form symbioses with some tree species. This symbiosis allows a higher accumulation of heavy metals into its cell walls and fungal vacuoles (Brunner et al. 2008).

Recently, research on phytoremediation has illustrated the benefits of using species that have great biomass as a renewable energy source (Gomes 2012). In addition to the cost, phytoremediation offers a better approach in comparison with other techniques, less disturbance to the site, and permanent removal of the heavy metals.

Poplar is currently one of the most studied species, not only for its phytoremediation activity but also for its capacity to be a source of energy in biomass production. *Populus* species can also rapidly invade disturbed sites, and reproduce asexually – By sprouting from the root collar of cut trees or broken branches and are not a source of food for farm animals, therefore reducing the risk of heavy metals entering the food chain (Shim et al. 2013). Poplars are among the few commercially important trees, which also include *Salix*, *Eucalyptus*, *Alnus*, *Acacia*, and *Casuarina*, that form tripartite symbiotic associations with ectomycorrhizal (ECM) and arbuscular (AM) fungi.

Mycorrhizal may improve phytoremediation by improving the extraction of heavy metal, through biomass production, more tolerant plants, increase of soil area explored, increase fungal exudation (Smith and Read 2008; (Sheoran, Sheoran, and Poonia 2011; Vamerali, Bandiera, and Mosca 2010).

Table 1. Compilation of several studies on the effect of common mycorrhizal networks on plants.

Host plant	Mycorrhizal fungus	Col. Rate (%)	Network activity	Reference
<i>Cucumis sativus</i> L.	<i>Rhizophagus irregularis</i>	87	Competition between plants, P transferred to large plants	Merrild et al. (2013)
<i>Solanum lycopersicum</i> L.		90		
<i>Zea mays</i> L	<i>Funneliforms mosseae</i>	26.2	Transferred cadmium from maize to soybeans	Ding et al. (2022)
<i>Glycine max</i>		22.4		
<i>Trifolium pratense</i>	AMF (there is no specific species)	48	CMNs preferentially distributed more mineral nutrients to the nonparasitized neighboring.	Yuan and Kleunen (2021)
Oaks, <i>Salvia</i> , <i>Eriogonum</i> and <i>Keckiella</i>	<i>Cenococcum geophilum</i> Fr., <i>Cortinarius</i> , <i>Telamonia</i> ,	EM (Donor -72, receiver 74); AMF (Donor – 23, receiver – 19)	Water transport via CMNs is bidirectional and cycles diurnally, moving toward transpiring plants during the day, but toward dry soil areas at night	Warburton, Querejeta and Allen (2007)
<i>Lycopersicon esculentum</i> Mill.	<i>Glomus mosseae</i>	Treat. A (Receiver-36.2 and Donor 55.5), Treat C (Receiver-48.7 and Donor-42.7) Treat D (Receiver	plants can 'eavesdrop' on defence signals from the pathogen-challenged neighbours through CMNs to	Song et al. (2010)

		36.1 and Donor 60.3)	activate defences before being attacked themselves	
<i>Tagetes tenuifolia</i>	AMF	61.4	CMNs expand the bioactive zones of allelochemicals in natural environments	Barto et al. (2011)
<i>Vicia faba</i>	<i>Glomus intradices</i>	x	Foliar defence chemistry	Babikova et al. (2013)
<i>Lycopersicon esculentum</i> Mill.	<i>Funneliformis mosseae</i>	Treat A (Donor 59.7, Receiver 38.1), treat C (Donor 55.4, receiver 41.5), Treat D (Donor 46.4, receiver 38.2)	Defence response to leaf-chewing caterpillar	Song et al. (2014)
<i>Casuarina cunninghamiana</i>	<i>Pisolithus tinctorius</i>	X	Transferred element 14NH ₄ ⁺	He (2002)
<i>Eucalyptus maculata</i>	<i>P. tinctorius</i>	X	14NH ₄ ⁺	He (2002)
<i>Glycine max</i>	<i>G. masseae</i>	X	Transferred 14 NH +4	He (2002)
<i>Medicago truncatula</i>	<i>Rhizophagus irregularis</i>	AM +Zn (Donor 52.4, receiver 46.4) AM – ZN (Donor 45.6, receiver 48)	Tranferred Zn	Cardini et al.,(2021)

<i>Quercus agrifolia</i> Nee	<i>Cenocuccum</i> <i>geophilum</i> , <i>Cortinarius</i> , <i>Boletus</i> <i>amygdalinus</i> , <i>Boletus dryophilus</i>	Total % EM (Donor 72, receiver 74.7), Total % AM (Donor 23.4, receiver 19.7)	Transferred water	Egerton- Warburton, (2007)
<i>Pinus densiflora</i>	<i>P. tinctorius</i>	X	Transferred 14 C	Wu et al., 2001
<i>Festuca ovina</i>	<i>Glomus sp.</i>	X	One-way and two- way transfer 13C	Graves et al. 1997
<i>Quercus agrifolia</i>	<i>Cenococcum</i> <i>geophilum</i> and <i>Cortinarius spp.</i>	20-49	Transferred	Querejeta et al. (2003)
<i>Calamagrostis</i> <i>epigejos</i>	<i>Glomus mosseae</i> , <i>G. claroideum</i>	Sand – G. mosseae (22, 14, 32, 37) Sand – G. claroideum (43, 30, 39, 26)	Transferred between plants 14 C	Malcova et al., 2001
<i>Pinus sylvestris</i>	<i>P. involutus</i>	X	Transferred between plants 15NH+4	Ek and Huss- Danell, 1995
<i>Erythronium</i> <i>Americanum</i>	<i>Field condition</i>	X	Transferred between plants, 14C	Lerat et al., 2002
<i>Plantago</i> <i>lanceolata</i> <i>Festuca ovina</i>	<i>Glomus</i> <i>caledonium</i> <i>Unspecific</i> <i>inoculum</i>	(30-50) non- specific and (15- 20) specific	Transferred 14C	Francis and Read, 1984

<i>Pseudotsuga menzeisii</i>	<i>Unspecific ectomycorrhiza</i>	X	Transferred 14C	Simard et al. 1997
<i>Betula papyrifera</i>				
<i>Arachis hypogaea</i>	Field soil with roots	40-60	Transferred 14NH+4	Moyer-Henry et al. 2006
<i>Sida spinosa</i>				
<i>V. radiata</i>	<i>G. caledonium</i>	With plastic film (rice 4 and mung bean 48.6), nylon-net (rice 33, mung beans 65)	Transferred 15NH+4	Li et al. 2009
<i>O. sativa</i>				
<i>Pseudotsuga menziesii glauca</i>	<i>Rhizopogon var. vinicolor</i>	In situ seedlings (0.5 um) donor 79, planted 87; (35 um) donor 88, planted 88; (250 um) donor 73, plated 88 (no mesh) donor 92, planted 87	Transferred carbon	Teste et al. 2010

1.9 Hypotheses, objectives, and thesis structure

The literature reviewed above (Table 1) shows the need for more detailed knowledge on (1) the common mycorrhizal network under Cadmium stress, (2) the metal transport between plants, under common mycorrhizal network, (3) the potential of this system extract metal out of the soils. Such information can be critical for phytoremediation, as well as microorganisms that can be efficiently applied in bioremediation processes.

Overall aim:

To understand the physiological basis of the common mycorrhizal network under cadmium stress its singularities as well as the potential of several saprotrophic fungi species on remediating soil contaminated by cadmium.

General hypotheses:

The common mycorrhizal network increases the amount of cadmium in plants.

Objectives:

- To screen for ectomycorrhizal fungi species in which Cadmium impacts their extracellular carbon cycling enzymes.
- Verify the accumulation of Cd in poplars and leeks when they are under a common mycorrhizal network.
- To investigate the potential of a common mycorrhizal network to transfer metal between poplars.
- To assess in the literature the potential of saprotrophic fungi species on their capacity to remediate Cd contaminated soil.

This thesis is divided into 6 chapters, a summary of each chapter is provided below.

Chapter 1: Literature Review

This chapter contextualizes the state of the art of the common mycorrhizal network and evidence of transfer substances.

Chapter 2: Cadmium stress causes differential effects on growth and the secretion of carbon degrading enzymes in four mycorrhizal basidiomycetes

This chapter aimed to evaluate the response of 3 ectomycorrhizal and a feremycorrhizal fungus in vitro on their extracellular carbon enzyme activity under Cd stress.

Hypotheses: Cd toxicity would hinder growth and the activity of four C degrading enzymes in different fungal strains, according to their tolerance to Cd stress.

This chapter has been published at Mycosciences (2021): <https://doi.org/10.47371/mycosci.2020.12.002>

Chapter 3: Common mycorrhizal networks mediate cadmium accumulation, glomalin production and soil enzyme activity in co-cultures of poplars and leeks

The main objectives of this chapter were to investigate the common mycorrhiza network, between poplars and leek, under Cd contamination.

The hypotheses were: (1) When mycorrhizal poplars are in co-culture pots, they accumulate less Cd (2) CMN enhances glomalin production when under soil contaminated by Cd; (3) CMN enhance carbon liberating enzymes, under soil with Cd contamination.

Chapter 4: Cadmium transfer between common mycorrhizal networks

Chapter 4 has the main experiment of this thesis in which poplar trees in symbiosis with arbuscular mycorrhizal fungus *Rhizophagus irregularis* facilitated to create a common mycorrhizal network with another poplar tree were assessed, in order to identify if under Cd contaminated soil they could transfer metal between them.

Hypotheses were: (1) CMNs can transfer Cd between poplars; (2) Nutritional concentration of the receiver and the donor plants are different; (3) Plant growth of the receiver and the donor plants are different.

Chapter 5: The role of saprotrophic fungi in environmental remediation, particularly in the context of cadmium contamination

This chapter is a review of several saprotrophic fungi and their potential to remediate soil contamination.

Chapter 6: General discussion

The main findings are discussed in the wider context of practical phytoremediation.

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Chapter 2

2. Cadmium stress causes differential effects on growth and the secretion of carbon degrading enzymes in four mycorrhizal basidiomycetes

2.1 Introduction

Secretion of enzymes by soil borne mycota is a common feature of symbiotic fungi that associate with woody roots. Ectomycorrhizal fungi (ECMF) form symbiosis with approximately 2% of vascular plants, they are defined by the presence of a Hartig net and mantle, (Brundrett & Tedersoo, 2018), the latter being formed around the root tip (Landeweert, Hoffland, Finlay, Kuyper, & Breemen, 2001). This mycorrhizal symbiosis is based on a reciprocal exchange of solutes; the fungus provides nutrients and water to plants in return for sugars from the phytobiont (Smith & Read, 2008). A newly described non-colonising symbiosis Feremycorrhiza (FM) in which the fungal hyphae inhabit rhizospheric soil but do not penetrate the plant roots (Kariman, Barker, & Tibbett, 2018), presents a remarkable capacity for biological dissolution of soil minerals and nutrient mobilization (Kariman, Barker, Finnegan, & Tibbett, 2012; Kariman, Barker, Jost, Finnegan, & Tibbett, 2016). ECMF also possess the capacity to access carbon (C) from soils by decomposing moribund organic matter and are known to metabolize simple organic compounds (Finlay, Frostegard, & Sonnerfeldt, 1992). Although ECMF has a modest ability to decompose organic matter, a restricted supply of plant photosynthates may increase enzyme production for obtaining carbohydrates from soil organic matter (Courty, Breda, & Garbaye, 2007; Courty, et al., 2010). However, this saprotrophic nature and organic matter degradation of ECMF is mostly associated with scavenging for other nutrients, such as N and P (Tibbett, Sanders, Minto, Dowell, & Cairney, 1998; Shah et al., 2016; Nicolas et al., 2019).

Some ECMF are facultative symbiotic and may display saprotrophic characteristics under various conditions, which puts them in a “biotrophy-saprotrophy continuum” (Kusuda et al., 2006; Koide, Sharda, Herr, & Malcolm, 2008). ECMF were found to possess genetic potential to produce Class II peroxidases – extracellular enzymes that are efficient in lignin decomposition (Bödeker et al., 2014). This saprophytic role in ECMF can be affected by heavy metal contamination in soils (Bellion, Courbot, Jacob, Blaudez, & Chalot, 2006), which is a pressing concern due to the potential hazard to environmental health and food safety. Heavy metals may alter the process of carbon cycling performed by soil microorganisms, mostly by inducing changes in their metabolism. According to Dahm and Strzelczyk (1996), Pb, Zn, Cd, and Cu inhibit the general enzymatic activity of the ECMF *Hebeloma crustuliniforme*, and Cd interferes with

pathways resulting in cellular damage because of its strong affinity for the sulphhydryl residues (Gallego et al., 2012). Therefore, heavy metals in soils may decrease the rate of decomposition of organic matter, as a result of the decrease of microbial activity.

Cadmium (Cd) is one of the most hazardous metals in the environment (ATSDR, 2017), it is toxic to living organisms at low concentrations (Alloway, 2012) and has high mobility in soils (Lei, Zhang, Khan, Qin, & Liao, 2010). In contaminated soil, symbiosis with ECMF can improve metal tolerance in the host plant by enhancing the plant's nutritional status and growth (Krznaric et al., 2009). Direct and indirect effects on the fungal performance are expected in polluted soils, as studies have shown that Cd affects soil microbiota (Chen et al., 2014; Landi, Renella, Moreno, Falchini, & Nannipieri, 2000) and the growth of ECMF (De Oliveira & Tibbett, 2018). However, the information about Cd impacts on the role of ECMF in soil carbon cycling and/or decomposition of organic matter is still very limited (Vivas, Barea, & Azcon, 2004; Johansson et al., 2008). There is a need for a better understanding of the influence of Cd on the secretion of carbon-degrading enzymes by ECMF. This study aims to determine the impact of Cd on the growth and secretion of extracellular enzymes by ECMF and FM fungi in axenic culture. We hypothesised that Cd toxicity would hinder the growth and the activity of four C degrading enzymes in different fungal strains and that these would vary according to their tolerance to Cd stress.

2.2 Material and methods

Toxicity assays were conducted in vitro using three ECM and one FM species, all isolated from non-polluted environments (Table 1). These species were selected from our in-house collection due to our understanding of their expected behavior in vitro. The three ECMF species and one FM, were: *Hebeloma subsaponaceum* (MycoBank no.:160633), *Hebeloma* sp. (MycoBank no.: 17723), *Scleroderma* sp. (MycoBank no.: 19309) and the FM species *Austroboletus occidentalis* (MycoBank no.: 129957) (Table 1). Nine 5 mm circular plugs were removed from the edges of actively growing fungal colonies (2 wk old) and transferred to Petri dishes containing 25 mL of modified Melin-Norkrans (MMN) liquid medium (Marx, 1969). A solution of CdCl₂ was added to the medium to reach the final concentrations of 1 and 3 mg/L Cd, while no Cd was added to the control (0 mg/L). Concentrations were based on previous toxicity experiments with Cd and ECM fungi (De Oliveira & Tibbett, 2018). The fungal cultures were incubated in the dark at ±22 °C for 28 d, each treatment had four replicates. After the growth period, the liquid medium was filtrated using a vacuum pump, with paper filters (no 1;

Whatman, Standard; Merck, England) to separate the mycelium from the liquid culture. The culture filtrate was collected for the determination of enzyme activities.

Table 2.1. Strains of mycorrhizal basidiomycetes selected for this study

Strain	Species	Isolated: from/	
		under	Origin/ Habitat
<i>W</i>	<i>Hebeloma subsaponaceum</i>	Boreal Forest	Boreal Forest, Norway
<i>D</i>	<i>Hebeloma</i> sp.	Pine trees	Pine trees, France
<i>Sc Hu</i>	<i>Scleroderma</i> sp.	Woodlands	Eucalypt, woodlands, Western Australia
<i>AB</i>	<i>Austroboletus occidentalis</i>	–	Eucalypt, woodlands, Western Australia

The activities of four hydrolytic enzymes were determined using fluorogenic substrates. The enzymes, substrates, and their functions are described in Table 2. The substrates were dissolved in deionized water, 4-MUB- β -D-glucopyranoside (EC 3.2.1.21) 67.7 mg/L DI H₂O, 4-MUB- β -D-xylopyranoside (EC 3.2.1.37) 61.7 mg/L DI H₂O, 4-MUB-N-acetyl- β -D glucosaminide (EC 3.2.1.50) 75.9 mg/L DI H₂O, and 4-MUB- β -D-cellobioside (EC 3.2.1.91) 100 mg/L. To determine the enzyme activities, 96-well black flat bottom microplates were used, with each well containing 50 μ L of culture filtrate, 100 μ L of the respective substrate, and 50 μ L of a modified universal buffer (Turner, 2010). Aliquots (50 mL) were adjusted to pH 5.5 with 1.0 M HCl or 1.0 M NaOH, then diluted to 100 mL with deionized water and stored at 4 °C (for up to 2 wk).

Table 2.2 Description of the enzymes and substrates used in the present experiment.

Substrate	Enzyme	General function
4-MUB- β -D-glucopyranoside	β -glucosidase (BG) EC 3.2.1.21	Hydrolysis of β -glucosyl residues to release β -D-glucose, the final step in cellulose hydrolysis
4-MUB- β -D-xylopyranoside	β -Xylanase (XYL) EC 3.2.1.37	Hydrolysis of cellulose from plant cell wall
4-MUB-N-acetyl- β -D-glucosaminide	N-Acetyl-Glucosaminidase (NAG) EC 3.2.1.50	Hydrolysis of glycosidic (N-acetyl-B-glucosaminide) bonds in chitin
4-MUB- β -D-cellobioside	β -D-Cellubiosidase (CB) EC 3.2.1.91	Hydrolysis of cellulose

Control wells were prepared for each substrate, containing buffer and pure MMN media (without enzymes). Blank wells contained the culture filtrate and buffer only (but no substrate). Standard curves were performed using buffer, 1 nmol methylumbelliferone (MU), and MMN medium without Cd addition. Microplates were covered with aluminum foil and incubated for 1.5 h at 26 °C, the fluorescence was determined immediately on a multidetection plate reader SpetroMax i3x (Molecular Devices), with excitation at 360 nm and emission at 460 nm. All enzyme activities were expressed as μmol activity per gram of dry fungi per 1.5 h (μmol 1.5 h⁻¹ g DW⁻¹).

Statistical analyses were performed on the dry weight (DW) and enzyme activities using R[®] software (R core team 2017). All data had homogeneous variances (Levene test, $p > 0.05$), but were not normally distributed. There were a couple of trials to transform the data but we did not reach reasonable numbers. Therefore, the non-parametric Kruskal Wallis test was applied, and when results were significant ($p < 0.05$), the Dunn test was used to discriminate the differences among treatments.

2.3 Results/Discussion

Our results (Figs. 1, 2) did not confirm the hypotheses tested and there was a wide variation of carbon-degrading enzyme activities amongst strains under Cd stress, and the results did not relate to the level of Cd tolerance. The biomass produced by *Scleroderma* sp. (ECMF) increased with the Cd concentration (Fig. 1C). This suggests that Cd triggered mycelial growth in *Scleroderma* sp., which could be a hormesis effect, in which small amounts of a toxic substance increase growth – possibly by activation of defensive mechanisms. This effect has been verified in both plants and fungi exposed to different heavy metals (Collin-Hansen, Andersen, & Steinnes, 2005; Morkunas et al., 2018; Carvalho et al., 2020). In an experiment, Baldrian and Gabriel (2002) also found that under Cd exposure, a wood-rotting fungus *Piptoporus betulinus* forms a dense layer of hyphae, which does not happen without Cd. In contrast, in the work from Oliveira and Tibbett (2018), using the same strain of *Scleroderma* was unaffected by both 1 and 3 mg/L Cd in terms of biomass. *Scleroderma* sp. is known to be a genus frequently found in contaminated areas (Colpaert, 2008), therefore may be tolerant to heavy metal toxicity (Hancock, Ernst, Charneskie, & Ruane, 2012). Unlike *Scleroderma* sp. *A. occidentalis* growth suffered a significant impact under Cd exposure, with a sharp biomass decrease as Cd concentration increased (Fig. 1D), indicating a high sensitivity to Cd stress.

The impact of Cd in ECMF and FM enzyme activity is shown in Figure 2. The enzyme activities were not consistent, β -glucosidase had a significant increase under Cd exposure (3 mg/L) in *A. occidentalis*, while *H. subsaponaceum* had a decrease in activity under the same Cd concentration (Fig. 2A). In contrast to our hypothesis, an increase in enzyme activity due to metal stress has been observed before. Martino et al. (2002), demonstrated the positive influence of heavy metal (Zn) in the secretion of polygalacturonases (PG) in the medium by ericoid mycorrhizal fungi (*Oidiodendron maius*). Moreover, membrane damage caused by metal stress can lead to enzymes being released from the cytoplasm into the growth medium, which can also explain the increase of enzyme activities found for *A. occidentalis*, under Cd exposure (Fig. 2) (Gadd et al., 2012). For instance, Wang, Yi, Liu, Han and Yi (2017) found that Cd exposure resulted in the collapse of mitochondrial membranes in yeasts, while cytoplasmic damage to mantle hyphae following exposure to aluminum has also been observed (McQuattie & Schier, 1992). Tibbett, Sanders, Grantham and Cairney (2000) also emphasizes the possibility of mistakenly measuring cytoplasmic intracellular enzymes if cells are accidentally damaged during the filtration process.

β -D-cellulobiosidase activity increased in *Scleroderma* sp. and β -xylanase was higher in *Hebeloma* sp. under Cd 3 mg/L (Fig. 2B, C). A similar increase was also observed in an ericoid mycorrhizal fungi (*Oidiodendron maius*) under Cd and Zn, which promoted the activity of pectinolytic enzymes that may play a direct role in the avoidance of heavy metal toxicity and/or influence fungal performance indirectly by increasing nutrient acquisition (Martino et al., 2002). However, the significance of this response is still not clear, and may be a stress response by increasing the enzyme as a way to survive. For instance, an increase in the release of extracellular enzymes has been reported in the bacteria *Erwinia* spp. due to general DNA damage by stress factors (Barras & Chatterjee, 1994).

All enzyme activities were negatively affected by Cd in *H. subsaponaceum* (3 mg/L), which means that their secretion of carbon-degrading enzymes were more sensitive to Cd under our study conditions. For this strain, the result matched our hypothesis.

As the experiment was conducted in vitro and only exposed to one C compound (glucose), it is not possible to infer that there would be similar enzyme activity within soil organic matter. The experiments conducted by Zak et al. (2019) show that adding glucose to a growth medium can increase the secretion of carbon-degrading enzymes. We presume the glucose triggers the same response from the fungus as it would if it were photosynthates from a host. The partner would then modify the organic matter through oxidative decomposition enabling organic nutrients such as N to be released (Nicolas et al., 2019).

ECMF are weak saprotrophs with a limited decomposition capacity that can facilitate oxidative decomposition, depending on the type of environment (Shah et al., 2016). The activities of the ECMF enzymes tested here do not necessarily mean the fungi are independently saprotrophic, i.e. that they can degrade the components of plant cell walls only. These enzymes are however part of the process of breaking down complex organic matter. They may do this by releasing organic nutrients including N, and thereby facilitating the decomposition by other organisms in the soil.

The experiment demonstrated that Cd exposure resulted in neutral or positive effects in the biomass of three ECMF strains, while effectively decreased the growth of FM fungus *A. occidentalis*. Carbon-degrading enzyme activities varied under Cd stress, and there was not a consistent decrease as hypothesized. This study shows that there are many uncertainties concerning Cd stress and the secretion of carbon-degrading enzymes in mycorrhizal fungi in vitro. Further research should explore how this occurs in a soil matrix and under field conditions.

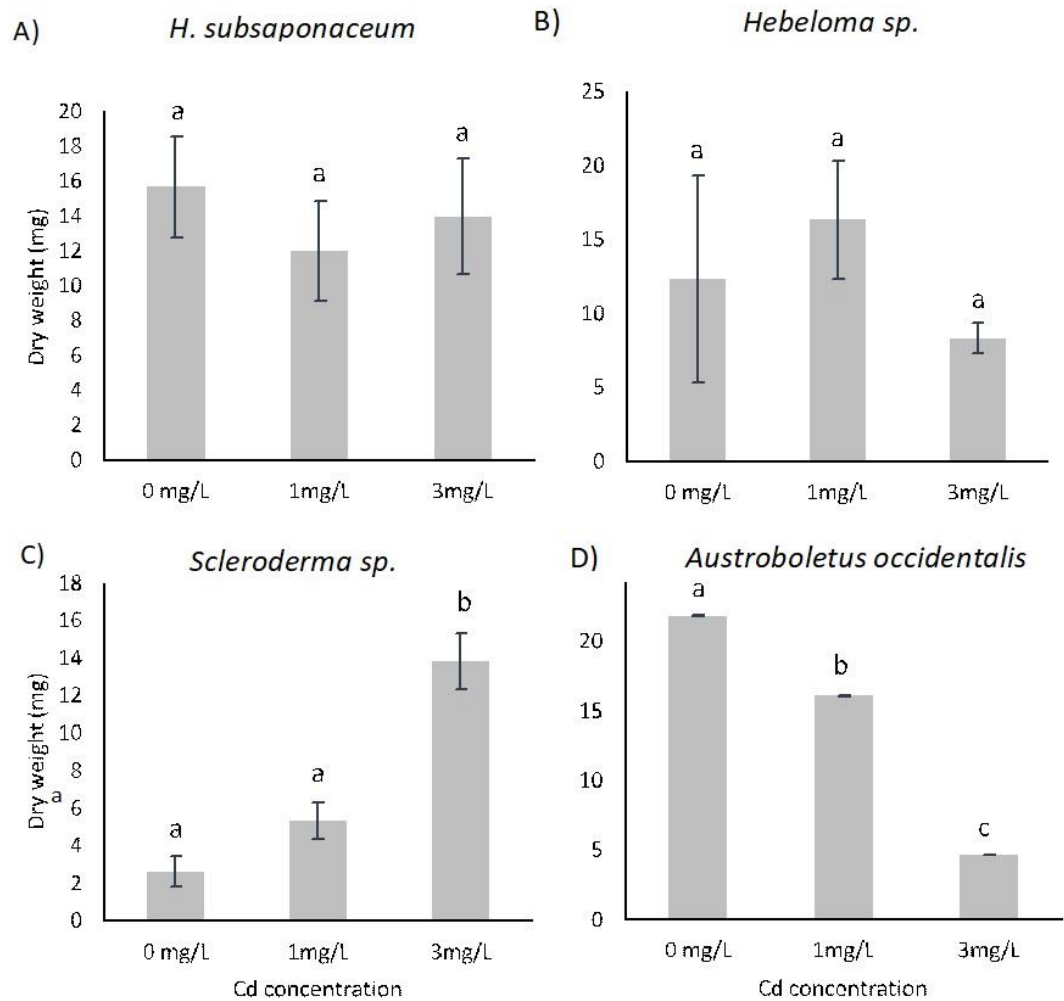


Fig. 2.1 - Dry weight of three ectomycorrhizal fungi species and one feremycorrhiza species after growing under Cd exposure for 28 d. A: *Hebeloma subsaponaceum*, (MB 160633), B: *Hebeloma sp.* (MB 17723), C: *Scleroderma sp.* (MB 19309) and D: *Austroboletus occidentalis* (MB 129957). Different letters represent significant differences by Dunn test ($p < 0.05$).

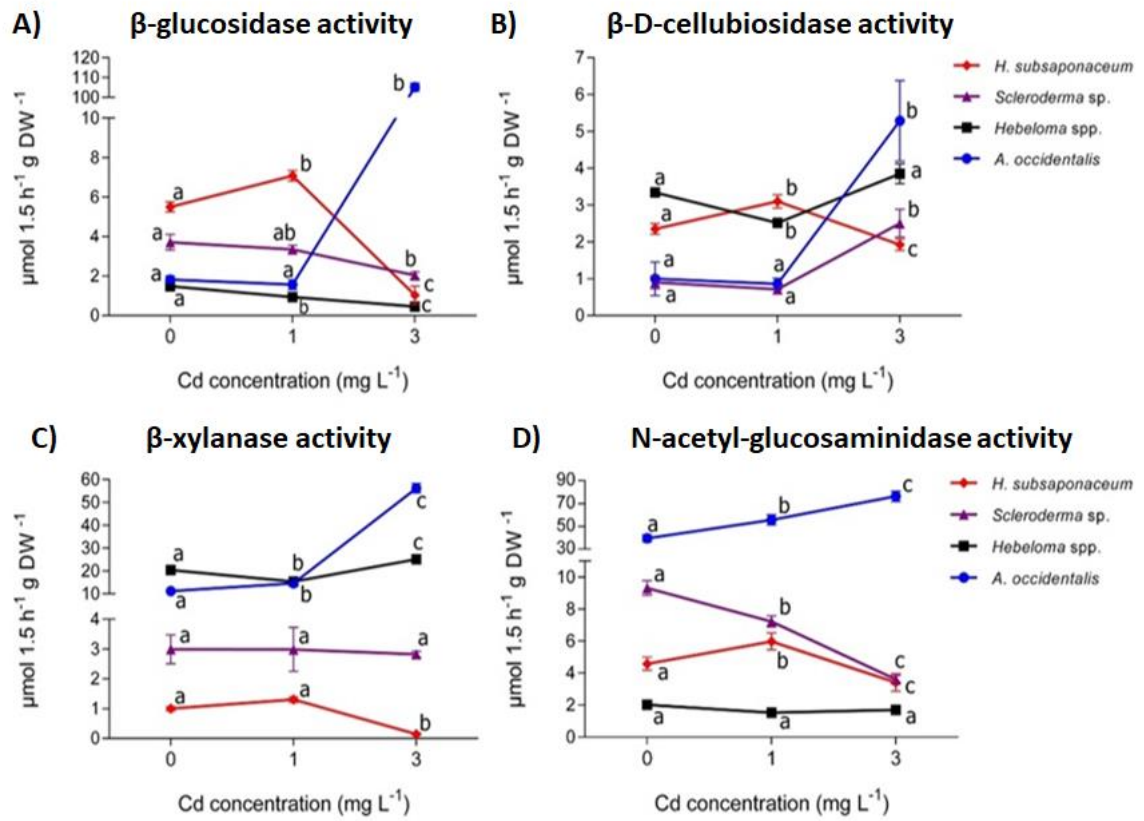


Fig. 2.2 - Activities of four extracellular enzymes produced by *Hebeloma subsaponaceum* (MB 160633), *Scleroderma* sp. (MB 19309), *Hebeloma* sp. (MB 17723) and *Auroboletus occidentalis* (MB 129957) grown under three different Cd concentrations for 28 d. A: β glucosidase, B: N-acetyl-glucosaminidase, C: β -D-cellubiosidase, D: β -xylanase. Different letters represent significant differences between Cd treatments within the same species (Dunn test, $p < 0.05$).

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Chapter 3

3. Common mycorrhizal networks mediate cadmium accumulation, glomalin production and soil enzyme activity in co-cultures of poplars and leeks

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) establish symbiotic associations with a majority of terrestrial plants to form underground common mycorrhizal networks (CMNs) that connect neighbouring plants (Selosse et al., 2006). They create a fungal link between different species of plants and different developmental stages (Dicke and Dijkman, 2001). As AMF are obligate symbionts and depend on the carbon (C) from their partner in exchange for increased nutrient uptake, it is understandable that mycorrhizal fungi have a strategy to ensure connectivity to plants and between plants (Bago, Pfeffer and Sharchar-Hill, 2000; Giovannetti et al. 2004). Similar to nutrients, water is taken up by fungal hyphae and delivered to host plants, and can be transported by the common mycorrhizal network from wet areas to dry areas, as shown by an experiment which used a fluorescent dye in order to see the movement along hyphae (Querejeta et al. 2003). Another study also showed that water transport within and on the outside of AMF hyphae (Kakouridis et al. 2022). Moreover, it has been demonstrated that the induction of defences in one plant can induce defences in a second plant if they are sharing a CMN (Babikova, et al. 2013, Song et al., 2010). Therefore, mycorrhizal colonisation, interplant resources and signal fluxes (carbon, water, nitrogen, phosphorus, micronutrients, stress chemicals and allelochemicals) through CMN have the potential to alter plant behaviour (Gorzalak et al., 2015).

Arbuscular mycorrhizal fungi form extraradical mycelium networks that spread from colonized roots into the surrounding soil, and their extension depends on the AMF species (Giovannetti and Avio, 2002; Mikkelsen, Rosendahl, and Jakobsen, 2008a, b) and the associated plant species (Giovannetti, et al., 2001). The AMF takes up phosphorus (P) nitrogen (N), sulphur (S) and various trace elements from the soil, and transfers these nutrients to the intraradical mycelium where the nutrients are exchanged for C from the host. The plant transfers up to 20% of its photosynthetically fixed C to the AM fungus (Wright et al., 1998) and the fungus uses these C resources to maintain and to extend its hyphal network in the soil.

CMNs can be formed by one individual fungus or when several conspecific fungal individuals connect by hyphal anastomoses (Mikkelsen et al., 2008a). In both cases, CMN can transfer nutrients to several host plants simultaneously (Lekberg, Hammer, and Olsson, 2010; Merrild, et al., 2013; Van Der Heijden and Horton, 2009). CMNs are dynamic and can turn over rapidly (Read et al., 2005), and a study has shown that hyphal length can be reduced even by the presence of other soil organisms, such as protozoa (Bonkowski et al., 2001). CMN also has the potential to transfer defence signals from a pathogen-infected or insect-attacked plant to a healthy neighbouring plant (Song et al. 2010) and lately, it was discovered that CMN has also the capacity to transport heavy metals (Ding et al., 2022).

It is speculated that the CMN is likely to provide some protection to plant's survival from most soil hazards (Garg & Bhandari, 2014), and many reports are uncovering the AMF-induced effects on the build-up of metals in plants (Souza et al., 2012). Heavy metals (HMs) can be immobilized in the fungal hyphae of internal and external origin (Ouziad et al., 2005). Externally, AMF produces a glycoprotein called glomalin, or glomalin-related soil proteins (GRSP), which is widely studied for its implications in the C storage, sequestration of potentially toxic elements such as heavy metals, and its role in soil stability (Rillig et al., 2001, 2007), and may be responsible for a significant percentage of metal retained or bound to the fungal walls. Internally in the cytosol, HMs are bound by a number of chelators (metallothioneins, glutathione), decreasing toxicity, while membrane transporters actively reduce the metal concentration by pumping metals out of the cytosol (Luo et al. 2014; Gonzales et al. 2009).

In the face of a possible effect of CMN on plant interactions, there are many theories. In this study, we focused on the source-sink theory in which resources or in this case toxic substances (Cd) would move in a concentration gradient. This could lead resources/substances to be distributed more equally among partners involved in the network (Heaton et al. 2012). That was the reason for our first hypothesis. Following the source-sink, Cd would present less concentrated in mycorrhizal poplar pots when they are in the presence of other mycorrhizal plants (leeks).

Heavy metals can affect plant physiology (Tibbett et al., 2021; Salazar et al., 2012), essential enzymatic activities, (Muradoglu et al., 2015), and plant growth (Athar and Ahmad, 2002). Biochemical mechanisms of metal toxicity to fungi are likely similar to those in plants, although the specific metals, enzymes, and other cellular components involved vary, as do toxicity thresholds (Del Val et al. 1999a, b). Plants can uptake metal and translocate from roots to the aerial parts, increasing the risk of HMs entering the food chain. Cadmium (Cd) is particularly

problematic because it is mobile in soils, toxic at low concentrations, affects plants and animals, and can leach into groundwater (Tibbett et al., 2021).

There are not many evidences in the literature regarding the CMN can transfer heavy metals (Ding et al., 2022). However, this still needs to be further explored. This partnership between plants could help each other's ability to cope with soil contamination. One of the questions that needs to be addressed is if the metals can be transported through CMNs creating an equilibrium (quantity of metal) between those partners, and if these networks can improve plant tolerance to toxic metals.

In order to test this partnership between plants we used one tree species (*Populus trichocarpa*), one herb species (*Allium porrum*), and one AMF species (*Rhizophagus irregularis*). Although HMs are known to be taken up by mycorrhizal fungi, there is a paucity of evidence that they are transferred or influenced by CMNs, in this chapter we will examine whether there is any evidence for such influence.

In this chapter, the fate of Cd in CMN was experimentally examined by determining its uptake in poplars and leeks, with and without AMF, when grown as single plants and together. When grown together, with or without CMN. The experiment was set up in a way to identify the main difference between Cd uptake within plants when in CMN, by having a single plant treatment as a form of partial control. In order to understand the CMN, we tested the following hypotheses: (1) When mycorrhizal poplars are in co-culture pots, they accumulate less Cd (2) CMN enhances glomalin production when under soil contaminated by Cd; (3) CMN enhance carbon liberating enzymes, under soil with Cd contamination. As proved by Silva et al. 2021 some mycorrhizal carbon secretion enzymes can be enhanced by Cd, however, there are many doubts about this particular area, so we tested in this study if the CMN would influence this dynamic presented here. The extent and directions of CMN effects on the fate of Cd were achieved by comparing outcomes of single plant treatments with dual plant treatment, when mycorrhizal or bare rooted.

3.2 Material and Methods

Plant material, treatments and experimental design

The growth substrate used in the experiment was formed by mixing 2.5 kg of sand and 0.5 kg of TerraGreen® clay (American Granules Plain, OIL-DRI, UK) (4:1, total of 3 kg per pot). The substrate was autoclaved for one hour at 121 °C to kill off any native mycorrhizal propagules. The experiment was conducted in a glasshouse located at the University of Reading.

The two plant species used were *Populus trichocarpa* (black cottonwood) and *Allium porrum* (leek) Musselburgh variety. The poplar cuttings were acquired from Nicky's Nursery, England, UK. *P. trichocarpa* was chosen because it is a well-known species that tolerates Cd stress (De Oliveira and Tibbett, 2018), and *A. porrum* because it is a crop which has a unique root system and is very responsive to mycorrhizal infection (Eason et al., 1999), facilitating the discrimination between both root systems during harvest (Figure 3.1. and Figure 3.2).



Figure 3.1. Photo from the experiment in the glasshouse, plants were 10 weeks old.

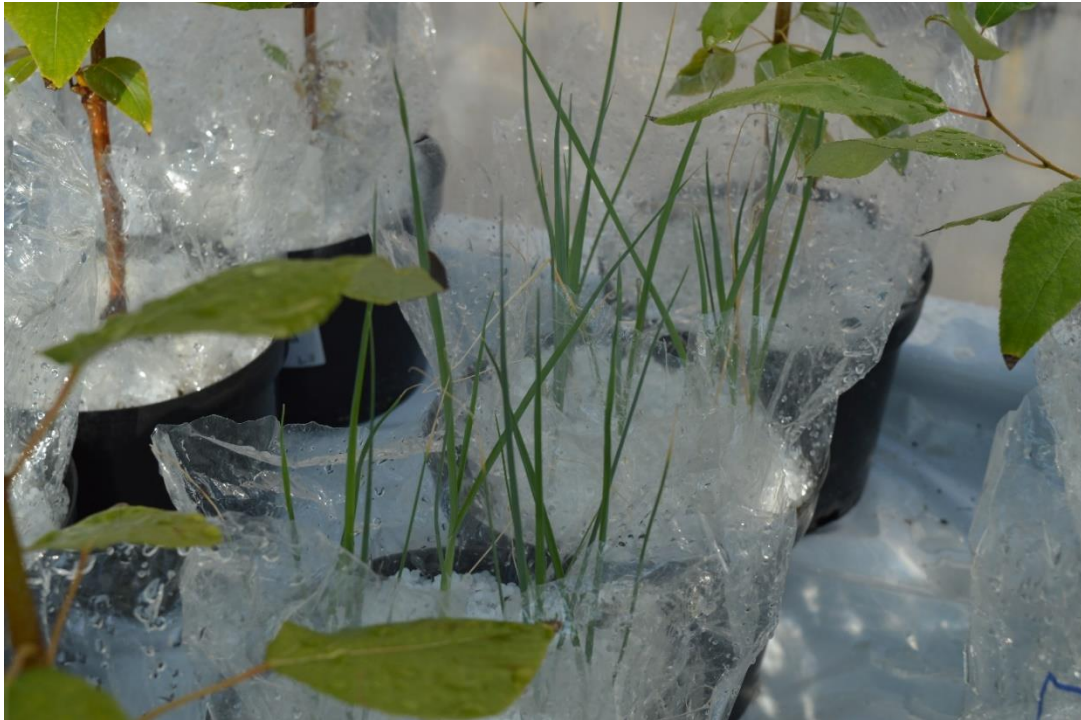


Figure 3.2. Photo in detail of the pots with leeks and the protection between pots in order to avoid fungal contamination between treatments.

The *Populus trichocarpa* cuttings (60 cm each) originated from the south of England and were planted on the 30th of April of 2018, and leeks were germinated from seeds. There were 12 treatments with 5 replicates each, resulting in 60 pots. These were divided into three sub-compartments dealing with (a) poplar responses, (b) leek responses and (c) combined poplar and leek CMN responses. The treatments were: A) Poplar's interactions: 1) Poplar with mycorrhizas (AM) + Cd, 2) Poplar with mycorrhizas without Cd (to verify the different behaviour of the mycorrhizal plants when exposed to Cd), and 3) Poplar without mycorrhizas (NM) + Cd, 4) Poplar without mycorrhizas without Cd; B) Leek interactions: 5) Leek with mycorrhizas + Cd, 6) Leek with mycorrhizas without Cd, 7) Leek without mycorrhizas + Cd, 8) Leek without mycorrhizas and no Cd; C) CMN interactions: 9) Poplar + leek with mycorrhizas + Cd, 10) Poplar + Leek with mycorrhizas without Cd (this treatment will enable us to see if plants have a different metal uptake pattern when under a supposed CMN), 11) Poplar + Leek without mycorrhizas +Cd, 12) Poplar + Leek without mycorrhizas and no Cd (Figure 3.3).

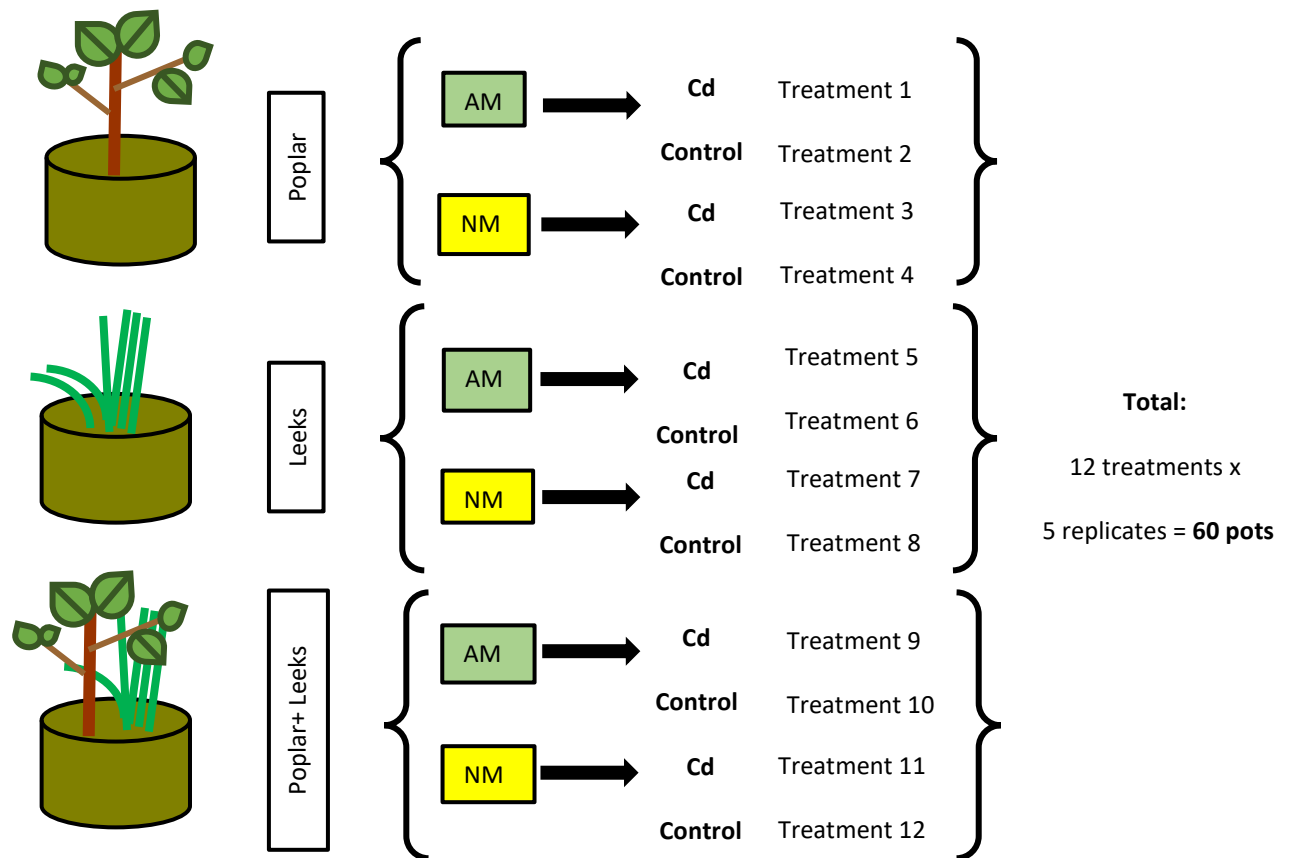


Figure 3.3 Schematic representation of the 12 treatments in this experimental design. M: mycorrhizal plants, NM: non-mycorrhizal.

To inoculate the plants with AMF (*Rhizophagus irregularis*), 300 g of soil from a pot culture of mycorrhizal *Plantago* sp. was used (containing fungal propagules, spores and root fragments). The inoculum was placed around the substrate in a circular area close to the cuttings or around the leek seeds. Single poplar cultures had just one cutting per pot, and single leeks culture had 10 seeds per pot; co-cultures had both (1 poplar + 10 seeds), as shown in Figure 3.4.

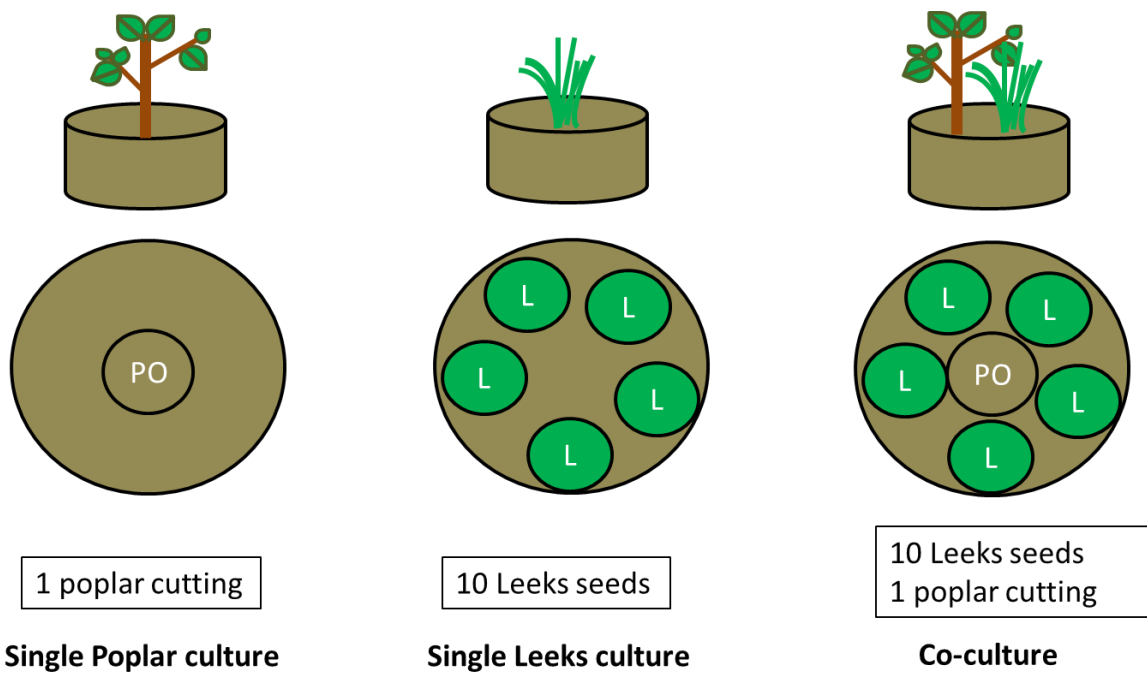


Figure 3.4 Schematic of the pots in the experiment. One poplar cutting was placed in the centre of the pots and leeks (10 seeds) were sown around it.

During the development of the plants, 10 mL of a modified Long Ashton's solution was used for fertilisation, applied three times (macronutrients: $(\text{NH}_4)_2\text{SO}_4$ (4 mM), K_2SO_4 (2 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.5 mM), NaNO_3 (8 mM), FeEDTA (0.1 mM); micronutrients: H_3BO_3 (2.86 mg L^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.81 mg L^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 mg L^{-1}), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.025 mg L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22 mg L^{-1})), according to Kariman et al. (2014). Cadmium was applied gradually via CdCl_2 solution, each application was made with 10 mL of 9 mg kg^{-1} Cd on three different days, reaching a total application of 27 mg kg^{-1} . Plants were watered three days a week to reach 70% of soil water capacity, by the procedure of weighing each pot. The average temperature recorded in the glasshouse during this period was 26°C (± 2.4), and plants were grown for three months. Harvest was performed by removing all plants from the substrate, and two soil samples were collected from each pot for further evaluation. Poplars were separated into leaves, stems, and roots, while leeks were separated into shoots and roots, poplars are bigger plants with stems well-formed while leeks were small in comparison. For the co-culture treatments, leeks were not split into shoots and roots due to their very small size, and thus the whole plant was analysed. Roots were rinsed with distilled water and immersed in a solution of 0.05 mM CaCl_2 for 30 minutes to remove surface adhering metals (Marmioli, Imperiale, Maestri, and Marmioli, 2013). Plant tissues were dried in the oven at 70°C for 5 days and weighed to quantify the biomass content (dry weight) for each treatment.

Plant digestion and metal determination

The dried biomass samples were ground into a fine powder. Each sample was weighed (0.05 g) in a glass tube, for quality control a blank tube was added (with no plant samples) as well as tubes with a reference material (IEAE – Cabbage leaves with 0.120 mg/kg of Cd), of a known Cd concentration. It was also placed control samples in the analyses. The samples were placed in a heating block at 110°C for 8 hours in 5 mL HNO₃ (< 69% Trace Select®). After digestion, 200 µL of the extract was diluted into a solution of 2% HNO₃ + 5 ppb Rh (internal standard) to a volume of 10 mL. Determination of Cd concentrations was performed via ICP-MS (KED mode - kinetic energy discrimination, Thermo Scientific™ iCAP™ Q ICP-MS) (Huang et al. 2004; De Oliveira et al., 2020). Due to very low biomass production, we were not able to determine Cd concentrations in leek plants under co-cultures.

Carbon enzymes

Enzyme activities were determined using fluorogenically labelled substrates (Courty et al, 2005; Sanallah et al., 2016). Four fluorogenic enzyme substrates based on 4-methylumbelliferone (MUB) were used: MUB-β-D-cellobioside for β-D-cellobiosidase (CB), MUB-β-D-xylopyranoside for xylanase (XYL), MUB-N-acetyl-β-D-glucosaminide for N-acetyl-β-D-glucosaminidase (NAG), MUB-β-Dglucopyranoside for β-glucosidase (BG). 2.75 g of soil with 92 mL of buffer were mixed. Soil suspension was pipetted into a 96-well microplate containing substrate and modified universal buffer. Fluorescence was measured immediately after incubations of soil suspension for 1.5 h at 35 °C on a multidetection plate reader SpectroMax with excitation with a wavelength of 350 nm and an emission of 460 nm.

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 mycorrhizas (Giovannetti and Mosse 1980).

Glomalin-related soil proteins (GRSP)

The BRSP, operationally defines as Bradford-reactive soil protein (Rillig, 2004), was extracted from soil subsamples (four per sampling pots) as easily extractable glomalin (EEG) and

as total glomalin (TG) as described by Wright and Upadhyaya (1998). EEG was extracted from 1 g of ground dry-sieved soil with 8 ml of 20 mM citrate, pH 7 at 121 °C for 30 min. TG was obtained by repeated extraction from 1 g of ground dry-sieved soil with 8ml of 50 mM citrate, pH 8 at 121 °C for 60 min. After each autoclaving cycle, the supernatant was removed by centrifugation at 500 rpm for 20 min and stored. TG extraction cycles were repeated until the glomalin content of the supernatant was under the detection limit of the method (ca. 2µg mL⁻¹). Extracts from each cycle were pooled, centrifuged at 10000 rpm for 10 min to remove soil particles and then analysed. The protein content in the supernatant was determined by Bradford assay with bovine serum albumin as the standard. The concentration of GRSP was extrapolated to mg g⁻¹ by correcting for the dry weight of coarse fragments including in the extraction of soil.

Plant nutrient analyses

Poplar and leek tissue samples were used in this study. Fresh samples were dried at 75°C for 10 hours in an oven and then ground with a Cyclone Mill to pass a 1.0-mm sieve. One gram of ground sample in a porcelain crucible was ashed in CMF for 16 hours at 450°C. The same sample was ashed in a CEM MAS-300 microwave for 20 and 40 minutes at the same temperature. The ash was dissolved in 5 mL 20% HCl, followed by 20 mL of hot water, and brought to 100 mL with deionized water. Phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), and boron (B) were determined in the extracts by ICP spectrometry (iCAP 6000), with two technical replicates (Zhang and Dotson, 1994).

Statistical analyses were performed using R[®] software (using R version 4.3.0). When all assumptions were met, three-way ANOVA was performed (Mycorrhizal plants (M,NM), Cd (Cd, -Cd), culture (Single, co-culture)), followed by Tukey test ($p < 0.05$). If data had homogeneous variances (Levene test, $p > 0.05$), but were not normally distributed (Shapiro-Wilk test), then the non-parametric Kruskal Wallis test was applied, and when results were significant ($p < 0.05$), the Dunn test was used to discriminate pairwise differences.

3.3 Results

Colonisation and biomass production

In both species, colonisation by *R. irregularis* was consistently between 40 and 60%, with no significant effect from being in single or co-cultures, or from Cd exposure (Fig. 3.5). No colonisation was detected in roots from non-inoculated pots (NM).

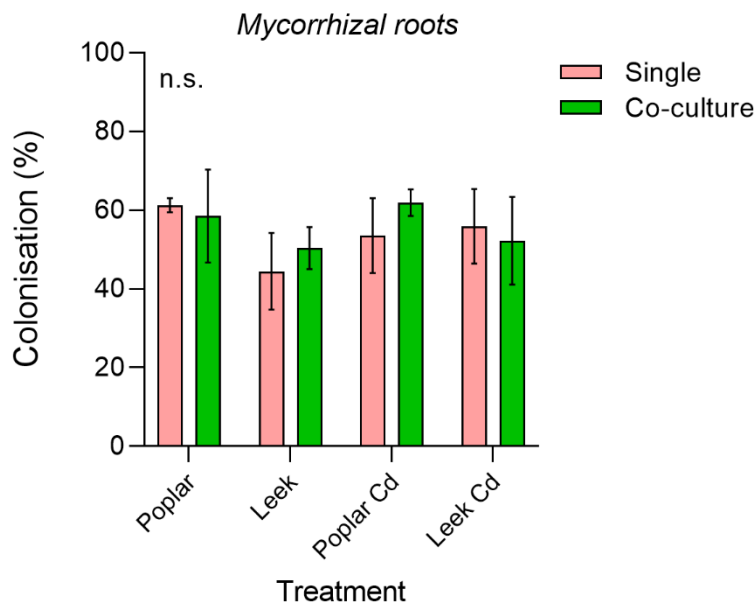


Figure 3.5. Colonisation percentages of poplar and leek roots by *R. irregularis* after 3 months growing individually (single) or together in the same pot (co-cultures), with and without Cd contamination. No significant (n.s.) differences were found ($p > 0.05$) after two-way ANOVA (Single and co-culture).

Leaf dry weight was not affected by Cd exposure, mycorrhization or culture type in poplars (Fig. 3.6a), as it was the case for stem or total dry weight (Fig. S3.1). In terms of root biomass, however, the NM roots were overall 37% larger than in colonised poplars, regardless of contamination and culture type (Fig. 3.6b).

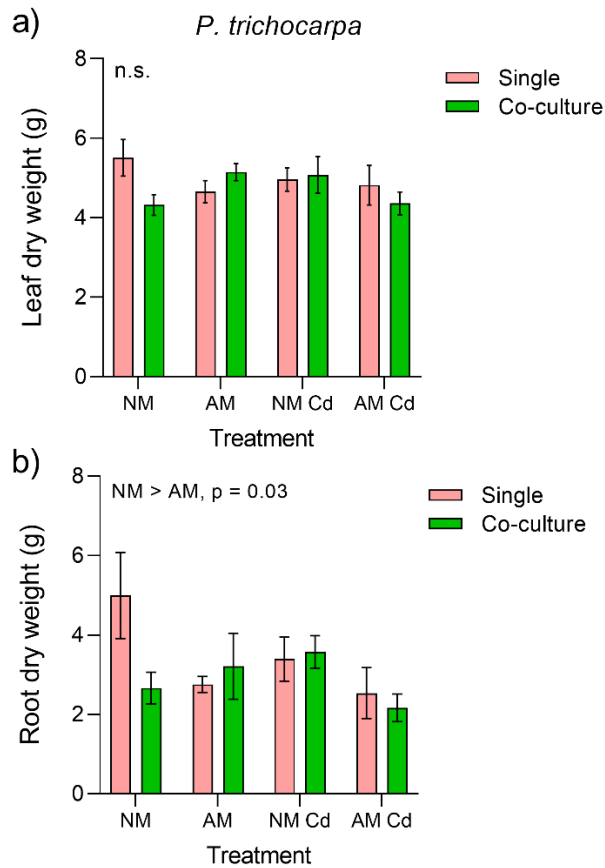


Figure 3.6. Leaf (a) and root (b) dry weight of poplar plants after 3 months growing individually (single) or together with leeks (co-cultures), with (AM) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=4) and standard errors. No significant (n.s.) differences were found ($p > 0.05$) after three-way ANOVA in leaves (NM, M; Single/co-culture; Cd/-Cd), but the main effect from mycorrhization was detected in roots ($p = 0.03$).

Similarly, in leeks, AM plants were significantly smaller than NM when soil was not contaminated by Cd (Fig. 3.7). But the strongest effect was from the co-culture system, where leeks total biomass was substantially lower when cultivated around a poplar seedling. Due to their extremely low biomass, Cd was unfortunately not determined in leeks from co-culture pots (< 0.5 g).

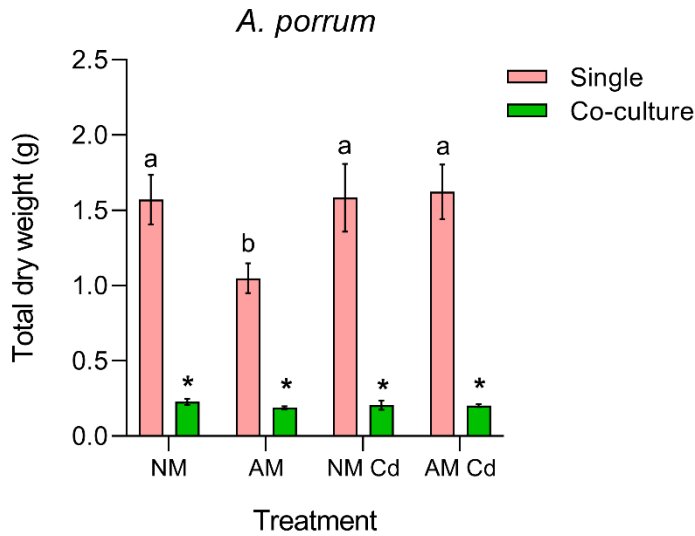


Figure 3.7. Total dry weight of ten leek seedlings after 3 months growing by themselves (single) or around a poplar plant (co-culture), with (AM) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=4) and standard errors. Different letters represent significant differences between plants in single cultures. Asterisks represent significant differences between culture types (Three-way ANOVA, Tukey test, $p < 0.01$).

Cadmium accumulation and GRSP contents

Foliar Cd concentration was generally below 1 mg kg^{-1} in poplars from non-contaminated pots, but significantly higher under Cd contamination, from 3.5 to 5.7 mg kg^{-1} (Fig. 3.8a). AM poplar plants also presented 34% lower Cd concentration than NM poplars, showing a significant effect from mycorrhization in this species (Fig. 3.8a). In roots, much higher Cd (102 mg kg^{-1}) was found in NM poplars growing in contaminated pots in co-culture with leeks (Fig. 3.8b), an effect that was attenuated when these co-cultures were under symbiosis, with Cd concentrations decreasing to 37 mg kg^{-1} .

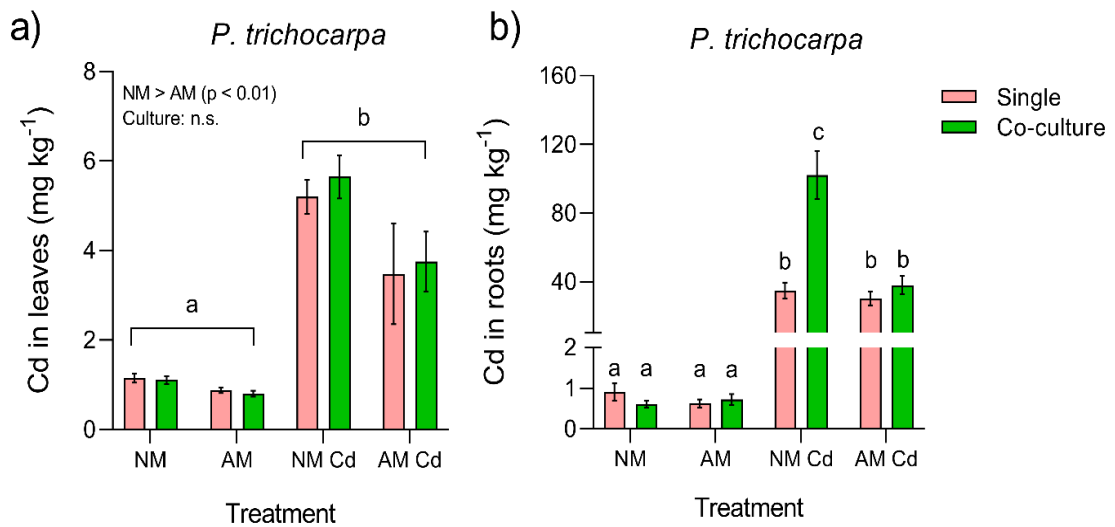


Figure 3.8. Cadmium concentration in leaves (a) and roots (b) of poplar plants after 3 months growing individually (single) or together with leeks (co-cultures); with (AM) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average ($n=5$) and standard errors. Different letters represent significant differences between treatments (Three-way ANOVA, $p < 0.05$).

The concentration of GRSP was assessed in both NM and AM pots, but because they were close to 0 in the former, it was decided to perform a two-way ANOVA only in the mycorrhizal pots, in order to detect effects from culture types and Cd contamination. Indeed, it was clear that Cd exposure had a significant effect in enhancing GRSP production (Fig. 3.9). Moreover, different cultures produced different amounts of GRSP, with AM leeks in single cultures producing almost 3 times more GRSP than AM poplars. In co-cultures GRSP was lower than in single leek pots, but still significantly higher than in single poplars (Fig. 3.9).

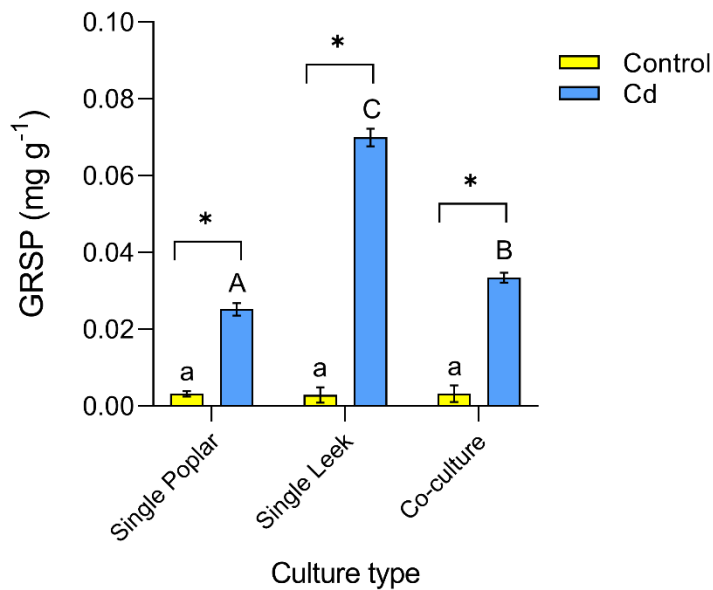


Figure 3.9. Concentration of glomalin-related soil proteins (GRSP) from mycorrhizal pots, with or without Cd contamination, and under different culture types: single poplar, single leek or co-culture. Bars represent the average (n=4) and standard errors. Different uppercase letters represent significant differences between culture types in contaminated pots and lowercase between Control pots; while asterisks denote Cd effects within each culture type, after Two-way ANOVA, and Tukey test, $p < 0.05$. ***Single Leek means that it was just leeks (10) in the pot, single Poplar means just one poplars in the pot and Co-culture was Poplars + Leeks in the same pots.***

Despite higher GSRP production in single AM leek pots, their shoots still had significantly higher Cd concentrations than NM leeks (Fig. 3.10). Which suggests that GSRP release is associated with internal Cd concentrations and not external soil Cd. Spearman correlations among variables from AM leeks showed a significant ($p < 0.05$) and positive relationship between soil GSRP and Cd concentrations in shoots ($r_s = 0.83$) and roots ($r_s = 0.87$). Similar results were also observed in AM poplars, with positive correlations found between GSRP and Cd in leaves ($r_s = 0.90$), stems ($r_s = 0.83$), and roots ($r_s = 0.82$). It is worth mentioning that such relationships were not detected between GSRP and other elements (e.g. P, N, Mg, Ca, Fe, Zn, Cu), in neither species.

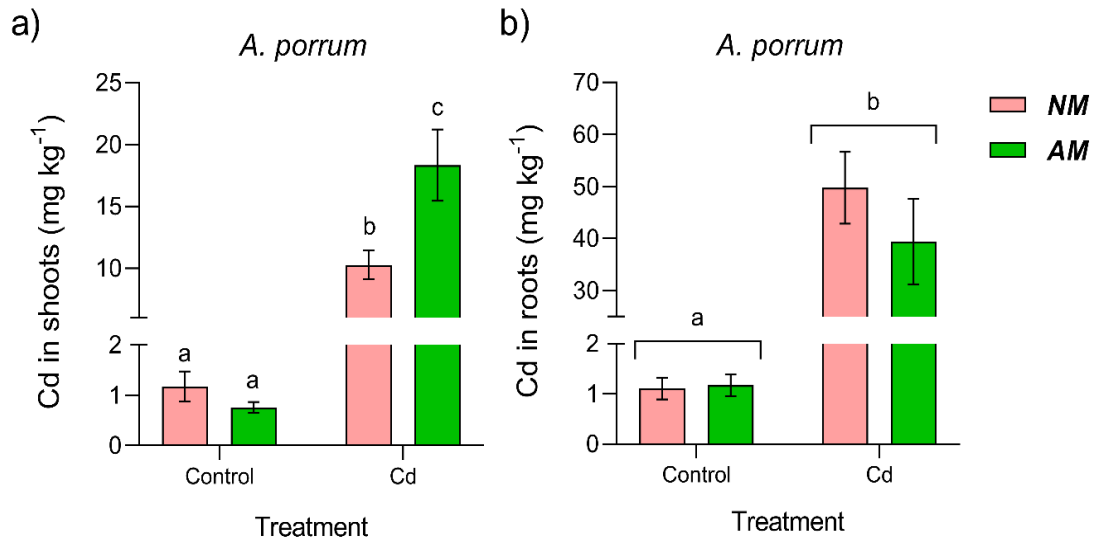


Figure 3.10. Cadmium concentration in shoots (a) and roots (b) of leek plants after 3 months growing under Cd contamination with (AM) and without (NM) mycorrhizal symbiosis. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments (Two-way ANOVA, $p < 0.05$).

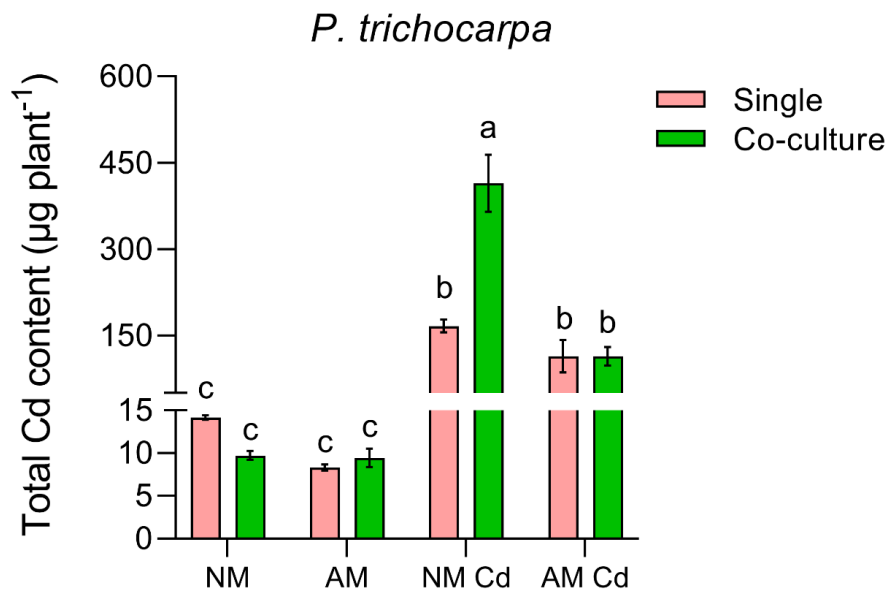


Figure 3.12. Total cadmium concentration *P. trichocarpa* after 3 months growing under Cd contamination with (AM) and without (NM) mycorrhizal symbiosis. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments (Two-way ANOVA, $p < 0.05$).

Total Cd content in poplars (μg per plant), follows the same pattern as root Cd concentrations (Fig. 3.8 b). It is shown that co-cultures lead to high Cd accumulation, but only

in NM treatments. Thereby co-cultures with AM leeks, provide positive effects such as GRSP and C-enzyme activities, without causing more Cd uptake.

Enzyme activities

Overall, the activity of enzymes involved in C cycling were mostly higher in pots containing leeks as a single culture (Fig. 3.11), in particular BG, CB, and XYL. For BG in particular, there is strong evidence that colonisation by AM leads to higher activity, except for single poplar pots (Fig. 3.11a). A similar effect was also observed for XYL, but only in single leek pots (Fig. 3.11d). Contrary to what was initially expected, Cd had little effect on the activities of most enzymes assessed here, especially in poplar pots, the exception being XYL, which was overall 50% lower in Cd-contaminated pots (Fig. 3.11d). Both CB and NAG appeared to be unaffected by Cd or mycorrhization (Figs. 3.11b and 3.11c).

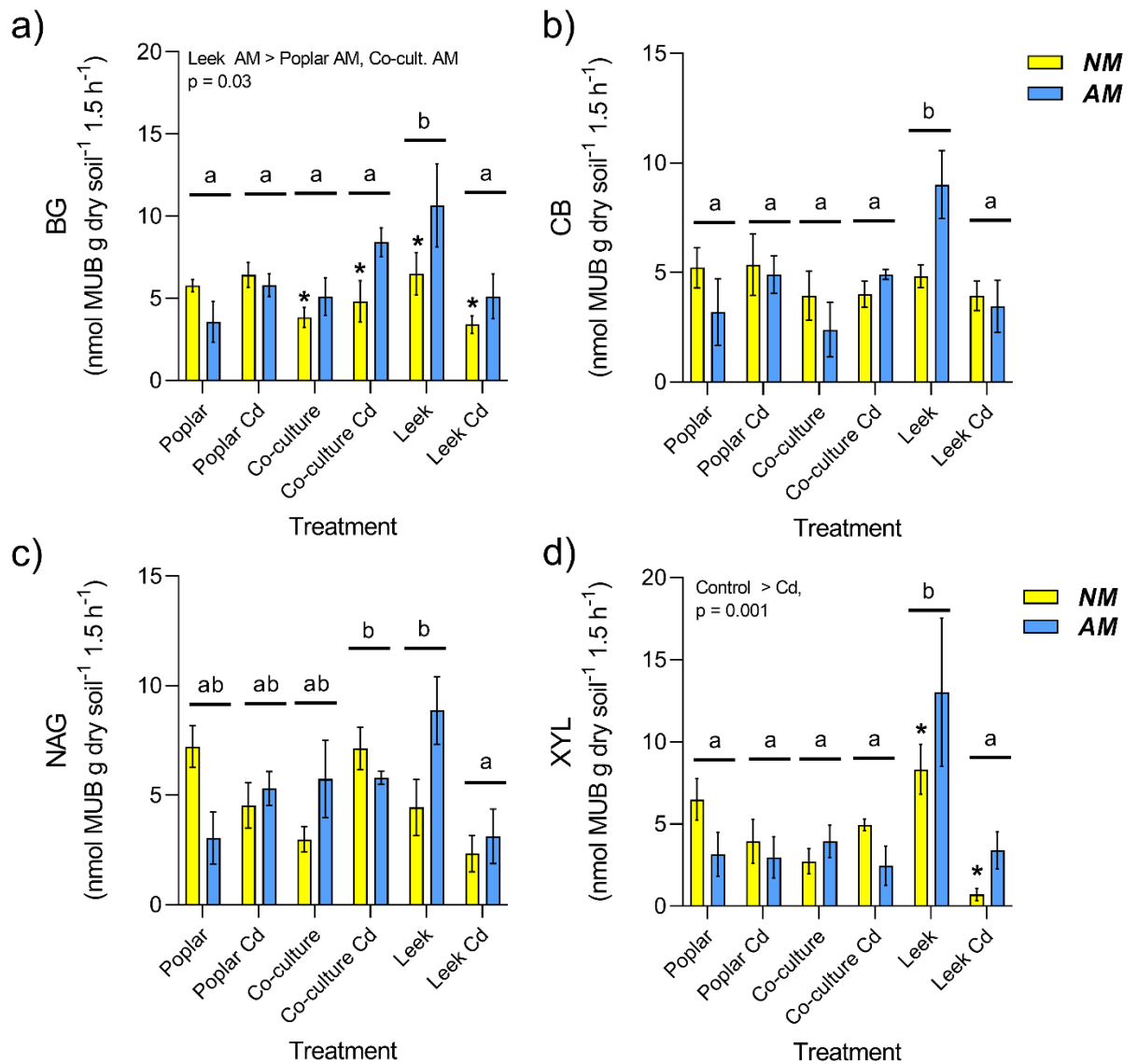


Figure 3.11. Activity of four different C cycling enzymes: a) β -glucosidase (BG); b) β -D-cellobiosidase (CB); c) N-acetyl- β -D-glucosaminidase (NAG); and d) xylanase (XYL) from poplar and leek pots growing in single or co-cultures, with (AM) or without (NM) mycorrhizal symbiosis, and in control or Cd contaminated conditions. Bars represent the average ($n=4$) and standard errors. Different letters represent significant differences between treatments, and asterisks denote differences between NM and AM treatments in any given condition, after three-way ANOVA (Tukey test, $p < 0.05$).

3.4 Discussion

Colonisation and biomass production

The colonisation rates of *R. irregularis* in *P. trichocarpa* are consistent with the ones reported by De Oliveira et al. (2020), from 46-50%, which was also not affected by Cd addition (81 mg kg^{-1}). Colonisation in *A. porrum* roots was also similar to the one found in leeks growing in soil contaminated by lead (Pb) and copper (Cu), around 50% (Mozafar et al., 2002). Poplar leaf biomass was not affected by Cd exposure, mycorrhization, or culture type, this consequently shows a tolerance to Cd as observed by De Oliveira and Tibbett (2018), stem and total dry weight follows the same pattern (Fig. S3.1, Fig. S 3.2). Interestingly, in the literature leaves are more sensitive to Cd toxicity than roots, as photosynthesis might be suppressed due to the disruption of the photosynthetic apparatus, reduction of the amount of chlorophyll and decrement of stomatal conductance (Shi et al., 2020).

Non-mycorrhizal roots were overall 45% larger than colonised roots, regardless of contamination and culture type. Decreases in roots and shoots while plants are mycorrhizal are commonly reported (Tran and Popova, 2013), the fungus mycelium can reach wider areas in the soil, explaining why mycorrhizal roots tend to be shorter. Mycorrhizal leeks presented the same pattern, they were significantly smaller compared to NM in no contaminated soil.

The greater effect in biomass was in leeks total dry weight when in the co-culture system, being substantially lower when cultivated around a poplar seedling. It might be because of competition aspects, even though there were ten leeks seedlings and only one poplar sharing the pot. It is well known that poplar's characteristics contributing to their potential for soil remediation, are also responsible for their high competitiveness, such as deep root systems, large and fast biomass production, and high water use (Bradshaw et al. 2000; Rodinson et al. 2009; Marmiroli et al. 2011; Bhargava et al. 2012). Consequently, poplars are known to be strong competitors that rapidly invade disturbed sites (Sebastiani et al. 2004). Moreover, a report from Weremijewicz et al. (2016) that CMN can amplify competition, with preferential mineral nutrients being allocated to the large host plants, thus, a similar situation might be happening in our experiment.

Cd accumulation

Foliar Cd concentrations found here in poplar leaves are similar to the results observed by De Oliveira et al. (2020), in which NM and AM seedlings accumulated 8.5 and 5.0 mg kg^{-1} Cd, respectively, despite the soil being contaminated with 81 mg kg^{-1} Cd. However, this is in stark

contrast with the values of around 40 mg kg⁻¹ Cd found in poplar leaves under 27 mg kg⁻¹ Cd (De Oliveira and Tibbett, 2018), the same as in the present work. This could be due to the contamination protocol: instead of one single pulse of 27 mg kg⁻¹ Cd (De Oliveira and Tibbett, 2018), we split the contamination across three different days. This could have allowed for an effective down-regulation of metal transporters in roots, such as *HMA4*, which was associated with restricted Cd translocation to shoots under excessive concentrations (De Oliveira et al., 2020).

A higher amount of Cd was found (102 mg kg⁻¹) in NM poplars growing in contaminated pots in co-culture (Fig. 3.8b), and when they were in symbiosis the Cd concentration decreased significantly to 37 mg kg⁻¹. This might occur because the co-culture pots had 11 individuals (1 poplar, 10 leeks), and consequently, there are more root exudation, leading to rhizosphere acidification and Cd availability and uptake (Bali, et al. 2020). However, the main point which brings our attention is that the effect is not significant when these 11 seedlings are under CMN. This could be due to: (1) Poplar transferring Cd (offloading) to the surrounding leeks; which could be related to the source-sink theory (Heaton et al., 2012). (2) Higher GRSP (glomalin) in soils cultivated with mycorrhizal leeks (Fig. 3.9); in which Cd would be more immobilised in the soil; as discussed by Rillig et al. (2001, 2007) and/or (3) AM symbiosis could have decreased organic acid exudation, not enhancing Cd availability as much as in NM co-cultures. AM symbiosis is known to alter carbohydrate metabolism in host roots, affecting exudation patterns, qualitatively and quantitatively, in some cases decreasing root permeability and exudation (Jones et al., 2004).

These results are also found by Chen et al. (2016) which show, that when *P. deltoides* are colonized by mycorrhizae the amount of Cd decreases in leaves. Leeks had higher Cd concentrations than poplars, regardless of mycorrhization. Mycorrhizal symbiosis decreased Cd concentration in leek shoots by around 40% but did not affect root concentrations (Fig. 3.10 a, b). A similar response was reported previously for some individuals of *P. deltoides* colonised by *R. irregulares* (Chen et al., 2016) while in *P. nigra*, Cd concentrations were not affected by mycorrhization (Mrnka et al., 2012).

The Cd accumulation in AM poplars when in co-culture did not decrease compared to single AM poplars, therefore we must reject our hypothesis that when under CMN, Cd would be lowest in poplars. Initially, we speculated that AM poplars in co-culture would 'offload' Cd excess towards leeks via their CMN. However, because we were unable to determine Cd in co-cultured leeks, we could not address this phenomenon.

GRSP production (glomalin) under Cd contamination

Glomalin is a glycoprotein produced by AMF (Gadkar and Rillig, 2006), and some studies indicate that it can bind and sequester some heavy metals, such as Cu, Cd, Pb, and Zn (Gonzalez-Chavez et al., 2004; Chern et al., 2007; Vodnik et al., 2008). Cd exposure had a significant effect in enhancing GRSP (glomalin-related soil protein) production (Fig. 3.9). Single cultured AM leeks produced almost 3 times more GRSP than AM poplars in single pots (Haoqiang et al., 2019). This result could be because: (1) there are more seedlings in leek treatments (10) compared to only one poplar in the pot. Consequently, there were more colonised roots, leading to higher GRSP production; and (2), AM leeks are able to produce more glomalin in general (Milleset et al., 2009). The fact that in co-culture, GRSP decreased in comparison to single leeks, suggests that both reasons are reasonable, as in co-culture leeks were very small, therefore decreasing their contribution towards GRSP contents, but remaining higher than in single poplar pots.

Cadmium has been shown to increase GRSP production in *Sorghum bicolor* colonised by AM fungus *Claroideoglomus etunicatum*, an effect linked to protection against heavy stress (Babadi et al., 2019). Here we see a similar response in both leeks and poplars, however despite higher GRSP under Cd treatments, AM leeks presented significantly higher Cd than NM leeks (Fig. 3.10). This suggests that what triggers GRSP production is not necessarily Cd availability in soil solution, but the plant internal concentration. Indeed, we found that Cd in shoots was significantly and positively correlated to GRSP in soils (Yinong, Jiazheng Xu, 2022). This may also explain why AM poplars produced much less GRSP, as these plants presented much lower Cd concentrations, where glomalin release would be less necessary.

Carbon enzymes and nutrients

In general, the activity of enzymes involved in the C cycling was mostly higher in pots containing leeks in a single culture (Fig. 3.11). This may be partially explained by the high amount of glomalin in single leek pots. Wu et al. (2012) found a correlation between glomalin and β -glucosidase (BG) activity, while Bai et al. (2009) found a link between glomalin with other different soil enzymes.

Xylanases are hemicellulolytic enzymes, which are responsible for the degradation of the heteroxylans constituting the lignocellulosic plant cell wall (Bellion et al., 2006). This enzyme is significantly lower (50%) under Cd concentrations, which could be explained by how toxic Cd can be affecting the organisms that are responsible for their production. The activity of -BG was

higher in pots with AM leeks compared with NM pots. This enzyme has a general function of hydrolysis of B-glucosyl residues to release B-D-glucose, the final step in cellulose hydrolysis and maybe the results showed could be explained by the presence of glomalin, or maybe AM plants attract other organisms. In general, all carbon enzyme activities were higher in pots with leeks in a single culture, especially under AM symbiosis; it was also verified that leeks could increase some enzyme activities in the soils through Elfstrand, Bath, and Martensson, (2007) study. In a recent meta-analysis Qin et al. (2019) have shown that AMF can significantly increase the activity of many different soil enzymes, including C-releasing enzymes, particularly in pot conditions.

Bai et al (2009) found a significant correlation between GRSP and soil enzyme activities. By sequestering C and slowing its turnover in soil, glomalin improves microbial activity and soil aggregation.

3.5 Conclusion

Cadmium concentration was affected by CMN in poplar roots, however, we could not tell whether the CMN was being used as a way of transferring Cd from poplars towards leeks in contaminated treatments, because we were unable to determine Cd in co-cultured leeks. GSRP was higher under Cd contamination, confirming the potential of this glycoprotein in influencing soil remediation and Cd immobilisation. Despite resource competition and higher Cd mobilisation in co-culture between poplars and leeks, the presence of CMN was shown to be beneficial, stimulating microbial C-releasing enzyme activities, whilst restricting Cd accumulation in poplars. However, future work is required to better understand the interactions between plants linked with CMN and their role when under metal contamination, as well as consideration of how CMN and metal contamination interact with the surrounding soil microbiota.

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3.7 Supplementary information

Supplementary Files (Chapter 3)

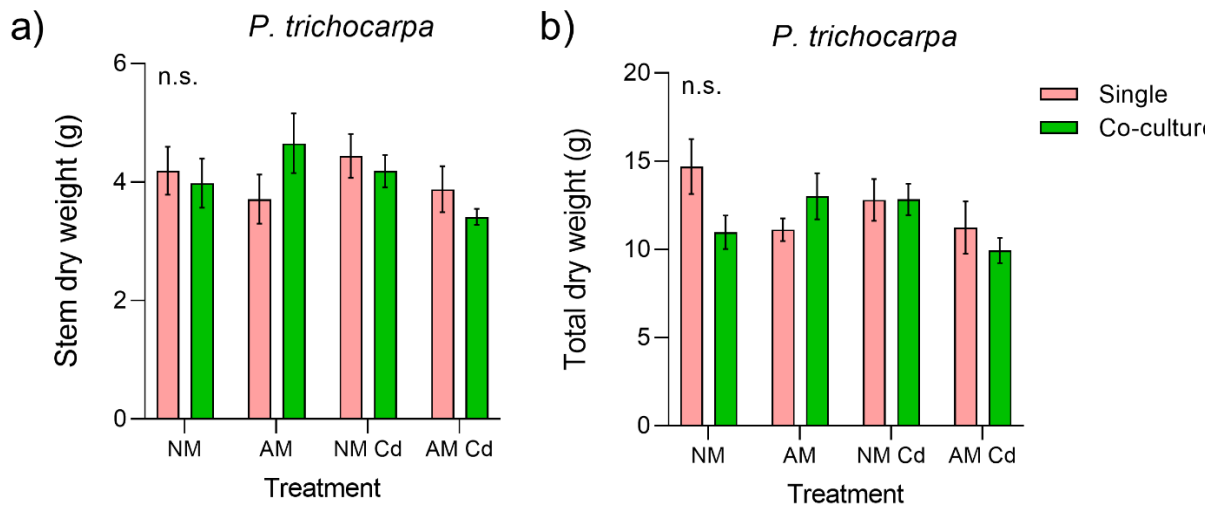


Figure S3.1. Stem (a) and total (b) dry weight of poplar plants after 3 months growing individually (single) or together with leeks (co-cultures), with (AM) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average ($n=4$) and standard errors. No significant (n.s.) differences were found ($p > 0.05$) after three-way ANOVA.

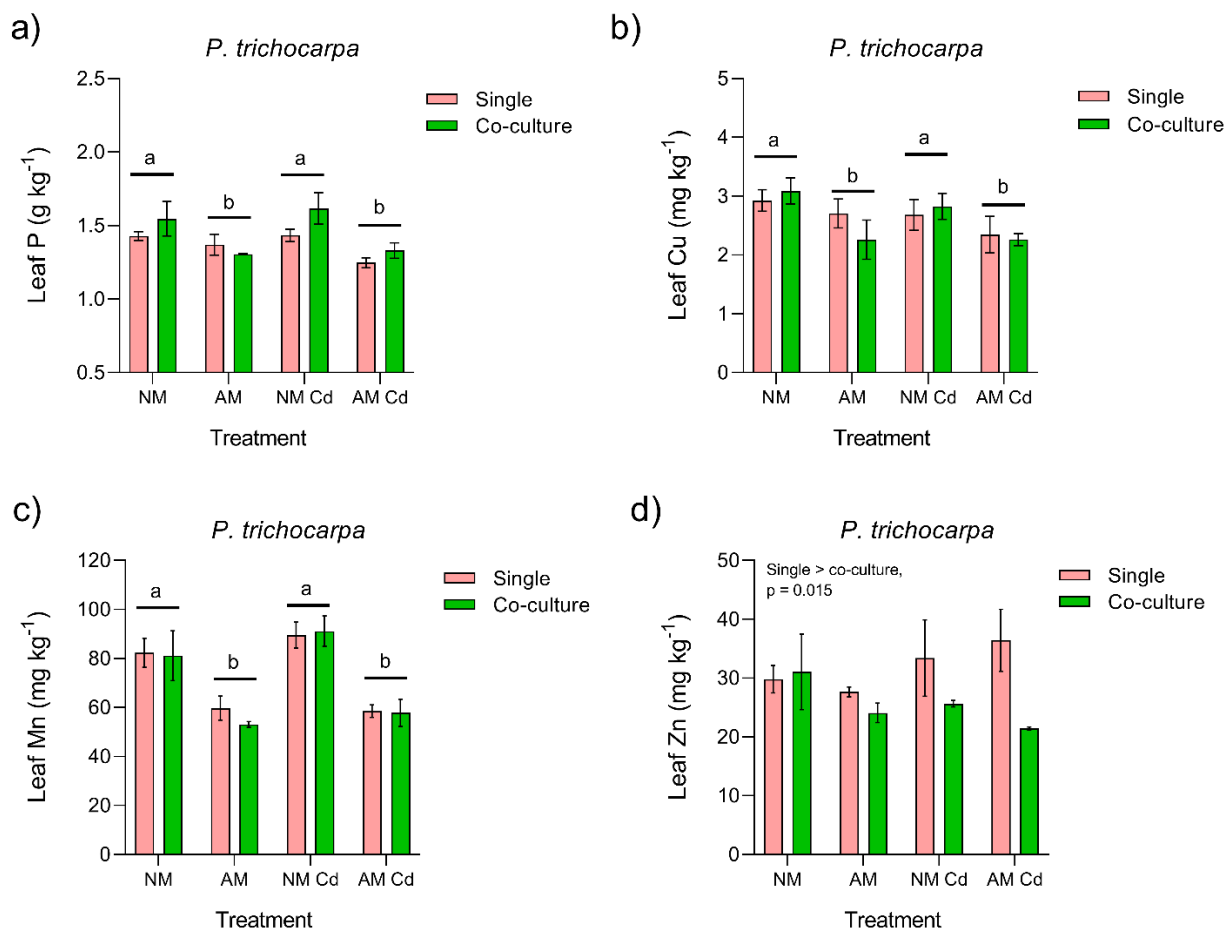


Figure S3.2. Foliar concentrations of P (a), Cu (b), Mn (c) and Zn (d) in poplar plants after 3 months growing individually (single) or together with leeks (co-cultures), with (AM) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=3) and standard errors. Different letters represent significant differences ($p < 0.05$) after three-way ANOVA and Tukey test.

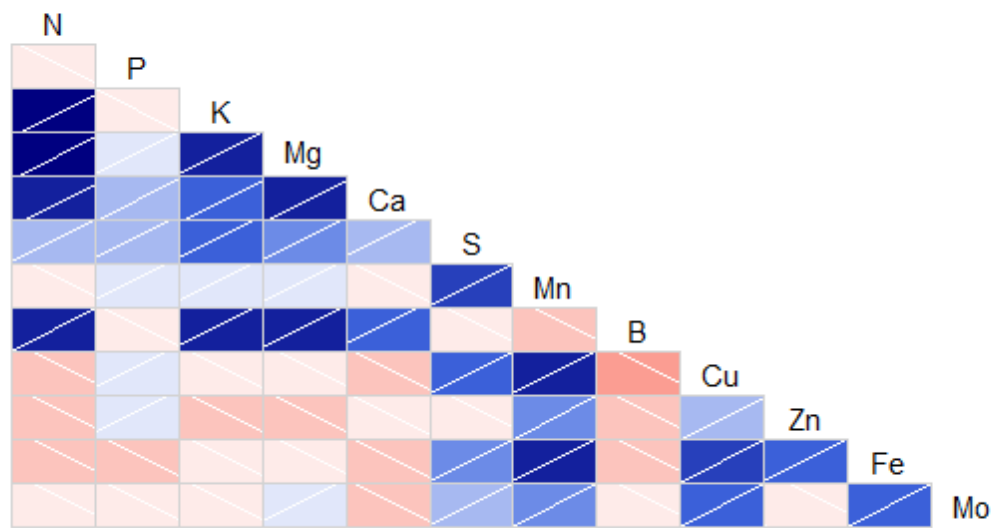










Figure S3.3. Pearson correlation matrix of nutrients of the plant composition. The significant values are showed ($p < 0.05$) in dark colours (Broadspec analysis). The colours represent the correlation among variables, positive correlations are displayed in blue and negative correlations in orange color. Color intensity is proportional to the correlation coefficients (1 , 0.75 , 0.5 , 0.25 , 0 , 0.25 , 0.5 , 0.75 , -1).

Table S3.1. Result of poplars leaves Bradford analyses (medium \pm SD).

Treatment	N(%)	P(%)	K(%)	Mg(%)	Ca(%)	S(%)	Mn (ppm)	B (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	Mo (ppm)
Single Poplar	0.74 \pm 0	0.14 \pm 0	1.38 \pm 0.1 a	0.45 \pm 0	0.93 \pm 0 ab	0.20 \pm 0 a	82.32 \pm 10.2 ab	23.53 \pm 4.2 a	2.92 \pm 0.3	29.82 \pm 4	123.84 \pm 26.1	0.48 \pm 0.1
Single poplar AM	0.80 \pm 0	0.13 \pm 0	1.22 \pm 0 a	0.47 \pm 0	1.05 \pm 0.1 ab	0.22 \pm 0 ab	59.7 \pm 8.4 ab	26.01 \pm 6.4 a	2.70 \pm 0.4	27.65 \pm 1.4	231.51 \pm 189	0.95 \pm 0.8
Single Poplar Cd	0.81 \pm 0.1	0.14 \pm 0	1.47 \pm 0.1 a	0.47 \pm 0	0.98 \pm 0 ab	0.22 \pm 0 ab	89.52 \pm 9.1 b	26.27 \pm 2 a	2.68 \pm 0.4	33.40 \pm 11.2	181.48 \pm 39.6	0.73 \pm 0.2
Single Poplar AM Cd	0.73 \pm 0	0.12 \pm 0	1.28 \pm 0.1 a	0.47 \pm 0	1.02 \pm 0 ab	0.19 \pm 0 a	58.51 \pm 4.4 ab	26.26 \pm 0.1 a	2.34 \pm 0.5	36.43 \pm 9.1	137.74 \pm 10.8	0.44 \pm 0.1
Co-culture	0.73 \pm 0	0.15 \pm 0	1.55 \pm 0 ab	0.47 \pm 0	1.07 \pm 0.1 ab	0.19 \pm 0 a	81.1 \pm 17.5 ab	29.6 \pm 6 ab	3.08 \pm 0.3	31.07 \pm 11.1	187.66 \pm 117	1.24 \pm 0.9
Co-culture AM	0.74 \pm 0	0.13 \pm 0	1.30 \pm 0 a	0.47 \pm 0	1.08 \pm 0 ab	0.22 \pm 0 ab	53.03 \pm 2.0 a	26.22 \pm 1.4 a	2.26 \pm 0.5	24.07 \pm 2.8	141.80 \pm 80	1.26 \pm 0.8
Co-culture Cd	0.73 \pm 0	0.16 \pm 0	1.65 \pm 0 ab	0.49 \pm 0	1.16 \pm 0 b	0.20 \pm 0 ab	91.18 \pm 10.7 b	29.54 \pm 1.2 ab	2.82 \pm 0.3	25.60 \pm 0.9	175.50 \pm 64.8	1.01 \pm 0.5
Co-culture AM Cd	0.67 \pm 0 a	0.13 \pm 0	1.40 \pm 0 a	0.50 \pm 0 ab	1.12 \pm 0 ab	0.21 \pm 0 ab	57.83 \pm 9.6 ab	31.9 \pm 3.0 ab	2.26 \pm 0.1	21.44 \pm 0.4	124.0 \pm 21	0.75 \pm 0.2
Single leek	1.20 \pm 0 b	0.15 \pm 0	2.83 \pm 0.3 c	0.7 \pm 0 b	0.95 \pm 0 ab	0.29 \pm 0 ab	69.25 \pm 18.1 ab	77.82 \pm 20.1 c	3.17 \pm 2.5	21.62 \pm 13.4	318.90 \pm 271	4.76 \pm 2.6
Single leek AM	0.80 \pm 0.1 a	0.09 \pm 0	2.13 \pm 0 b	0.70 \pm 0.1 b	0.91 \pm 0.1 ab	0.22 \pm 0 ab	58.36 \pm 17.8 ab	66.1 \pm 13.1 c	2.54 \pm 2.6	19.10 \pm 14.6	861.70 \pm 1194	6.14 \pm 7.1
Single leek Cd	1.35 \pm 0.2 b	0.09 \pm 0	2.83 \pm 0.2 c	0.64 \pm 0 ab	0.88 \pm 0.1 ab	0.34 \pm 0 b	60.74 \pm 9.4 ab	63.91 \pm 9.9 c	1.63 \pm 2.7	10.72 \pm 12.1	162.1 \pm 212	1.96 \pm 0.7
Single leek AM Cd	1.15 \pm 0 b	0.10 \pm 0	2.81 \pm 0.4 c	0.65 \pm 0.1 ab	0.84 \pm 0.1 a	0.25 \pm 0 ab	62.52 \pm 6.7 ab	55.39 \pm 16.8 bc	3.30 \pm 0.3	21.32 \pm 3.4	363.7 \pm 337	1.18 \pm 0.8

Chapter 4

4. Cadmium transfer between common mycorrhizal network

4.1 Introduction

Heavy metal (HM) pollution is one of the major threats to the environment (Pourret and Bollinger, 2018). Heavy metals can degrade the quality of water, air, soil, and food and is a threat to human health due to their persistence, high toxicity, and bioavailability (Kim et al., 2015; Lu et al., 2018; Geng et al., 2019). These metals are absorbed by plants and can accumulate in edible organs, then transfer to animals via food chains (Tibbett et al., 2021), leading to a frequent exposure of humans to HMs (Mishra et al., 2019).

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 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 also severely affect plant physiology (Salazar et al., 2012), interfering with many physiological activities such as carbon assimilation and photosynthesis (Farooq et al., 2016). It can compete with essential metallic elements for transport and thereby induce mineral deficiencies, or even displace zinc (Zn), calcium (Ca), iron (Fe), or other metals from proteins (Heyno et al., 2008; Janik et al., 2010). It also competes with other ions in the soil matrix, such as Ca^{2+} and Zn^{2+} due to their chemical similarities and the same uptake pathways in plants (Clemens 2006; Kirkham 2006). Cd contamination in modern agricultural ecosystems has been continually increasing, which poses potential hazards to both food safety and human health.

Plants can accumulate Cd to various extents and in some cases, these concentrations reach levels that may be phytotoxic (Hatata and Abdel-AL 2008). Consequently, it can cause detrimental effects at morphological, structural, biochemical, and physiological levels. However, some soil microorganisms can enhance plant tolerance to heavy metals, such as arbuscular mycorrhizal fungi (AMF). They establish a symbiosis with plant roots improving the host's nutritional status, growth, and stress resistance while maintaining an extensive hyphal network that explores the soil matrix (Miransari 2011; Smith and Read, 2008). Plant colonization by AMF can mitigate HM phytotoxicity by reducing metal uptake and translocation from roots to shoots (Li et al., 2016), decreasing heavy metal accumulation in leaves or in fruits and grains (Wang et al, 2020).

Those fungi can also establish an extensive hyphal network. The extraradical fungal hyphae can colonize the roots of neighbouring plants of the same or different species to form common mycorrhizal networks (CMNs) (Johnson and Gilbert, 2015). This CMN system is likely to provide some protection to plant survival, as confirmed by Ding et al. (2022), who showed cadmium has been transferred between maize and soybean plants via common mycorrhizal networks. CMNs benefit host plants in many ways, such as increasing fungal vigor at the expense of other plants, in which the transfer may be bidirectional between plants, with a net flux toward one plant (Selosse et al. 2006). CMNs can also increase the inequality between individuals (Allsopp and Stock, 1992; Shumway and Koide, 1995; Facelli and Facelli, 2002) which means that in plant populations, large individuals will in general access a disproportionate share of the growth-limiting resources and suppress the small individuals (Weiner, 1990).

In order to understand if the CMN works differently when one plant is under stress (in this study Cd concentration in the soil) and the mycorrhizal neighbour plant are in different conditions (there is no stress), what happens with the nutritional concentration between receiver and donor are unknown. As some studies showed CMN can affect on the bargain power of the fungal partner dependent on the environment (Bucking et al, 2016; Weiner, 1990).

In this study, CMNs were established between poplars exposed to Cd contamination and the neighbouring untreated poplar plants using interconnected pots. We hypothesized firstly that (1) CMNs can transfer Cd between poplars; (2) Nutritional concentration of the receiver and the donor plants are different; (3) Plant growth of the receiver and the donor plants are different. We also analysed the content of Cu, phosphorus (P), Zn and C:N ratio inside the plants to identify if there were any influence of Cd on these elements. There was evidence in the literature that arbuscular mycorrhizal enhances cadmium uptake in *Phragmites australis* depending on the phosphorus concentration. Consequently, this study also tries to speculate the influence of Cd in the P concentration in poplars.

4.2 Material and Methods

Plant material and pre-growth

Cuttings of *Populus trichocarpa* 'Trichobel' were obtained from Nicky's Nursery, England UK, and were kept refrigerated at 4 °C until the experiment started. Cuttings were trimmed (15 cm, including two nodes) and transplanted into undrained plastic pots. The growth substrate used in the experiment was prepared by mixing 750 g of sand and 250 g of terra green clay (4:1), for a 1 kg pot. The substrate was autoclaved for one hour at 121 °C to kill off any native

mycorrhizal propagules. The experiment was conducted in a glasshouse located at the University of Reading. *P. trichocarpa* was chosen because it is a well-known species that has rapid growth, tolerates Cd stress and is able to form AM symbiosis (De Oliveira et al., 2020).

To inoculate the plants with AMF (*Rhizophagus irregularis*), 110 g of soil from a 4 months pot culture of mycorrhizal *Plantago* sp. was used (containing fungal propagules, spores, and root fragments). The inoculum was placed around in a circular area close to the cuttings. For pots with no mycorrhizal fungus, we added 110 g of dead inoculum of *R. irregularis* fungi (autoclaved) to provide the same substrate structure.

After inoculation, poplar cuttings were planted in a two-pot system (Figure 4.1), in which they were connected by a 5cm pipe, with a 30 mesh in both sides of the pipe to make sure the poplar roots could not go through from one pot to another, just the hyphal will be able to go through.

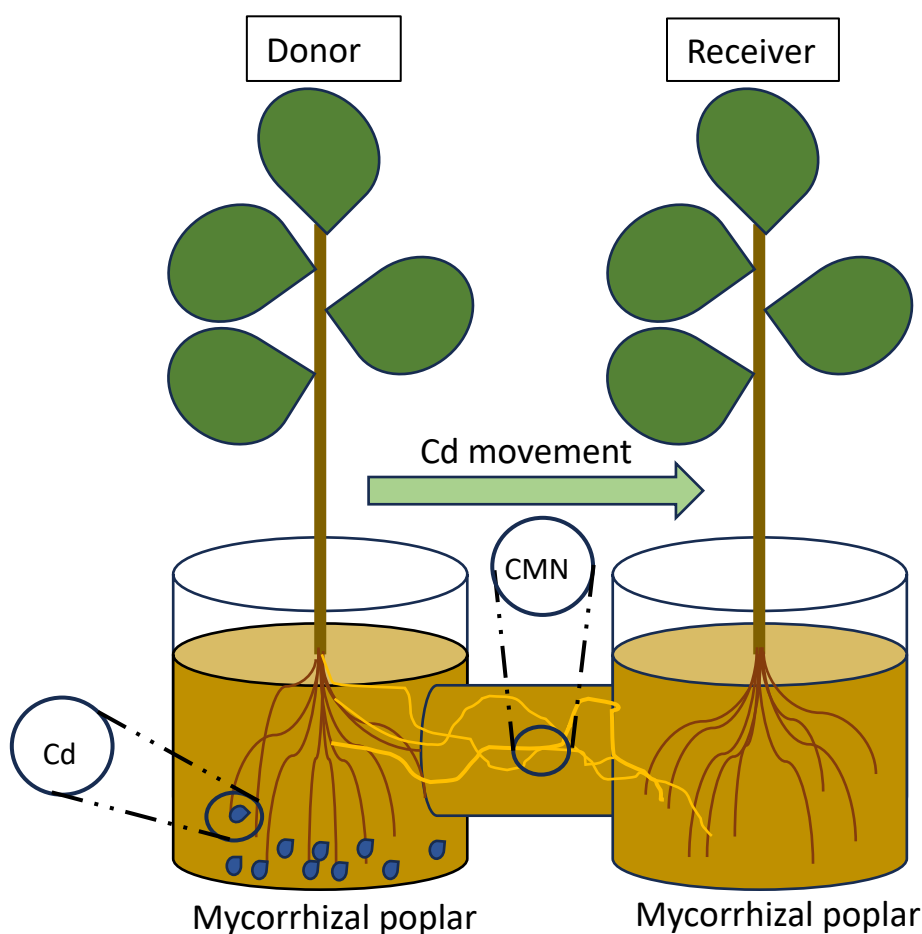


Figure 4.1 Poplar cuttings growing in a two-pot (1 kg each) system connected by a pipe (3 cm) containing a nylon mesh (30 μm) in both ends to avoid root contact, allowing only the hyphae to grow through. One side was contaminated by 27 mg/kg Cd, which we called donor plants.

During plant development, a modified Long Ashton's solution was used for fertilisation, applied every 15 days (10 mL per pot), macronutrients: $(\text{NH}_4)_2\text{SO}_4$ (4 mM), K_2SO_4 (2 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.5 mM), NaNO_3 (8 mM), FeEDTA (0.1 mM); micronutrients: H_3BO_3 (2.86 mg L^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.81 mg L^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 mg L^{-1}), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.025 mg L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22 mg L^{-1}), according to Kariman et al. (2014). Cadmium was applied gradually over three days with 10 mL aliquots of a CdCl_2 solution, each application was equivalent to 9 mg kg^{-1} Cd, reaching a total application of 27 mg kg^{-1} . Plants that received the CdCl_2 solution were called 'donors' and the ones on the connected pot, which did not have CdCl_2 solution, were called the 'receivers'. The treatments were: a) NM-NM: Non-mycorrhizal donors and non-mycorrhizal receivers; b) MP-MP: mycorrhizal donor and mycorrhizal receiver; c) (NM-NM)Cd: Non-mycorrhizal donor exposed to Cd (CdCl_2 solution) and a non-mycorrhizal receiver

(without Cd); d) (MP-MP)Cd: mycorrhizal donor exposed to Cd and a mycorrhizal receiver (without Cd). The code throughout the text will be simplified as: NM, MP, NM+Cd and MP+Cd.

Plants were watered three days a week to reach 70% of soil water capacity, by checking the soil moisture with a moisture meter. The temperature average recorded in the glasshouse during this period of four months was 23.5°C (+/-2.4). Harvest was performed by removing the whole plants from the substrate, and two samples of the substrate were collected from each pot for further evaluation. Poplar cuttings were separated into leaves, stems, and roots. Roots were rinsed with distilled water, and immersed in a solution of 0.05 mM CaCl₂ for 30 min to remove surface adhering metals (Marmiroli et al., 2013). Plant tissues were dried in an oven at 70°C for five days and weighed for biomass determination.

Mycorrhizal colonisation

Poplar root sub-samples were cleared in KOH solution (10% w/v) KOH at room temperature for 10 days, and then stained in a 5% (v/v) black ink vinegar solution (Vierheilig, Coughlan, Wyss, & Recherche, 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 mycorrhizal colonisation (Giovannetti and Mosse 1980).

Plant digestion and metal determination

The dried biomass samples were ground into a fine powder. Samples were digested (500-1000 mg) in nitric acid using DigiPrep digestion block, and mineral contents in the extracts were determined via ICP-OES (Perkin Elmer AVIO 500). Standard and Quality Control solutions were made up of stock solutions (NIST). The samples had initially 50% nitric acid and then after digestion, they were made up to either 25 mL or 50 mL with Millipore water, depending on the weight of the sample used.

The following system shows how the pots and the tube were assembled and the outcome of Cd application in this experiment. Donor plants received the Cd solution in 3 doses, these results were analysed after 4 months in the glasshouses at University of Reading.

Determination of total N and C using an elemental analyser

This method relates to the analysis of the total elemental N and C content of botanical materials. The equipment used in this method was Thermo Finnigan Elemental Analyser (FlashEA 1112 Series) and a Mettler MT5 Microbalance. The detection limit of this method for a

15 mg sample ranges from a minimum of 0.03% N and 0.02% C, to a theoretical maximum of 100% dry weight for N or C. The Sample (15 mg) contained in a tin capsule was dropped into a combustion reactor maintained at 900°C. The container melted and the tin promoted a 'flash reaction' in a helium atmosphere temporarily enriched with pure oxygen. This momentarily raised the temperature in the reactor to ~2000°C combusting the sample. The combustion products were carried by a constant flow of helium through an oxidation catalyst, copper oxide and platinised Alumina. CO₂, N₂, NO_x, and H₂O then flow into a reduction reactor containing copper wires held at 680°C, where excess oxygen was removed and any nitrogen oxides were converted into nitrogen gas. Water was then absorbed by magnesium perchlorate. A chromatographic column held at 400°C then separated the CO₂ and N₂ into defined peaks, and the relative amounts were determined using a thermal conductivity detector. The reference materials used were a range of primary and secondary reference materials with certified C and N contents, are used to calibrate the Reference and QC Check materials used by this method.

Statistical analyses

Statistical analyses were performed using R[®] software (using R version 4.3.0). When all assumptions were met, Three-way ANOVA was performed (M, NM; Cd, -Cd; Donor, Receiver), followed by Tukey test ($p < 0.05$). If data had homogeneous variances (Levene test, $p > 0.05$), but were not normally distributed (Shapiro-Wilk test) then the non-parametric Kruskal Wallis test was applied, and when results were significant ($p < 0.05$), the Dunn test was used to discriminate the differences among treatments.

4.3 Results

Root colonisation

The results showed that all mycorrhizal treatments MP-MP and (MP-MP)Cd had consistent colonisation by *R. irregularis* around 60 and 70%. Colonisation was slightly higher under Cd contamination, and in both cases, donor and receiver plants had similar colonisation rates (Fig. 4.2). There was no colonisation detected in roots from non-inoculated pots.

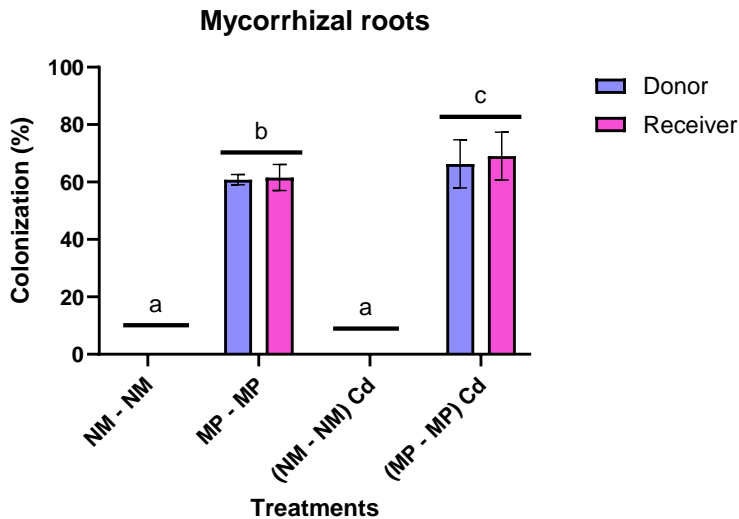


Figure 4.2. Colonisation percentages of poplar roots by *R. irregularis* after 4 months with and without Cd contamination, with and without mycorrhizal inoculation. Bars represent the average (n=6) and standard errors. Different letters represent significant differences between treatments ($p < 0.05$) after two-way ANOVA.

Cadmium accumulation

Results showed a foliar Cd concentration of around 1 mg kg^{-1} and above for donor plants in both Cd treatments NM + Cd, MP + Cd. However, there were no statistical differences between them and either for receivers whose values were below 0.4 mg kg^{-1} . In roots, the concentration of Cd was higher ($1.50 - 2.25 \text{ mg kg}^{-1}$) particularly in donor plants, they showed a significant difference between Cd treatments (Figure 4.4). On the other hand receiver plants showed a low Cd concentration for all Cd treatments. Non-mycorrhizal roots (Donor) exposed to Cd, accumulated 50.8% more Cd compared with their leaves, and similar behaviour in receiver plants, with higher Cd in roots than its leaves (Figure 4.3). The content of Cd in leaves also confirms that treatment NM -NM and MP-MP do not differ statistically within them as expected because they do not have any Cd in them.

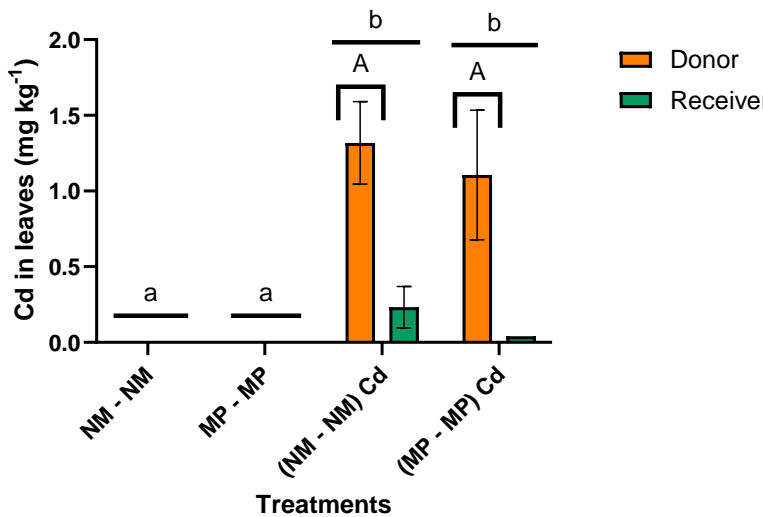


Figure 4.3. Cadmium concentration in leaves of poplar plants after 4 months growing individually; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different lowercase letters represent significant differences between treatments; while capital letters denote differences between donor and receiver plants, after Two-way ANOVA, and Tukey test, $p < 0.05$.

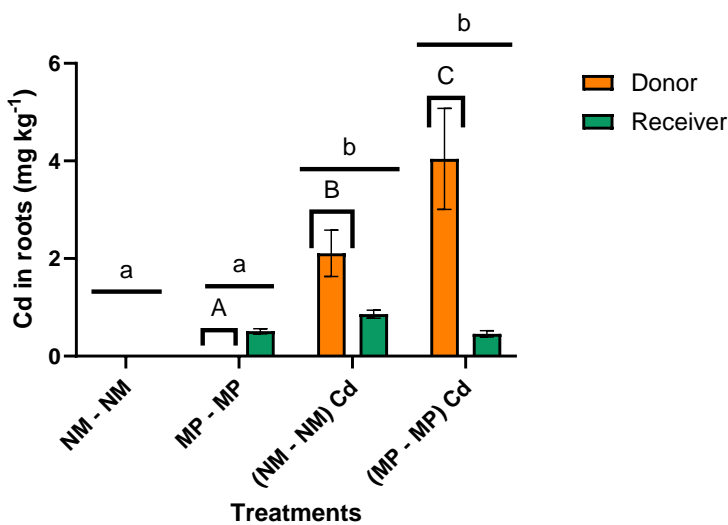


Figure 4.4. Cadmium concentration in roots of poplar plants after 4 months growing under Cd contamination with (AM) and without (NM) mycorrhizal symbiosis. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments (n=5) and standard errors. Different capital letters represent significant differences between plants (Donor/Receiver); after Two-way ANOVA, and Tukey test, $p < 0.05$.

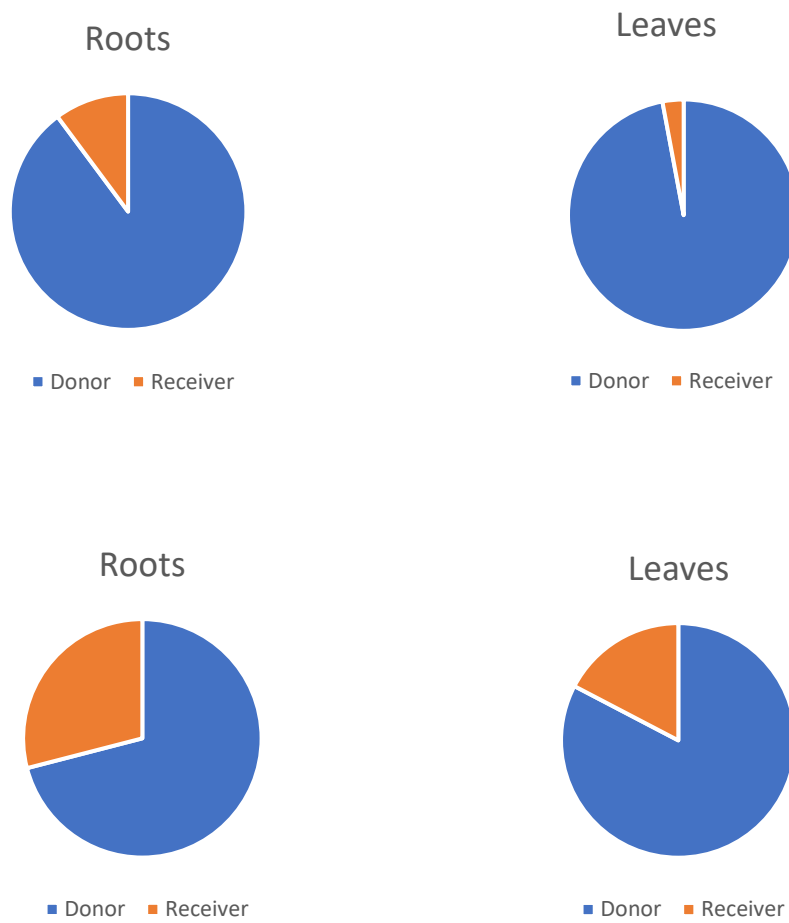


Figure 4.5 Amount of cadmium in poplar roots and leaves after four months with or without common mycorrhizal network. Donor refers to plants which had Cd application directly and the Receiver plant had no Cd additions in the pot. That is a representation from the (MP – MP) Cd treatment in comparison with (NM-NM) Cd.

Donor plants had much more Cd than the receiver plants at the end of the experiment (Figure 4.3/4.4/4.5). The content of Cd in treatments (NM – NM) Cd and (MP – MP) Cd is quite similar and does not show any statistical differences within them.

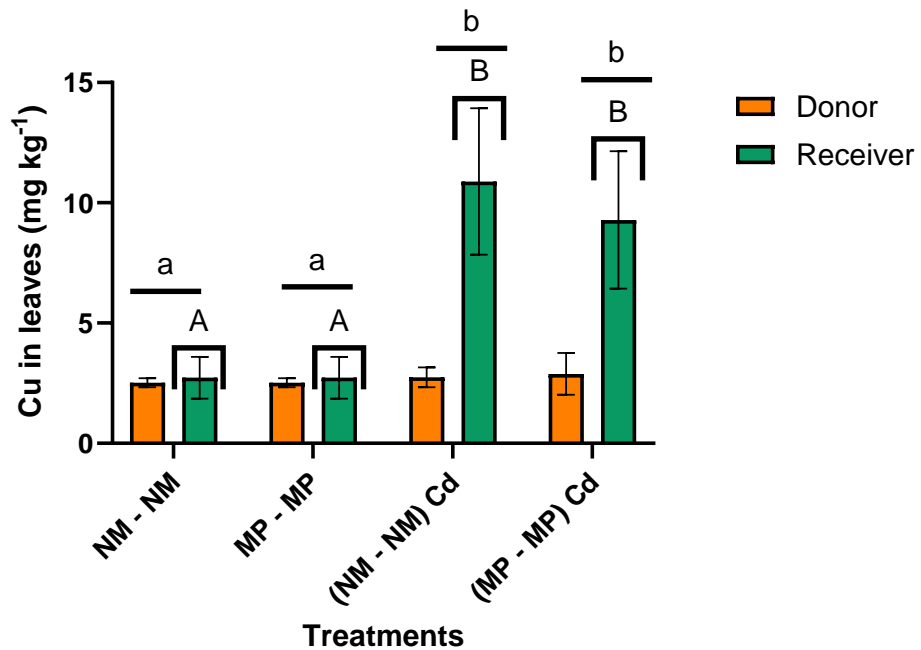


Figure 4.6. Copper concentration in leaves (a) of poplar plants after 4 months growing individually; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments; while capital letter denote Cd effects within each plant (donor/receiver), after Two-way ANOVA, and Tukey test, $p < 0.05$.

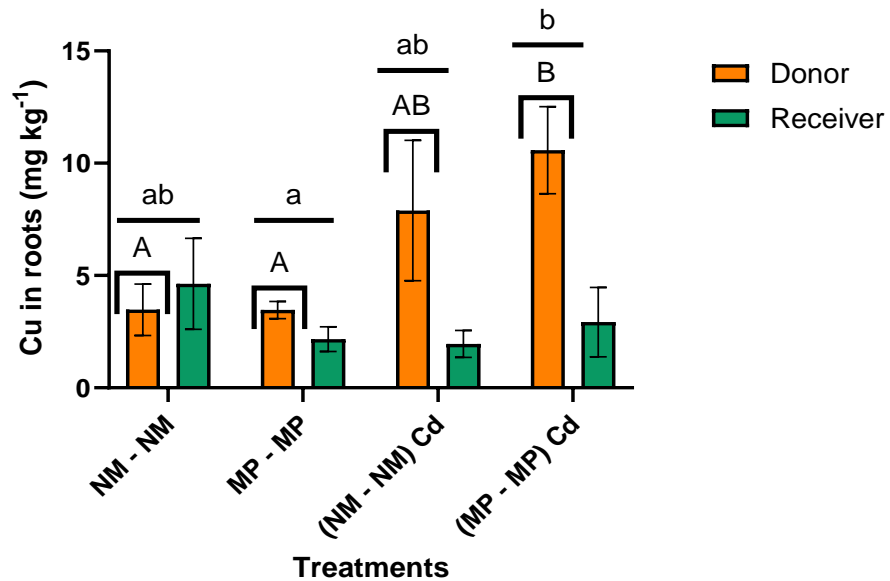


Figure 4.7. Copper concentration in roots (a) of poplar plants after 4 months growing poplars; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments; while capital letter denote Cd effects within each plant (donor/receiver), after Two-way ANOVA, and Tukey test, $p < 0.05$.

Copper was analysed under Cd stress. Cd contamination led to significantly higher Cu concentrations in donor roots, regardless of mycorrhization (Figure 4.6/Figure 4.7). However, in leaves, this synergistic effect was only observed in mycorrhizal plants. Another nutrient analysed was zinc (Zn), which is known to be chemically similar to Cd, and be carried out by similar transporters in plants, however, the results presented here did not show that as clearly as could for roots. There was a higher Zn accumulation in the receiver plant, when mycorrhizal and exposed to Cd. Treatments do not show any statistical differences for the accumulation of foliar Zn (Table 4.1/Table 4.2).

Table 4.1. Zinc concentration in Poplars leaves and roots (mean \pm SD).

Treatment		Zn mg kg ⁻¹	
	Plant	Leaves	Roots
NM-NM	Donor	33.79 \pm 0.3	18.41 \pm 4.4
	Receiver	31.99 \pm 0.2	20.27 \pm 5.4
MP-MP	Donor	29.3 \pm 3.5	12.87 \pm 1.1

	Receiver	30.2 ± 5.4	14.40 ± 0.7
(NM-NM) Cd	Donor	22.91 ± 7.1	19.49 ± 1.5
	Receiver	19.87 ± 4.0	22.97 ± 4.0
(MP-MP) Cd	Donor	32.91 ± 10.22	20.10 ± 3.6
	Receiver	41.25 ± 4.2	28.67 ± 18.5

ANOVA (P-values)			
Factors			
Treat		NS	*
Plant		NS	NS
Rep		NS	NS
Treat*Plant		NS	NS

Plant means (Donor and Receiver). Asterisks indicate significant differences (main effects, interactions) detected by two-way ANOVA (* $p < 0.05$). df: degrees of freedom, NS: not significant ($p > 0.05$).

The statistical difference (Tukey test) showed in roots were in the treatment MP from treatment MP+Cd. Which the only difference between them is the presence of Cd, which might be the explanation for why the amount of Zn is higher in this treatment (Table 4.1/Table 4.2).

Table 4.2. Results of Tukey test for roots with Zn.

Zn Roots		
Treatments	Average	
NM - NM	13.639	a
MP - MP	17.744	ab
(NM-NM) Cd	21.137	ab
(MP-MP) Cd	24.388	b

There were no differences between treatments or plants in relation to P foliar concentrations (Table 4.3). However, Cd contamination increased P concentrations in roots,

especially in NM plants, in which root P essentially doubled, from 453 to 913 mg kg⁻¹ Cd (Table 4.3/ Table 4.4).

Table 4.3. Phosphorus concentration in poplar leaves and roots (mean ± SD).

Treatment	P mg kg ⁻¹		
	Plant	Leaves	Roots
NM-NM	Donor	1446 ± 0.4	650 ± 16
	Receiver	1516 ± 0.5	666.3 ± 13
MP-MP	Donor	1397 ± 14	420.1 ± 80
	Receiver	1304 ± 30	487.5 ± 74
(NM-NM) Cd	Donor	1336 ± 20	816.8 ± 29
	Receiver	1247 ± 37	1010 ± 15
(MP-MP) Cd	Donor	1487 ± 17	743.5 ± 22
	Receiver	1270 ± 48	678.2 ± 26
ANOVA (P-values)			
Factors			
Treat		NS	*
Plant		NS	NS
Rep		NS	NS
Treat*Plant		NS	NS

Plant means (Donor and Receiver). Asterisks indicate significant differences (main effects, interactions) detected by two-way ANOVA (* p < 0.05). df: degrees of freedom, NS: not significant (p > 0.05).

Table 4.4. Results of Tukey test for roots with P.

P Roots			
Treatments	Media		
NM - NM	453.78		a
MP - MP	658.20		ab
(MP-MP) Cd	711.39		bc
(NM-NM) Cd	913.64		c

Total N in leaves do not show any effect of Cd or mycorrhizal,(Figure 4.8) in contrast in the roots it follows the same pattern as P (phosphorus) for roots. It is shown Cd was promoting the increase of N accumulation in roots for treatment NM+Cd (Figure 4.9).

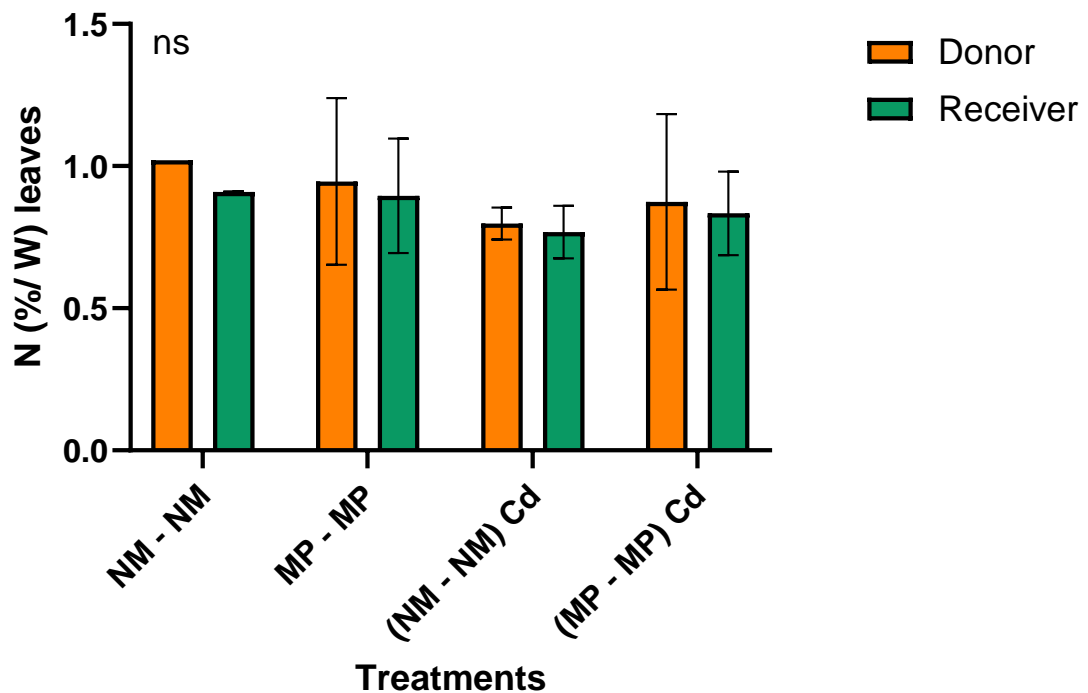


Figure 4.8. Nitrogen concentration in leaves (a) of poplar plants after 4 months growing poplars; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments; after Two-way ANOVA, and Tukey test, $p < 0.05$.

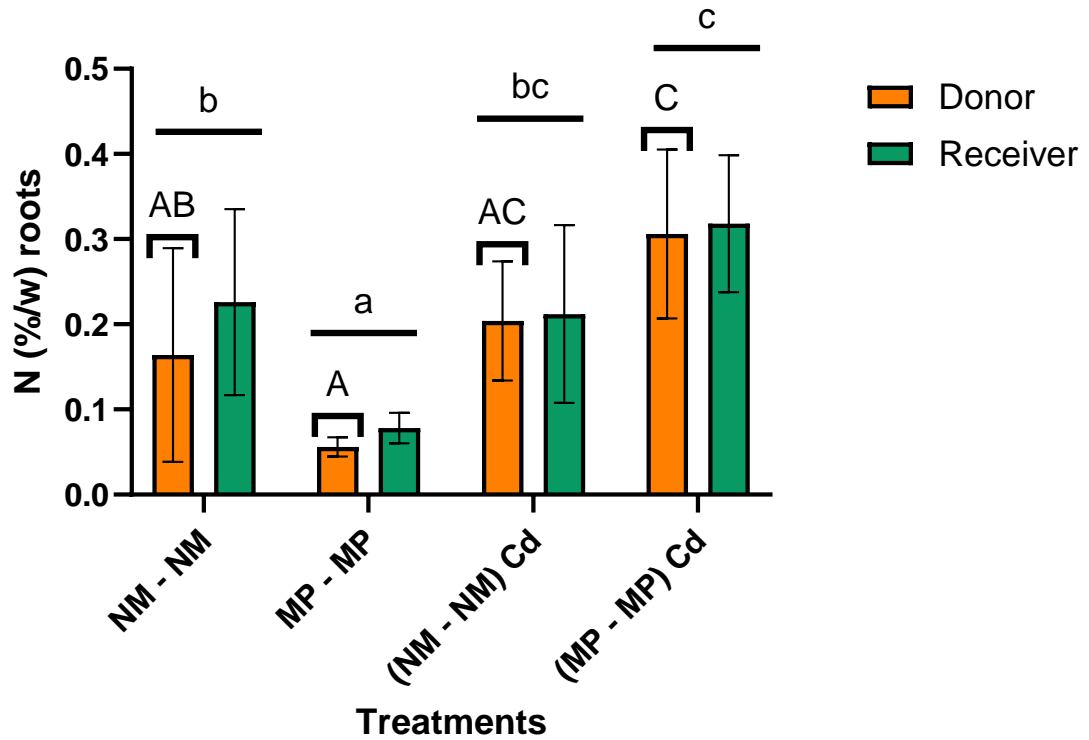


Figure 4.9. Nitrogen concentration in leaves (a) of poplar plants after 4 months growing individually; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments; while capital letter denote Cd effects within each plant (donor/receiver), after Two-way ANOVA, and Tukey test, $p < 0.05$.

Carbon concentration in poplars showed differences between treatments for leaves and roots. In leaves, the treatment that presented the greatest difference was NM and MP + Cd with a 2% increase. In roots, the treatments were significantly different particularly for donor plants. MP accumulated less C than MP+Cd (Figure 4.10/ Figure 4.11).

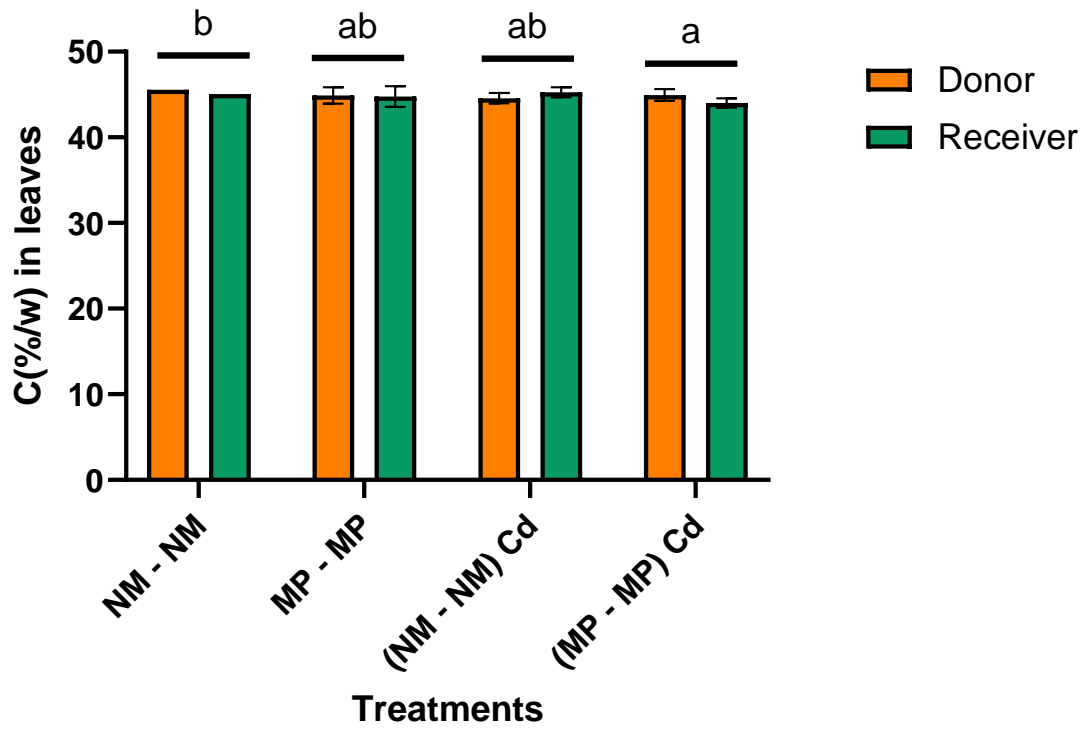


Figure 4.10. Carbon concentration in leaves (a) of poplar plants after 4 months growing individually; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments after Two-way ANOVA, and Tukey test, $p < 0.05$.

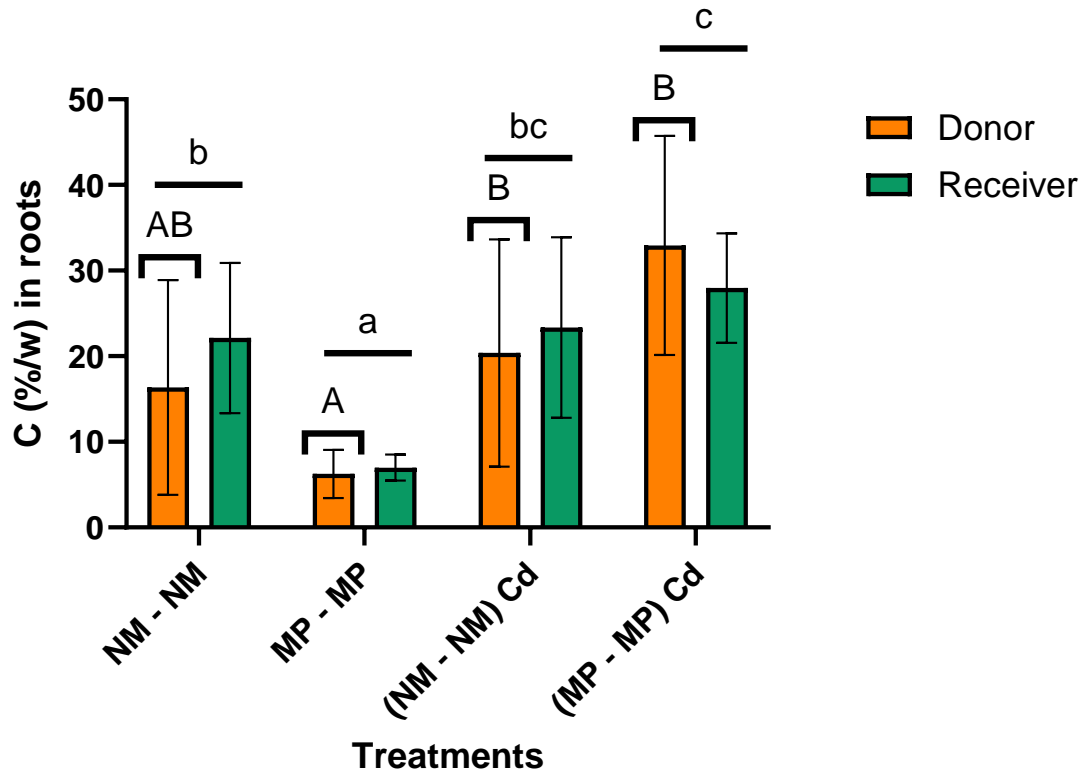


Figure 4.11. Carbon concentration in leaves (a) of poplar plants after 4 months growing individually; with (MP) and without (NM) mycorrhizal symbiosis or Cd contamination. Bars represent the average (n=5) and standard errors. Different letters represent significant differences between treatments; while capital letter denote Cd effects within each plant (donor/receiver), after Two-way ANOVA, and Tukey test, $p < 0.05$.

4.4 Discussion

The colonization rates for *R. irregularis* in *P. trichocarpa* were high in both plants (donor and receiver). They reached 60 to 67%, however, there were a statistical difference between treatments, MP – MP was slightly lower (10%) compared to (MP -MP) Cd. A similar effect was reported by Pawlowska and Charvat (2004), in which *Glomus. intraradices* responded with higher hyphal extension when exposed to Cd and Pb.

In (NM-NM)Cd treatment, we observed small amounts of Cd in receiver plants. This could be due to mass flow from one pot to another, as Cd is highly mobile in soils (Lei et al. 2010). Movement from the zone of heavy metal application could occur by diffusion, either as a free ion or as a complex, by mass flow with the water front, or by the movement of metal-laden particulates through open channels in the soil. Movement of heavy metals through channels may be extensive in unique situations such as extensive soil cracking during wetting and drying cycles, in our experiment as the substrate was sand and terra green there is a big chance to have happened those channels. Generally, diffusion of metal occurs over short distances and will play a major role in metal uptake by plants (Barber, 1974). However, mass flow is probably the principal means by which heavy metals move appreciable distances within soils (Dowdy and Volk, 1983). Consequently, we believe Cd might had been transported from one side through the other by the mechanisms cited above.

Treatment (MP-MP) shows a small amount of foliar Cd in the receiver plant. It is probably a little trace that it was present in the substrate, even the substrate used was the same for all pots in the experiment. Without human interference, Cd content in soils varies considerably according to the parent material, which bears 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).

The high amount of Cd in the treatment MP + Cd, which was not expected, leaves, and roots might be explained by the different mechanisms which mycorrhizal plants can achieve to create tolerance to metal toxicity. Those mechanisms are a dilution in a higher plant mass, detoxification by fungal or plant metabolites, and sequestration of metals within plant of fungal vacuoles (Hildebrandt et al. 2007). Arbuscular mycorrhizal fungi act as a metal sink, reducing local concentrations in soils and creating a more suitable environment for plants growing in metal-contaminated soils (Gohre and Paszkowski, 2006), their association can alter plant metal

uptake (Toler, Morton, and Cumming, 2005; Andrade et al. 2008) by increasing or reducing metal in plant tissues.

As a consequence of physiological changes, AM plants have a great performance under metal stress conditions (Paradi, Bratek, and Lang et al. 2003). The mycorrhizal Cd treatment showed greater root Cd concentrations in donor plants. The Donor's plants were exposed to a higher amount of Cd, we presume that those mycorrhizal plants had a role of detoxifying by accumulating Cd in the fungal vacuoles (De Oliveira et al., 2020). Consequently, the amount of Cd that was able to be transferred to the receiver plant was much less than that in the donors. Kariman et al. (2016) showed that mycorrhizal fungi can induce tolerance against metal toxicity by the reduction of minerals, binding phytochelatin to the toxic substance, or accumulating peptide-metal complexes inside fungal vacuoles (Meharg and Macnair 1992). In treatment (MP-MP)Cd, receivers presented much less Cd in leaves and 89% in the roots compared to donors of the same treatment, which also could be explained by Gonzalez-Guerrero et al. (2008) work, which showed that results obtained from electron-dispersive X-ray spectrometry (EDXA), such as Zn, Cu and Cd accumulated in the cell wall and electron-dense granules in the cytoplasm of the fungi while their cytoplasm itself was essentially free of these elements). In addition, vesicles might serve as storage compartments for heavy metals (Turnau, 1998; Weiersbye et al. 1999), as it might be a complementary explanation for the results shown above (Cd content in treatment MP + Cd, figure 4.12).

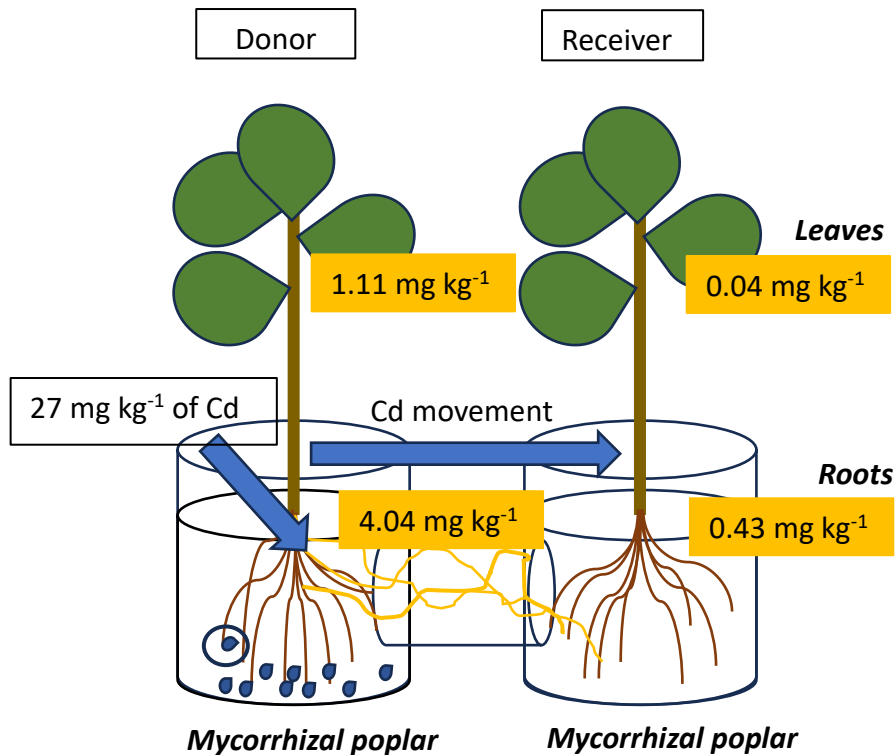


Figure 4.12. Poplar cuttings growing in a separated pots in a substrate (1 kg) connected by a pipe (3 cm) containing a mesh (30 μm) in both ends to avoid root contact, allowing only the hyphae to grow through. One side was contaminated by 27 mg/Kg Cd, which was in donor plants. The results showed here are the average from the treatment (MP-MP)Cd.

Cd and Zn share a similar uptake pathway in plants (Clemens, 2006) and may be geochemically similar, but by being inherently different in terms of essentiality for plant or fungal development, it becomes apparent the AMF influence will not be the same when in terms of toxicity avoidance. However, in the treatments (NM-NM)Cd and (MP-MP)Cd, Zn concentration was higher in the receiver instead of the donor, which was expected the opposite regarding the Cd concentration in donor pots.

Phosphorus is known to have a large impact on AMF, it has also an important influence of Cd tolerance and plant uptake. AMF can improve the growth of plants through mechanisms such as root chelation fixation, vacuolar sequestration, and cell wall fixation, and enhance antioxidant capacity under Cd stress (Jia et al., 2020). AMF has been noted as being sensitive to the amount of P in the environment where P also affects the absorption and transport of heavy

metals. Both coexist in nature, and their interaction impacts the accumulation of Cd in host plants. You et al. (2022) found that P uptake was positively regulated by Cd in AMF, increasing Cd accumulation in *Phragmites australis* when P is more available. The mechanism of AMF in enhancing Cd uptake in *P. australis* depends on the P concentration. However, from our results, Cd significantly increased P in roots, regardless of AMF presence.

It is described in the literature that P reduced Zn and/or Cu in mycorrhizal plants. Brunetto et al., (2019) proved that adding P fertilization on soil reduces Cu transport from roots to shoots. Mycorrhizal plants are able to extend their mycelium further in the soil and consequently, very important for nutrients that have narrow diffusion zones around roots, such as P, Zn, and Cu, and those elements are mostly increased by mycorrhizae. This might be one of the explanations of a such low level of Cu was found in the leaves of donors, the amount of P that we had to apply for fertilizer was just enough to keep the plant alive, however could have assisted the mycorrhizal of fixing it and helped to avoid Cu (when. Interest, the amount of Cu in roots followed a similar pattern for receivers. Cu showed lower values in Donor plants, another explanation could be that Cd also interacts somehow with Cu promoting more Cu availability for the leaves of the receivers and for the roots of the Donors.

Heavy metal can reduce available pools of nutrients in soils, by inhibiting the mineralization processes, and then consequently litter decomposition rate in ecosystems, generally, decreases (Derome, 1998, 2000). For instance, nitrogen is the most limiting nutrient for plant growth. However, Cd did not show a significant difference between treatments for leaves or for roots (Figure 4.9, 4.10) which in Blandez, Bolton and Chalot (2000) confirms the effect of heavy metal in N uptake by plants.

The results showed that *P. trichocarpa* was not affected by the Cd levels used over the time of the exposure of this study, however, it is not possible to prove the transport of Cd between donor and receiver plants was by CMN or by soil diffusion because Cd was also detected in non-mycorrhizal receivers. Further investigations are needed to verify if under a higher concentration of Cd (level of toxicity/threat to life) mycorrhizal *Populus trichocarpa* would behave in a different fashion when under a common mycorrhizal network.

The hypotheses suggested initially were: (1) CMNs can transfer Cd between poplars. After our results, the first hypothesis still cannot be proved, we faced many variations along the way, and consequently, we cannot accept it. (2) Nutritional concentrations of the receiver and the donor plants are different. The second hypothesis cannot be accepted because there is a

great variation between nutrient concentration in donor and receiver plants. Lastly (3) Plant growth of the receiver and the donor plants are different. The third hypothesis was also not accepted, because there were not many evidences of a concrete result.

4.5 References

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Chapter 5

5. The role of saprotrophic fungi in environmental remediation, particularly in the context of cadmium contamination

5.1 Introduction

Heavy metals are widely recognised as significant environmental pollutants due to their ability to accumulate in biological systems and their non-biodegradable nature. The toxicity of heavy metals largely depends on the specific type of metal and its concentration in the environment. However, certain heavy metals play crucial roles in microbial and plant physiology. For instance, elements such as copper (Cu), iron (Fe), manganese (Mn), cobalt (Co), molybdenum (Mo), zinc (Zn), and nickel (Ni) are vital for fungal metabolism and plant growth. Nonetheless, their beneficial effects depend on their concentrations. Excessive amounts can pose significant ecotoxicological threats. High levels of heavy metals in the environment can induce toxicity symptoms in plants, disrupt biological processes, and adversely affect microbial activities, highlighting the dual role of heavy metals as both essential nutrients and potential environmental hazards (Adriano, 2001).

The presence of heavy metals in soil can significantly disrupt many physiological processes in plants, insects, bacteria, or fungi (Rajapaksha, Tobor-Kaplon, and Bååth, 2004; Babich and Stotzky, 1977; Oliveira and Pampulha, 2006; Khedhairi, Rokayan, and Misned, 2001, Silva 2021), eventually altering the composition and structure of entire ecological communities (Moffett et al., 2003). Environmental stress induced by heavy metals typically decreases the diversity and activity of soil microorganisms. This reduction, in turn, impacts ecosystem processes dependent on the activity of these microorganisms (Su et al., 2023). For example, heavy metals have been shown to impair the growth and extracellular enzyme production capabilities of fungi (Gadd, 1993; Baldrian, 2003; Silva, 2021), which in turn disrupts the cycling of carbon and nutrients.

Cadmium (Cd) is identified as a priority hazardous substance and ranks among the top 20 contaminants (ATSDR, 2012). Notably, it is a non-essential element for living organisms. It is characterized by a high solubility in water, which facilitates its mobility within various environments (Pinto et al., 2004). Cd is considered one of the most detrimental metals in the environment due to its capacity to affect human health and other living organisms at relatively low concentrations (Lei et al., 2010). Numerous studies have consistently demonstrated that

soil microbial diversity diminishes in areas with increasing levels of Cd pollution (Wang et al., 2007; Azarbad et al., 2013; Chen et al., 2014).

Soil fungi play a significant role in mitigating heavy metal toxicity to plants and influencing their accumulation and transport. For example, research by Sun et al. (2017) demonstrated that the addition of *Mucor circinelloides* resulted in a 45.77% decrease in Pb accumulation in *Solanum nigrum* compared to treatments involving only the plant. Moreover, the fungal fruiting bodies may accumulate and store high levels of toxic elements (Svoboda et al., 2006). Fungi represent a unique group of organisms with the ability to biodegrade the substrates on which they grow. However, studies such as those by Gabb (2004) and Kubrova et al. (2014) have shown that metal accumulation in fruiting bodies is generally dependent on the species' metabolism and is significantly influenced by the chemical composition and properties of the substrate, which provides their nutrients.

In forest ecosystems, the soil fungi consist of two dominant groups, the saprotrophic and ectomycorrhizal fungi (Awad et al 2019). The saprotrophs are free-living organisms that obtain carbon and other nutrients through the decomposition of organic matter, utilising extensive and specialised enzymatic systems. On the other hand, ectomycorrhizal fungi form symbiotic relationships with tree roots, receiving carbon from their host plants. In return, they have the capability to acquire additional nutrients, especially nitrogen and phosphorus, for their use and to supply these to their plant partners in exchange for carbon.

While it is widely acknowledged that mycorrhizal fungi enhance plant resistance to various stresses, including protection against heavy metal toxicity, there remains much to uncover, particularly regarding the impact of Cd on saprotrophic fungi (Baldrian et al., 2000; Ceci et al., 2018). Research has highlighted the potential for certain saprotrophic species to remediate heavy metals, yet the diversity within saprotrophic fungi presents challenges for a comprehensive exploration. As crucial facilitators of nutrient cycling in terrestrial ecosystems, saprotrophic fungi play a pivotal role. They are the primary agents in plant litter decomposition, and their hyphal networks, which extend across the soil-litter interface, serve as conduits for the distribution of nutrients (Watkinson et al., 2006).

This chapter presents a comprehensive literature review of various saprotrophic fungi species, including a few of the most dominant species in the forest ecosystems: *Trichoderma asperellum*, *Trichoderma viride*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Lentinus edodes*, *Ganoderma lucidum*, and *Purpleocillium lilacinum*. Part of our own research is

included to emphasise the fungal growth pattern under low and high Cd contamination. The goal is to deepen our understanding of these species and their responses to environments contaminated with Cd. Research on the impact of heavy metals on soil fungi encompasses diverse methodologies, allowing for a comparison of the effects of heavy metals on saprotrophs. This investigation will encompass both laboratory and field experiments to provide a comprehensive understanding of fungal responses to heavy metal contamination. Laboratory experiments focusing on heavy metal accumulation in liquid media will offer insights into the fungal uptake capacity. Meanwhile, field experiments, which more closely mimic natural conditions, will also be considered. However, a notable challenge in field experiments is the difficulty in isolating fungal responses to heavy metals from the influence of plant interactions (Baldrian, 2003). This complexity underscores the need for careful experimental design to discern the specific effects of heavy metals on fungi.

5.2 Interaction of saprotrophic fungi with Cadmium in the soil environment

Cadmium is among the most extensively researched trace elements in forest soil fungi. In the forest soil saprotrophic fungi, Cd concentrations range from 0.10 to 7.5 mg kg⁻¹ (Svoboda et al., 2000; Malinowska et al., 2004; Mendil et al., 2005; Cocchi et al., 2006; Yamaç et al., 2007; Kojta et al., 2011; Chudzynski et al., 2013; Petkovšek and Pokorný, 2013). Cadmium content in the fruiting bodies of fungi is generally reflective of the metal concentration in the soil. Nonetheless, other factors can also influence Cd levels in fungi, such as soil pH, organic matter content, the mineral composition of the bedrock, as well as the species and ecology of the fungi (Malinowska et al., 2004; Melgar et al., 2009). Compared to mycorrhizal fungi, saprophytic fungi exhibit higher metal contents due to their accelerated decomposition and catalase activity (Kuusi et al., 1981; Alonso et al., 1999) and possess an effective mechanism for the absorption of heavy metals from their surroundings (Turkekul et al., 2004).

Saprotrophic fungi are essential to terrestrial ecosystems, playing a crucial role in the carbon cycle and other nutrient transformations. These organisms are pivotal in the degradation, utilisation, and transformation of organic and inorganic materials, through their production of extracellular enzymes (Baldrian, 2008). Additionally, they contribute to the formation and mineralization of humic substances and assist in the mobilization of metals bound to organic matter (Baldrian, 2008; Siric et al., 2016). However, metals such as Cd can disrupt these processes by inhibiting enzyme activity. This inhibition occurs through the binding of Cd to thiol groups at the active sites of enzymes or through the irreversible inactivation of aromatic

amino acid residues, a result of oxidative damage from reactive oxygen species (Baldrian, 2003; Gadd, 2010; Dighton, 2016; Liu et al., 2017). Although many studies highlight the detrimental effects of metals on extracellular enzymes, with heavy metals generally acting as inhibitors of enzymatic activity (Silva, 2021), there are studies where metals have been observed to have no impact effects on microbial metabolism (Sandrin and Maier, 2003; Sandrin and Hoffman, 2007; Almeida et al., 2013; Bourceret et al., 2016). Given their dependence on extracellular enzymes for nutrient uptake, saprotrophic fungi are particularly vulnerable to heavy metal toxicity, underscoring the complex interplay between these organisms and their environment (Baldrian, 2003).

5.3 *Trichoderma asperellum*

Trichoderma asperellum belongs to the phylum Ascomycota, class Sordariomycetes, and family Hypocreaceae. It is recognized for its capacity to combat other microbes through the secretion of extracellular enzymes and antibiotics. This species, along with others in the *Trichoderma* genus, has been widely utilized in agriculture for its beneficial properties (Watanabe et al., 2006). Recently, it has also been identified as an effective agent for heavy metal remediation. *T. asperellum*, in particular, has demonstrated significant bio-adsorption capabilities, showing promise in adsorbing and immobilizing heavy metals from contaminated environments (Maurya et al., 2019). Among various fungal species investigated for their ability to scavenge heavy metals, *T. asperellum* stands out, with a reported efficacy of 88.9% in Cd removal (Ayangbenro and Babalola 2017). Despite its potential, heavy metals still pose a threat to its growth and development. Research by Maurya et al. (2019) highlighted that the mycelial growth and sporulation of *T. asperellum* were inhibited by more than 30% in the presence of lead (Pb) and Cd, emphasizing the complex relationship between these fungi and environmental contaminants.

A study by Hoseinzadeh, Shahabivand, and Aliloo (2017) demonstrated the capability of *T. asperellum* to survive at high heavy metal concentrations (200 mg L⁻¹) and absorb heavy metals from aqueous solutions, noting that this capacity increases at higher pH levels compared to lower pH environments. Analysis of metal removal efficacy revealed a species-specific correlation between fungi and Cd removal capabilities. The findings highlighted that *Trichoderma asperellum* exhibits a remarkable proficiency in eliminating Cd ions, achieving a removal rate of 76.17% (pH = 9). In a separate investigation, Syed et al. (2023) employed a novel approach utilising *Pseudomonas fluorescens* and *Trichoderma* sp. as metal-tolerant microbial

inoculants. This strategy effectively reduced the levels of antioxidant enzymes that would typically rise under Cd stress (Syed et al., 2023).

T. asperellum, recognized for its biocontrol and plant growth-promoting capabilities, has been shown to increase maize resistance against biological and Cd stresses. In a comprehensive study, *T. asperellum* demonstrated the highest tolerance to Cd compared to seven other fungal genera. Maize seedlings inoculated with *T. asperellum*, after exposure to varying Cd concentrations, exhibited significant improvements in plant height, root length, fresh and dry weight, chlorophyll content, and antioxidant enzyme activity (Mengting et al., 2021). Furthermore, inoculation led to a decrease in Cd content in the aboveground parts of the plant while increasing it in the underground parts. This process facilitated the redistribution of Cd, enhancing its accumulation and promoting the transfer from shoot to root. Notably, *T. asperellum* also induced the redistribution of Cd from leaves to roots, evidenced by a decrease in the Cd transfer coefficient in maize seedlings under 100 and 200 mg Kg⁻¹ Cd concentrations (Mengting et al., 2021).

5.4 *Trichoderma viride*

Trichoderma viride, also a member of the Hypocreaceae family, has been shown to effectively remove substantial quantities of Cd from media across all pH levels, with Pb and Zn also being removed, albeit to a lesser extent. The optimal removal efficiency for all three metals was observed at a pH of 6, with Cd removal being notably high across various pH levels, followed by Pb, and Zn showing the least removal efficiency. Hlihor et al. (2015) demonstrated that *T. viride* could withstand Cd concentrations as high as 200 mg L⁻¹. The growth of *T. viride* was observable up to concentrations of 75 ppm Cd, beyond which it gradually decreased until reaching 200 ppm, at which point no spores were visible (Sahu et al., 2012).

There is a clear relationship between the concentration of metal introduced into the medium and its accumulation in the mycelium, a phenomenon particularly evident with Cd, where absorption rates ranged from 64.56% to 100%. Research focusing on the growth of *T. viride* in Cd (II) solutions and its capacity for bioaccumulation revealed that the living biomass could fully bioaccumulate Cd (II), achieving 100% removal from a 50 mg/L solution at a pH of 6.0 (Hlihor et al., 2015). However, biosorption and bioaccumulation are significantly affected by factors such as pH, the amount of biomass used, the duration of contact, and temperature (Hlihor et al., 2015).

Taken together, these findings indicate that *T. viride* has potential as biosorbent for heavy metals, including Pb, Hg, and Cd (Simonovicova et al, 2002). However, the specific tolerance strategies or resistance mechanisms utilised by *T.viride* remain less explored.

5.5 *Pleurotus ostreatus*

Pleurotus ostreatus, a white-rot fungus, is distinguished by its remarkable capacity to decompose lignin and a range of structurally related organic compounds. This ability is partly due to the necessity of certain heavy metals for fungal metabolism; for instance, copper and manganese are directly involved in the lignin degradation process in white-rot fungi. While these fungi have metabolic needs for trace metals, it's important to note that the very same metals can become toxic at concentrations only slightly higher than their metabolic requirements (Jellison et al., 1997).

Pleurotus ostreatus, a widely consumed and edible fungus, has shown evidence of accumulating significant amounts of heavy metals, such as Cd, within the fruiting body (Radulescu, Stihi, and Busuioc, 2010). This species, along with others in the *Pleurotus* genus, has been recognized for its potential in biodegradation of organic pollutants and accumulation heavy metals. Studies have demonstrated that a considerable amount of Cd can be sequestered within the hyphae, with the concentration increasing as the levels of heavy metals in the liquid culture rise. Across a broad spectrum of concentrations tested, the mycelial growth rate, colony diameter, and mycelial dry weight of the organism were all adversely affected or reduced with the increase in heavy metal concentrations (An and Zhou, 2008; Xuanzhen et al., 2017). The maximum amount of Cd adsorbed was recorded at 1.89 mg g⁻¹ of fungal biomass. The *Pleurotus* genus, which includes other edible macrofungi such as *P. pulmonarius*, *P. cornucopiae*, or *P. sapidus*, is considered promising for mycoremediation. This is due to their ability to survive in and accumulate heavy metals like Cd, Cu, Cr, Pb, Hg, and Zn from contaminated environments (Petkovsek and Pokorny, 2013).

Pleurotus ostreatus possesses the capability to degrade polycyclic aromatic hydrocarbons (PAHs), demonstrating the ability to oxidize dense PAH molecules containing up to six aromatic rings. This process effectively reduces their water solubility and, consequently, diminishes their toxicity (Barr and Aust, 1994; Bezalel, Hadar, and Cerniglia, 1996; Bumpus, Tien, and Wright, 1985). However, a significant challenge in bioremediation technology is the presence of mixed pollutants, i.e., heavy metals, which can impede the process. Particularly for

the white-rot fungi, these metals may adversely affect the activity of ligninolytic enzymes (Baldrian, Gabriel, and Nerud, 1996; Khedhair, Rokayan, and Misned, 2001), affecting their growth.

5.6 *Phanerochaete chrysosporium*

Phanerochaete chrysosporium, a white-rot fungus, is renowned for its broad capacity to degrade organic compounds and has been identified as a potential candidate for hyperaccumulation in the removal of heavy metals, demonstrating a significant ability for intracellular bioaccumulation (Xu et al., 2014). Over the past few decades, the utilisation of white-rot fungi for environmental remediation has grown, due to the increasing research and experiments targeting heavy metal contamination. Specifically, *P. chrysosporium* has been shown to achieve Cd remediation with absorption efficiencies up to 96.23% and adsorption capacities reaching 71.43 mg g⁻¹ (Noormohamadi et al., 2019), highlighting its potential in addressing heavy metal pollution.

In the study conducted by Zhao et al. (2016), *P. chrysosporium* exhibited an interesting response to Cd exposure. The growth of the fungus's hyphae progressively increased with the rising concentration of Cd, up to a certain point. Specifically, when the concentration reached 8 mg L⁻¹, the hyphal growth became inhibited, and their performance deteriorated. Remarkably, the colony's diameter in vitro showed an increase at a Cd concentration of 4 mg L⁻¹. Similarly, the dry mass of the mycelium exhibited an increase when exposed to 2 mg L⁻¹ of Cd, surpassing that of the control group. Higher Cd concentrations can impede the production of ligninolytic enzymes, directly affecting fungal growth and metabolic activity (Zhao et al., 2016a; Mora et al., 2005).

P. chrysosporium has been employed in composting as an effective strategy for reducing the bioavailability of heavy metals and for the degradation of agricultural wastes (Zhang et al., 2013). This research demonstrated that *P. chrysosporium* can expedite the composting process and contribute to the passivation of heavy metals. This effect is particularly evident in the transformation of organic materials, largely through the chelation process facilitated by organic humus. In addition to its use in composting, *P. chrysosporium* has been explored for wastewater treatment applications. Specifically, the use of rice straw inoculated with this white-rot fungus has been tested for the removal of Cd²⁺ from water. Zhao et al. (2016b) found that under optimal conditions - initial Cd²⁺ concentration of 150 mg L⁻¹, pH 5, and a temperature of 35°C - over 99%

of total Cd²⁺ was removed. Furthermore, it was observed that *P. chrysosporium* could withstand Cd²⁺ stress at concentrations up to 250 mg L⁻¹. Beyond this threshold, however, there was a significant decline in growth, reproduction, and the activity of ligninolytic enzymes, indicating a limit to *P. chrysosporium* tolerance to heavy metal stress.

5.7 *Lentinus edodes*

Lentinus edodes, another white-rot fungus, has also been identified as an effective agent for removing of toxic heavy metal ions from aqueous solutions (Bayramoglu and Arica, 2008; Zhan et al., 2014). Cadmium is recognized as affecting fungal growth, extracellular enzyme production (Baldrian et al., 2000), and the morphology of mycelium (Baldrian and Gabriel, 1997). These impacts were evident in the work of Hatvani and Mecs (2003), where an increase in mycelial density was observed in *L. edodes* in the presence of Cd. *L. edodes* shows a high potential in remediation systems for Cd-contaminated soil. Its efficiency is notably enhanced when combined with the addition of exogenous organic acids (Ma et al., 2019). For example, the addition of exogenous oxalic, malic, and citric acids increased Cd accumulation in *L. edodes* by 21.45%, 25.64%, and 59.19%, respectively (Ma et al., 2019).

Although renowned as the second most widely cultivated edible mushroom globally (Gao et al., 2015) and having a high nutritional value (Cao et al., 2013), *L. edodes* can readily absorb heavy metals, especially Cd, posing a risk to its use as food (Hatvani and Mecs, 2003). Managing Cd content and understanding the mechanisms involved in Cd accumulation in *L. edodes* is crucial. Research by Yu et al. (2020) explored a broad spectrum of expressed genes, identifying several potential candidate genes involved in Cd accumulation. This insight is invaluable for developing strains aimed at environmental pollution control.

5.8 *Ganoderma lucidum*

Ganoderma lucidum, belonging to the Phylum Basidiomycota, Order Polyporales, and Family *Ganodermataceae*, is a medicinal mushroom highly appreciated and extensively cultivated across China, Japan, Korea, Taiwan, Vietnam, Thailand, and Malaysia, among others (Chang, 1996). However, this species exhibits remarkable tolerance to heavy metals (Gupta et al., 2019). Research conducted by Ipeaiyeda, Adenipekun, and Oluwole (2020) demonstrated that *G. lucidum* could accumulate between 34.4% to 50% of Cd from contaminated soil within a

three-month incubation period. *G. lucidum* is capable of absorbing Cd from plant-derived cultivation substrates in polluted environments, which may result in growth alteration, reduced biomass, and Cd-contaminated fruiting bodies (Guo et al., 2016). Furthermore, Tham et al. (1999) observed that cadmium could significantly inhibit mycelial growth, with higher levels of cadmium leading to more rapid decreases in the fresh biomass of mycelia in liquid culture.

5.9 *Purpureocillium lilacinum*

Purpureocillium lilacinum, classified within the Phylum Ascomycota, Order Hypocreales, and Family Ophiocordycipitaceae, is recognized for its effectiveness as a nematode pathogen, targeting juvenile and female nematodes (Khalid et al., 2021). Research conducted by Deng et al. (2021) further reveals its potential in remediating Cd-contaminated soils, by facilitating increased Cd uptake in plants and contributing to the reduction of overall Cd levels in the soil. This saprotrophic fungus demonstrated resilience by thriving in a nutrient broth containing Cd concentrations as high as 12,000 mg L⁻¹, achieving a maximum removal efficiency of 70%. The study also found that, in conditions of low soil contamination, plants inoculated with the fungus showed a 112.8% increase in Cd concentration, whereas in highly contaminated soils, a decrease of 9.5% was observed. Furthermore, Gao et al. (2010) observed that *P. lilacinum* could enhance plant biomass by 30-45% and increase Cd concentration within the plants by 10-15%, showcasing its dual role in promoting plant growth and contributing to heavy metal phytoremediation.

5.10 *Mucor circinelloides*

Mucor circinelloides, classified within the Phylum Mucoromycota, Order Mucorales, and Family Mucoraceae, is a widespread fungus prevalent in various soil types, recognized for its role in environmental remediation as an effective biosorbent. It boasts a considerable biomass that exhibits a high capacity for absorption (Hou et al., 2013). *Mucor sp.* is noted for its enhancement of growth, water acquisition, and nutrient uptake, particularly under conditions of abiotic stress (Zhang et al., 2017).

Research by Zhu et al. (2015) demonstrated that *Mucor circinelloides* can withstand cadmium concentrations of up to 1000 mg kg⁻¹ on agar plates. However, growth is inhibited when Cd levels reach 1200 mg Kg⁻¹, highlighting *M. circinelloides* limit of tolerance. The cell wall serves as

a crucial defence, acting as the initial barrier to restrict the entry of heavy metals into the cellular system and preventing the excessive accumulation of toxic ions (Fu et al., 2011). Furthermore, as elucidated in the study by Li et al. (2021), extracellular immobilization of heavy metals by *M. circinelloides* involves a synergy of electrostatic interactions, complexation, ion exchange, and surface precipitation with the fungal cell wall's functional groups, providing a multifaceted approach to metalloid sequestration (Zhang et al., 2017; Li et al., 2021).

5.11 The growth of saprotrophic fungi under low and high Cadmium contamination

Four saprotrophic fungi, *Trichoderma asperellum*, *Pholiota squarrosa*, *Trichoderma viride*, and *Mortierella macrocystis*, were cultivated on agar plates supplemented with Cd-contaminated organic material, specifically the dried mycelium of *Hebeloma subsaponaceum*. *H. subsaponaceum* had been previously cultured in a modified Melin-Norkrans (MMN) liquid medium (Marx, 1969), with amendments of a CdCl₂ solution to achieve final cadmium (Cd) concentrations of 3 and 6 mg L⁻¹, in contrast to a control group without Cd (0 mg L⁻¹). The growth patterns of these fungi were analysed using digital images of the fungal colonies, with representative examples showcased in Figure 1.

The results showed that *Trichoderma asperellum* and *Mortierella macrocystis* did not show morphology alteration under Cd contamination (Table 1). *Pholiota squarrosa* and *Trichoderma viride* showed a contrasting pattern, with the highest, respectively, the lowest density under the highest level of Cd contamination present in the growth media (Table 1). However, only *T. viride* showed a clear pattern in mycelium decrease density with increasing Cd contamination level (Table 1). In contrast, the other species remained unaffected, with *Pholiota squarrosa* even exhibiting increased density under Cd stress. These observations highlight the diverse fungal responses to Cd contamination, suggesting species-specific mechanisms of tolerance or resistance to heavy metal pollution.

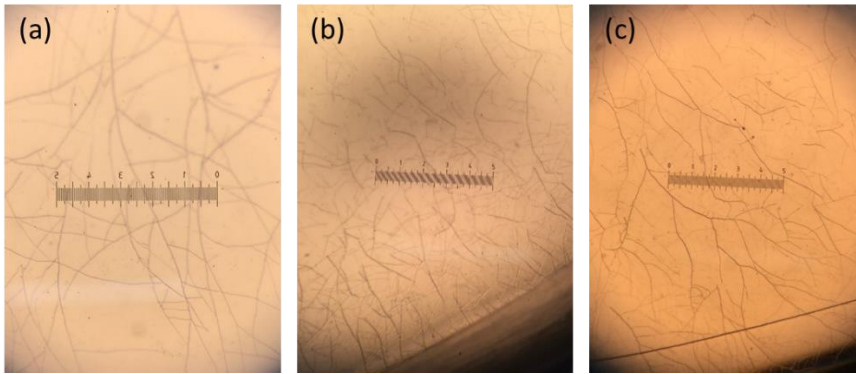
Table 1: Mycelial density measurement (calculated as the number of intersection points of hyphae with a straight line extending from the inoculum point). Sample size (N) = 4. Presented as mean (\pm standard deviation). Statistical analysis was conducted using the Kruskal-Wallis test.

Cd contamination	<i>Mortierella macrocystis</i>	<i>Trichoderma viride</i>	<i>Trichoderma asperellum</i>	<i>Pholiota squarrosa</i>
Control	17.25 \pm 3.10a	29.00 \pm 5.35b	30.50 \pm 6.19a	68.50 \pm 34.43ab
Low	22.50 \pm 1.73a	16.50 \pm 7.77ab	30.25 \pm 7.37a	41.50 \pm 12.12a
High	22.75 \pm 10.72a	12.75 \pm 7.50a	29.25 \pm 3.69a	61.00 \pm 6.32b

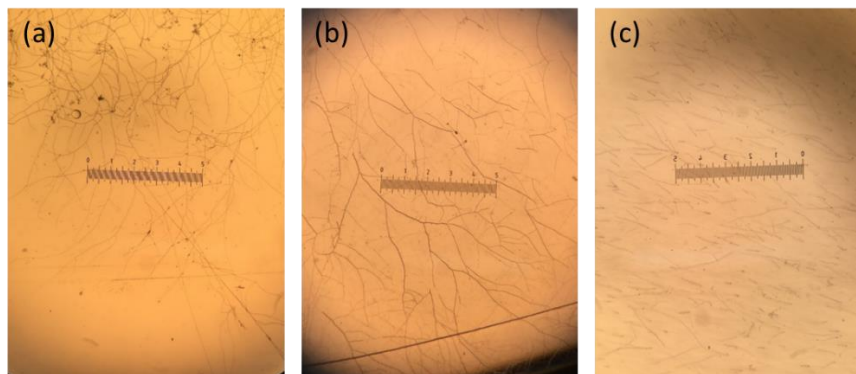
5.12 Conclusion

The saprotrophic fungi, including those discussed above, *Trichoderma viride*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Lentinus edodes*, *Ganoderma lucidum*, *Purpureocillium lilacinum*, and *Mucor circinelloides*, play a significant role in environmental remediation, particularly in the context of heavy metal contamination (Table 1). These species exhibit diverse mechanisms of action, from direct absorption and bioaccumulation of toxic metals like Cd to the degradation of organic pollutants and enhancement of plant growth and resistance under stress conditions. Their abilities range from the high tolerance and bioaccumulation capacities of *Trichoderma viride* and *Ganoderma lucidum* to the specialised enzymatic degradation processes of *Phanerochaete chrysosporium* and *Pleurotus ostreatus*. The synergistic effects of fungi like *Lentinus edodes* with exogenous organic acids demonstrate the complexity and potential for targeted remediation strategies. *Purpureocillium lilacinum* and *Mucor circinelloides*, with their high Cd tolerance and biosorption capabilities, further highlight the vast potential of fungal species in bioremediation efforts. Collectively, these fungi offer promising solutions for mitigating pollution and enhancing soil health and represent a vast area of potential for future research in environmental science and biotechnology.

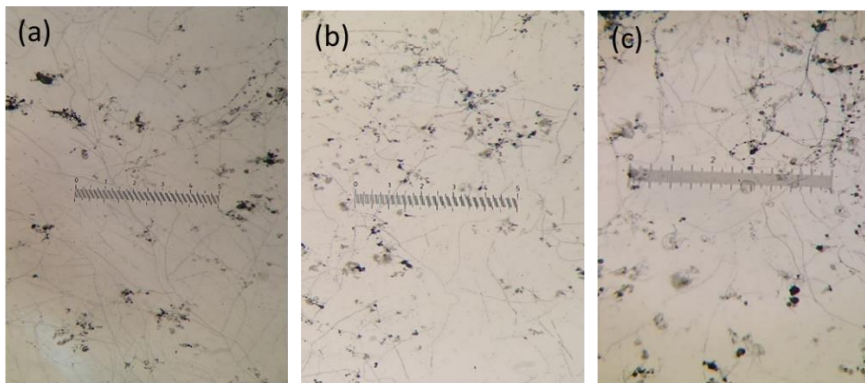
Mortierella macrocystis



Trichoderma viride



Trichoderma asperellum



Pholiota squarrosa

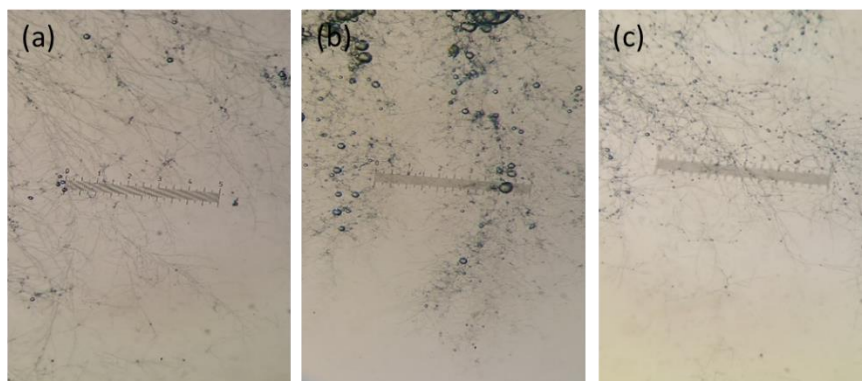


Figure 1: Illustrative depictions of saprotrophic fungal colonies cultivated on agar media with Cd-contaminated organic matter: control, no contamination (a); low contamination levels (b); and high contamination levels (c).

Table 2: Overview of saprotrophic fungi documented in the literature with potential for Cadmium (Cd) bioremediation

Saprotrophic fungi	Heavy metals (Concentration)	Main mechanisms	References
<i>Lentinus edodes</i>	Cd ²⁺ (25-600 mg/L)	Biosorption	Bayramoglu and Aria (2008)
<i>Phanerochaete chrysosporium</i>	Cd ²⁺ (2-32 mg/L)	Biosorption	Zhao et al (2016)
<i>Pleurotus ostreatus</i>	Cd (20 mg/L)	Biosorption	Chen et al (2018)
<i>Phanerochaete chrysosporium</i>	Cd(NO ₃) ₂ (10-500 mg/L)	Biosorption	Iqbal et al. (2007)
<i>Phlebia brevispora</i>	CdCO ₃ (1-100uM)	Metal in vacuoles	Sharma et al. (2020)
<i>Coriolus versicolor</i>	Cd(NO ₃) ₂ .4(H ₂ O) (1g/L)	Synthesize extracellular	Chen et al. (2011)
<i>Mucor circinelloides</i>	Cd(NO ₃) ₂ (0-50ml/L)	Biosorption	Li et al. (2021)
<i>Mucor circinelloides Trichoderma asperellum</i>	Cd ²⁺ (10-100mg/kg)	Immobilizing metal	Li et al. (2018)
<i>Trichoderma viride</i>	CdCl ₂ (5-200 ppm)	Biosorption	Sahu, Mandal and Thakur (2012)

<i>Pleurotus ostreatus</i>	CdCl ₂ (150ug/ml)	Binding in the cell wall	Favero, Costa and Massimino (1991)
<i>Trametes versicolor</i> , <i>Phellinus igniarius</i> , <i>Fomes fomentarius</i>	Cd(NO ₃) ₂ .4(H ₂ O) (0.05-0.50 mmol/L)	NA	Baldrian and Gabriel (1997)
<i>Purpureocillium lilacinum</i>	CdCl ₂ (1000-12000 mg/L)	Biosorption	Deng et al. (2021)
<i>Phanerochaete chrysosporium</i>	Cd(NO ₃) ₂ .4(H ₂ O) (15-35 mg/L)	Biosorption	Noormohamadi et al. (2019)
<i>Phanerochaete chrysosporium</i>	CdSO ₄ (150 mg/L)	Biosorption and chelation	Zhao et al. (2016 b)
<i>Pleurotus ostreatus</i>	CdCl ₂ H ₂ O (25-250ug/L)	Biosorption	Kocaoba and Arisoy (2011)
<i>Phanerochaete chrysosporium</i>	Cd (NA)	Biosorption	Kocaoba et al. (2009)
<i>Pleurotus ostreatus</i>	CdCl ₂ (25-250 ug/L)	Biosorption	Kocaoba and Arisoy (2018)
<i>Phanerochaete chrysosporium</i>	Cd(NO ₃) ₂ .4(H ₂ O) (1g/L)	Biosorption	Zuo et al. (2015)
<i>Phanerochaete chrysosporium</i>	Cd ²⁺ (50 mg/L)	Biosorption	Miao et al. (2021)
<i>Trichoderma asperellum</i> <i>Hypocrea nigricans</i>	Cd(NO ₃) ₂ .4(H ₂ O) (10-30ug)	Scavenging	Maurya et al (2019)
<i>Trichoderma asperellum</i> ,	Cd(NO ₃) ₂ .4(H ₂ O) (1-200 ppm)	Adsorption	Mohsenzadeh and Shahrokni (2014)

<i>T. harzianum</i> ,			
<i>T. tomentosum</i>			
<i>Saprolegnia delica</i> <i>Trichoderma viride</i>	CdCl ₂ (2ug/ml)	NA	Ali and Hashem (2007)
<i>Trichoderma viride</i>	CdSO ₄ (1-200ppm)	Sorption	Simonovicova, Sevc and Iro (2002)
<i>Lentinus edodes</i>	Cd ²⁺	Absorption	Zhang et al. (2013)
<i>Lentinus edodes</i>	CdCl ₂ . 2.5H ₂ O (10 mg/kg)	NA	Ma et al. (2019)
<i>Ganoderma lucidum</i>	NA	NA	Ipeaiyeda, Adenipekun and Oluwole (2020)

NA = Information not available in the cited reference.

5.13 References

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Chapter 6

6.1 General Discussion

This PhD project evaluated the effect of Cd on mycorrhizal (AM) *Populus trichocarpa* when they are in a common mycorrhizal network, and expanded our understanding of ectomycorrhizal fungi and saprotrophic fungi processes under soil contaminated by Cd. However, along the way by doing preliminary tests and with information from the literature, more questions started to emerge, which guided new directions in my research hypothesis. This chapter will cover the overall findings in this thesis, as well as some of the limitations and the future steps that could be investigated in the future for a clear understanding of some new questions about Cd on mycorrhizal.

This thesis has added some insights on phytoremediation issues and a common mycorrhizal network under Cd contamination, in the practical knowledge which is necessary for investigation and provides fundamental knowledge on metal plant uptake. The fundamental question on which the whole thesis was based was, does Cd can be distributed between plants when they are in a common mycorrhizal network (AM)? Once this system is established does it affect the physiology of the plants and fungi?

In the first experiment in the laboratory (Chapter 2), we developed a first screening check on the influence of Cd in carbon enzyme production by four mycorrhizal basidiomycetes. Our hypothesis where Cd toxicity would hinder growth and the activity of four C degrading enzymes in different fungal strains, and these would vary according to their tolerance to Cd stress. The results covered Cd exposure which presented a neutral or positive effect in the biomass of three ECMF strains, while effectively decreasing the growth of FM fungus *A. occidentalis*. All enzyme activities were negatively affected by Cd in *H. subsaponaceum* (3 mg/L), which means that their secretion of carbon-degrading enzymes was more sensitive to Cd under our study conditions. For this strain, the result matched our hypothesis however for the other stains tested (*Hebeloma* sp. (Mycobank no.: 17723), *Scleroderma* sp. (Mycobank no.: 19309), and the FM species *Austroboletus occidentalis* (Mycobank no.: 129957) there was a wide variation of carbon-degradation activities amongst them under Cd stress so the results did not confirm the hypotheses tested.

In chapter 3, our hypotheses were: (1) When mycorrhizal poplars are in co-culture pots, they accumulate less Cd (2) CMN enhances glomalin production when under soil contaminated by Cd; (3) CMN enhances carbon liberating enzymes, under soil with Cd contamination. I showed

that non-mycorrhizal poplars in co-culture pots were able to accumulate more Cd than mycorrhizal poplars. Cd contamination increased the production of GSRP (glomalin) in all mycorrhizal plants, particularly in leeks. Mycorrhizal fungus uses Glomalin as a possible mechanism of blocking toxicity to get into the plant and increased the amount of carbon in the soil (Wang et al., 2017). This finding contributes to the literature by emphasising the potential of phytoremediation by using leek as a support of other species in contaminated areas. However, ideally a similar plant to leek because for phytoremediation is desirable a plant that is not a direct source of food for humans or herbivores (Yadav et al. 2018).

In chapter 4, our hypotheses were (1) CMNs can transfer Cd between poplars; (2) Nutritional concentration of the receiver and the donor plants are different; (3) Plant growth of the receiver and the donor plants are different. We were not able to demonstrate that a common mycorrhizal network can be used to transfer cadmium from one plant of the same species to another. However, this is a new area of study that has been explored by paper research from Ding et al, 2022. That proves that plants in no contaminated soil can have heavy metal within them transported by common mycorrhizal networks.

In chapter 5, presented a comprehensive literature review of various saprotrophic fungi species and an emphasis on the fungal growth pattern under low and high Cd contamination. The goal of this chapter was to provide a deeper understanding of one of the most dominant species in forest ecosystems *Trichoderma asperellum*, *Trichoderma viride*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Lentinus edodes*, *Ganoderma lucidum*, and *Purpureocillium lilacinum* and their responses to environments contamination with Cd. Those organisms play a significant role in environmental remediation, particularly in the context of heavy metal contamination. *Trichoderma viride* and *Ganoderma lucidum* have high tolerance and bioaccumulation capacities, *Purpureocillium lilacinum* and *Mucor circinelloides*, also presented a high Cd tolerance and biosorption potential. All those examples, demonstrate how not just mycorrhizal and ectomycorrhizal have the capacity for soil remediation but also saprotrophic fungi. As they have a wider range of species, there is still a lot to be explore and identified for this matter. There are a few reviews published about it (Baldrian, 2003; Baldrian, 2010; Ceci et al., 2020) however not many for Cd in particular. Those results connect with the other chapter, expanding the knowledge of the potential fungi which present remediation capacity. There are a huge number of possibilities in the environment and the potential of those fungi to minimize the toxicity in plants is still on the horizontal to get to know it dipper.

Here, I integrated the findings of the existing knowledge of this field with the findings of all the experiments and their implications, summarizing with future research directions in this area:

1- *Does cadmium affect carbon enzymes produced by mycorrhizal?*

Basidiomycete have a very important role in the ecosystems, not only as partner for plants enhancing their growth by uptaking carbon in exchange to other nutrients but also as a decomposer. However, it is shown in this thesis that process can be interfered with by cadmium contamination affecting the whole carbon cycle (Chapter 2 and chapter 5), (Dahm and Strzelczyk, 1996). Enzymes that are produced by mycorrhizas, more specifically ECM, such as hydrolytic and oxidative extracellular enzymes which degrade soil organic matter (SOM) enable these fungi to look for N-bearing compounds such as chitin, proteins, and phenol-protein complexes which might be produced in a different fashion once under a cadmium contaminated environment (Phillip et al., 2013). The importance of C cycling by mycorrhizal is also emphasised by glasshouse experiments and field studies suggest that plants allocate between 20%, and sometimes up to 50%, of assimilates to EM fungi (Hobbie and Hobbie, 2008). This, adding the fact that most of all terrestrial ecosystems, and some agricultural ecosystems, are dominated by ECM trees, shrubs and herbs, indicates that mycorrhizal fungi probably play a key role in the global C cycle. Therefore, it would be worthwhile to examine the impact of heavy metal on the fungal ability to produce carbon enzymes.

Enzymes catalyse biochemical reactions and therefore are involved in all biological processes, as well as nutrient cycles. Activities of enzymes such as phosphatases, proteases, cellulases, chitinases and laccase were shown in saprotrophic fungi and in ectomycorrhizal fungi which is likely to contribute not only to the functioning of symbiosis but also to the nutrient mobilisation (Bruns and Shefferson 2004; Hibbett and Matheny 2009). A genome sequencing of mycorrhizal fungi has shown that arbuscular mycorrhizal fungi and ectomycorrhizal fungi have a restricted set of plant-cell wall degrading enzyme genes compared with orchid and ericoid mycorrhizal fungi (Yuhua et al. 2023). Although ectomycorrhizal fungi have a modest ability to decompose organic matter, a restricted supply of plant photosynthates may increase enzyme production for obtaining carbohydrates from soil organic matter (Courty, Breda, & Garbaye, 2007). In chapter 2, the ectomycorrhizal fungi tested were *H. subsaponaceum*, *Hebeloma sp* and *Scleroderma sp*. for B-glucosidase activity, B-D-cellubiosidase activity, B-xylanase and N-acetyl-gucosaminidase activity, all of them responsible for a particular stage of C degradation under Cd stress, the results varied and there are many uncertainties.

All these processes can be affected by heavy metals, particularly with Cd (Phillip et al., 2013). All heavy metal speciation determines its toxicity via bio-mobility and chemical complexation performing in the accumulation, bio-modification and compartmentalization inside the organisms (Beiyuan, et al, 2018). Cd is particularly dangerous because of their toxicity of low levels, their speciation was divided into five forms: exchangeable, carbonate-bounded, iron and manganese oxides-bounded, organic bounded and residual forms (Tessier, Campbell and Bisson, 1979). All those characteristics are connected to soil components, such as pH, cation exchange capacities, hydrous oxides, clay minerals, organic matter etc. Some reports are showing the enzyme activities of extraradical mycelium were more sensitive to Cd stress (Whitfield et al., 2003). In another study, the addition of Cd depressed alkaline phosphatase and succinate dehydrogenase activities, especially of *Glomus intraradices* (Li et al., 2009). In chapter 2, *Hebeloma subsaponaceum* had a decrease in enzyme activity under exposure of 3 mg/L of Cd. In addition, the AM *R. irregularis* in chapter 3, Cd decreased their activity of xylanase by 50%. Xylanases are hemicellulolytic enzymes, which are responsible for the degradation of the heteroxylans constituting the lignocellulosic plant cell wall.

Enzyme activities in soil polluted by metals are greatly affected (Landi et al., 2000). It is confirmed that heavy metals in soils, decrease the microbiological activity of soils and affect root systems (Johnson and Temple, 1964; Adachi and Tainosho, 2004). Yang et al., (2010) and Gunwal et al., (2014) found that heavy metals can inhibit arbuscular mycorrhizal fungi spore germination and hyphal extension in vitro, as well as reduce or eliminate arbuscular mycorrhizal fungi colonisation of plant roots in pot experiment (Audet and Charest, 2007).

Although a great part of the literature surrounding heavy metals describes negative effects on the soil microbiota, some studies have found significant increases in the root colonisation levels and length of extraradical mycelium even at high Cd concentrations (Krishnamoorthy et al., 2015). One of the species tested in the experiment (Chapter 2) *Scleroderma* sp. showed an increase in their biomass when in the presence of Cd, which is reported by another study a similar behaviour for a *Agaricus abruptibulbus* species (Meisch, Schmitt and Scholl, 1979). Chapter 2 also demonstrated that *A. occidentalis* had a significant increase of enzyme, β -glucosidase, when under Cd exposure.

Poplar was the species used in the majority of the experiments presented here in this thesis. That species can establish symbioses with ectomycorrhizal as well as arbuscular mycorrhizal fungi (Gherghel et al., 2014). This species is considered to be superior hardwood trees as a pioneer and as it is known as an important tool for phytoremediation (Robinson et al.

2000). They rely on mycorrhizal symbiosis, which can be extremely important for the establishment of these trees in contaminated soil (Colpaert 2008). As presented in the previous chapters carbon-degrading enzymes can be affected by cadmium toxicity (Da Silva, De Oliveira and Tibbett, 2021), consequently, this can bring to a real scenario of less biomass degradation. It does not mean that will happen in all types of environments because there are so many variables within soil conditions.

Less biomass degradation means less carbon loss. On one hand, it could be beneficial for carbon sequestration in contaminated areas, promoting an accumulation of biomass and carbon pool in soil, however in the other hand other organisms could be affected by the cadmium toxicity and present a different behaviour as suggested by Chen et al., 2014; Landi, Renella, Moreno, Falchini, & Nannipieri, 2000. This means that some organisms could increase their enzyme activity that breaks carbon molecules, so they could lose more carbon in contaminated soil (cadmium contamination). It is a vital subject that will demand more studies to understand deeply the influence of heavy metal (cadmium) in the carbon cycle out of a controlled environment.

2- Common mycorrhizal network as a potential to enhance phytoremediation

Common mycorrhizal networks (CMN) are known as a belowground system of interconnected hyphae and roots, they benefit host plants in many ways and transfer a net flux towards one plant (Selosse et al. 2006). However, there is still much to be found about their potential in the ecosystem as well as their role under extreme circumstances such as contaminated soil by high concentrations of heavy metals.

CMN has shown in these studies a very important behaviour, as not only being able to transfer nutrients between plants but also there is new evidence that plants in common mycorrhizal networks can be a tool for plants to manage the heavy metal content as present here in this thesis and in a different perspective by Ding et al. 2022. This study revealed mycorrhizal networks mediated the migration of Cd from maize to soybean plants, evidenced by decreased Cd contents in donor maize plants, while increased Cd contents in receiver soybean plants. The finding shows mycorrhizal networks mediated transfer of Cd between plants of different species as a promising approach as CMN is a channel to transfer toxic metals from plants to plants through intercropping, consequently minimizing the detrimental effect of metals on plants. This may affect the survival and behaviour of connected plants (Deslippe and Simard, 2011) as well as competitive and cooperative patterns, consequently influencing plant

diversity at local and regional scales (Simard et al., 2012). This is just the start of a discovery that will provide very important findings.

Chapter 3 also shows the benefit of mycorrhizal leeks, even if there are plants for consumption they possess the potential to extract cadmium out of the soil. This is an example that could be used for establishing a phytoremediation system in contaminated areas, for example, Poplars and leeks planted together to improve heavy metal (cadmium) uptake.

The amount of Cd applied in the soil in the present experiments was 27 mg/kg which according to the literature, it is easily handled by *P. trichocarpa*, this species has the potential of phytoextraction (F. Wu, Yang, Zhang, & Zhou, 2010). Consequently, poplar trees in this experiment did not show any toxicity symptoms in their leaves, as the most common symptoms of cadmium phytotoxicity are leaf chlorosis, growth inhibition and necrotic points, especially in the interveinal parts of the leaves (Laspina et al. 2005). Many studies indicated that various *Populus* species could tolerate and accumulate high concentrations of heavy metals such as cadmium (Cd), nickel (Ni), zinc (Zn), copper (Cu) and lead (Pb) in the aboveground tissues (Radojčić Redovniković et al., 2017; Tran and Popoca, 2013). Poplar trees are a great resource for phytoremediation, one of the reasons is that they can have both mycorrhizal roots, ectomycorrhizal and arbuscular mycorrhizae fungi.

6.2 Limitations of the research

This research was developed in the control environment, as well as using an inert substrate (mostly sand and TerraGreen R clay), which had to be autoclaved in order to evaluate the effects of a single mycorrhizal species. The experiments were developed all in the glasshouse and laboratory conditions, due to time constraints we were not able to establish the same experiment on the field. Consequently, in terms of practical application, those results need further complementation.

Experiments in the laboratory with ectomycorrhizal fungi under Cd contamination were done in petri dishes, which was a limitation however the idea was to isolate any variables to analyse enzyme activities. Keeping plants in pots, as discussed in our experiment in chapter 4 the pipe pretends to be a part of the soil, consequently, during the experiments some conditions have changed and do not allow us to analyse as we were expected.

6.3 Future research suggestions

Considering all the findings from this thesis and the unexplored ideas, knowledge gaps, as well as all the limitations from our methodological approach, we have several opportunities for future research.

Explore a wider range of carbon enzymes produced by ectomycorrhizal and arbuscular mycorrhizal fungi, to have a clear idea of the impact of cadmium in this production. Poplar studies in the field or in pots with soil collected from contaminated areas are very important in terms of practical remediation experiments, as tested by Cicutelli et al. (2014). This test would show a better prediction of the potential of *P. trichocarpa* and *A. porrum* when in common mycorrhizal symbioses to extract cadmium from the soil. However, there will have many factors that will need to adjust, as soil pH, soil nutritional status and microbiota, as they affect metal availability (Alloway 2013).

In chapter 4, I could have explored a common mycorrhizal network more in detail having a donor and a receiver, it would be necessary to explore more levels of Cd to verify if under threat (high concentrations of cadmium) condition how the CMN will behave when connecting two plants. One was contaminated and the other one was clean. This result would open up a completely different area of study, it would be so interesting to find out other functions of this fantastic system (CMN).

The quantity of Cd left in the soil and in the medium could have been evaluated. The result would be able to show how much the plant would extract from the soil, as well as how much the ectomycorrhizal fungi would extract from the medium. In Chapter 2, as an additional analysis would be important to quantify the amount of metal (cadmium inside the mycelium) as a way to understand how each species could accumulate (Cd).

GRSP (Glomalin-related soil protein) by arbuscular mycorrhizal are an important factor influencing metal uptake and availability to plant roots. They are glycoprotein called glomalin, which is widely studied for its implications in the C storage, and sequestration of potentially toxic elements such as heavy metals (Rillig et al. 2001; Khan et al. 2014). Evaluating this factor apart from the experiment in chapter 3 (which I have done) would be interesting to determine glomalin concentrations in mycorrhizal poplars in experiment of the chapter 4.

To finalise, the effect of dual symbiosis (ECM + AM) in modulating heavy metal uptake and the effects of multi-contaminated soils in *P. trichocarpa* uptake and tolerance. Exploring

other variables, would be important to understand better the potential of *P. trichocarpa* in phytoremediation.

6.4 Conclusion

Cadmium is highly persistent, toxic and represents one of the most severe environmental problems even in low concentrations it can seriously affect environmental quality and human health (Khalid et al. 2018). 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 extremely important for soil scientists, biologists, mycologists and all specialists in that field to explore even more the potential of this association (plants and fungi) in order to help the environment. 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 common mycorrhizal networks under cadmium contamination. The overall results are useful for better explaining the mechanisms by which mycorrhizal symbiosis can affect Cd uptake in woody plants and highlight the importance of mycorrhizal symbiosis in phytoremediation.

The knowledge gathered and generated in this thesis may serve to refine and enhance this technique. This work will be a small contribution to the understanding of the behaviour of cadmium within common mycorrhizal networks, as well as *P. trichocarpa* potential for phytoremediation.

6.5 References

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