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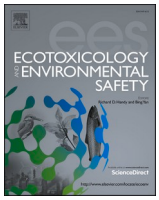

The role of common mycorrhizal networks in mediating cadmium accumulation, glomalin production, and soil enzyme activity in co-cultures of poplars and leeks

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ABSTRACT

Cadmium (Cd(II) ion) is a mobile metal, that can be toxic to plants and microorganisms, yet its effects on common mycorrhizal networks (CMNs) are seldom explored. Arbuscular mycorrhizal fungi (AMF) can improve Cd tolerance in plants by mediating uptake, sequestering Cd into hyphae and/or in exogenous glomalin proteins. Here, we examined how the AM fungus *Rhizophagus irregularis* affects Cd accumulation, glomalin production and microbial enzyme activity in single plant cultures and in interspecific co-culture conditions (CMN). A glasshouse experiment with *Populus trichocarpa* (poplars) and *Allium porrum* (leeks) was conducted including three factors: Contamination (Control vs Cd(II), 27 mg kg^{-1}), Mycorrhization (non-mycorrhizal vs AM) and Culture type (single vs co-culture, 1 poplar + 10 leek seedlings). We assessed biomass, Cd(II) uptake, glomalin concentration and carbon cycling enzyme activities. Poplar biomass was unaffected by Cd, and mycorrhization reduced foliar Cd by 34 %. Root Cd accumulation was highest in non-mycorrhizal poplars when co-cultured with leeks (102 mg kg^{-1}), suggesting increased Cd mobility from root exudation and acidification due to having multiple plants. However, root Cd decreased by 64 % under CMN, possibly because of hyphal binding and glomalin production, but we also propose this could be due to reduced exudation caused by AMF. Cd stimulated glomalin production, sometimes by 25-fold compared to controls, but had little effect on the studied soil enzymes. We conclude that i) Cd stimulates glomalin production in mycorrhizal plants, and ii) Non-mycorrhizal co-culture between leeks and poplars enhances Cd accumulation in poplar roots, but this effect is mitigated by CMNs.

1. Introduction

Soils and natural systems are under constant threat due to inputs of contaminants by anthropogenic activities such as urbanisation, mining, sewage discharges, waste disposal, and other industrial processes (Alloway, 2013). Unlike organic contaminants, heavy metals are not biodegradable and can persist in the environment, posing a long-term toxicity risk. Cadmium (Cd(II) ions) in particular is a non-essential metal that can be highly toxic to biota even at low concentrations in comparison to other metals such as lead (Pb(II)), zinc (Zn(II)), and nickel (Ni(II)) (Alloway, 2013). In addition to its toxicity, Cd is highly mobile in soils, being easily leached towards the groundwater or taken up by plant roots, transferred to vegetative parts, and ultimately entering the food chain (Tibbett et al., 2021).

Cadmium entry in plants occurs via different metal transporters in roots as the divalent cation Cd(II), by trans-membrane carriers responsible for the uptake of Zn(II), magnesium (Mg(II)), calcium (Ca(II)), iron (Fe(II)), and copper (Cu(II)) (Zulfiqar et al., 2022). This process is further enhanced by root exudation, which lowers soil pH, increasing Cd (II) availability in the rhizosphere (Tibbett et al., 2021; De Oliveira et al., 2016). Phytotoxicity of Cd in plants usually occurs at foliar concentrations above 10 mg kg^{-1} (Gallego et al., 2012). Cd stress hinders seed germination and plant growth, usually by causing oxidative damage in cell membranes, inactivating enzymes by ion substitution, and competing with other divalent nutrients, causing leaf chlorosis and tissue necrosis (Zulfiqar et al., 2022). Soil microorganisms are also sensitive to Cd exposure, which can decrease microbial biomass, as well as the activity of enzymes involved in organic matter and nutrient cycling,

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such as β-glucosidase and phosphatase (Wu et al., 2018; Silva et al., 2021).

Notwithstanding, some soil microorganisms can endure different levels of Cd exposure, and even improve plant tolerance to contamination, such as the case of arbuscular mycorrhizal fungi (AMF). Most terrestrial plants form a symbiotic association with AMF, these fungi are obligate symbionts and rely on photosynthates provided by the host plant (e.g. carbohydrates and lipids), usually provided in exchange for mineral nutrients such as Zn and phosphorus (P), as the extra-radical hyphae enhance soil exploration and access to nutrient sources (Smith and Smith, 2011). Studies have shown that inoculation of plants with AMF can enhance Cd immobilisation in plant roots, decreasing its translocation to aerial parts and reducing its toxicity (De Oliveira et al., 2020). The arbuscular mycorrhizal (AM) effect in Cd tolerance has been attributed to several mechanisms, such as improved plant nutrition and fitness, enhanced expression of metal detoxifying genes in the host, and the binding of Cd(II) ions in fungal cell walls, restricting its movement into the host (Gao et al., 2023; Kaur et al., 2023).

Another potential mechanism by which AMF can improve plant tolerance to Cd and other metals is through the release of glomalin proteins, also referred to as Glomalin-Related Soil Proteins (GRSP) (González-Chávez et al., 2004; Rillig, 2004). These GRSP are highly recalcitrant and water-insoluble glycoproteins produced by AMF hyphae and spores which, besides increasing soil aggregation and carbon storage, are known to bind metals reducing their availability for plant uptake (De Oliveira et al., 2016; Kaur et al., 2023; Bisht and Garg, 2022). Higher GRSP release has been observed in Cd-contaminated soils (Babadi et al., 2019), and more recent findings suggest GRSP may also function as a way of excreting heavy metals out of contaminated plants (Salazar et al., 2024). The release of GRSP by AMF hyphae, as well as the allocation of carbohydrates from the plant to the fungus generates a sink for carbon (C) in soils, stimulating microbial activity, and consequently the activities of nutrient and C-cycling enzymes (Xu et al., 2018; Agnihotri et al., 2022). In this sense, soil enzyme activities are often used as sensitive indicators of the effects of contaminants in soils, including Cd (Liu et al., 2024). Because Cd can reduce microbial diversity, biomass and activity (Silva et al., 2021; Wang et al., 2024), it is likely that the presence of AMF and release of GRSP attenuate this effect.

Arbuscular mycorrhizal fungi have an almost unrestricted range of host plants with which they can establish the symbiosis, allowing their extraradical hyphae to colonise multiple plants at the same time and fuse with hyphae from separate mycelia (self-anastomosis or with different AMF isolates), creating what is known as a common mycorrhizal network (CMN) (Giovannetti et al., 2004; Mikkelsen et al., 2008). These CMNs can interconnect multiple plant species and individuals simultaneously, creating a complex fungal system with significant influences on plant community composition and ecosystem productivity (Bever et al., 2010; Tedersoo et al., 2020). Among their reported effects, CMNs can allow plant communication, improve mineral nutrition and nutrient exchange, alter microbial composition, and improve seedling establishment (Figueiredo et al., 2021). The term CMN has been generally accepted as referring to any situation where one or more mycorrhizal fungi interact with two or more root systems, regardless of the degree of connectivity or implied function (Rillig et al., 2024), therefore here we will use the term CMN within this context, e.g. not requiring the verification of direct hyphal links between host plants.

CMNs are generally considered beneficial to the fungal partners due to having multiple sources of C that can be allocated towards fungal growth [27. 28]. However, to the interconnected plants, CMNs may have positive, neutral, or negative effects on the competitiveness between individuals and their growth (Weremijewicz et al., 2018; Awaydul et al., 2019). For instance, nutrients may be unevenly distributed between different plant species under CMNs, where nutrient transporters can be differentially expressed in the periarbuscular space (where exchange occurs) favouring a particular host plant (Walder et al., 2016). Alternatively, by allowing more access to soil nutrients and water via extraradical hyphae, symbiosis with AMF may ease plant-plant competition, providing nutrients to young seedlings unable to invest much C into the association, or by redistributing nutrients (e.g. P and N) between connected plants (Pickles et al., 2017; Wagg et al., 2015). Moreover, recent findings indicate that CMNs are able to transfer metals such as Cd between the connected plants (Ding et al., 2022), which may affect plant competition and metal accumulation.

A useful way of studying the effects of AMF under CMNs on both host and non-host plants is through experiments using co-culture systems, where different plant species grow together sharing the same resources. These systems may feature plants that are all able to form AMF associations, or only one mycorrhizal host. In a co-culture system between a mycorrhizal and a non-host plant species, where roots were kept separate by a mesh but not AMF hyphae, Mnasri et al. (2017) showed that inoculation of the mycorrhizal host enhanced Cd uptake in the neighbouring non-host plant. This suggests that the presence of AMF extraradical hyphae plays a significant role in heavy metal uptake in the surrounding root systems, even those of non-host plants.

Different species will have varying root exudation profiles and recruitment of microbial communities, which may result in pH shifts affecting Cd uptake and/or tolerance in plants (Song et al., 2024). For instance, *Beta vulgaris* had a significant decrease in root exudation of malate, citrate and oxalate when in co-cultures with mycorrhizal *Hordeum vulgare*, affecting mineral uptake (Hajiboland et al., 2020). Chen et al. (2019) observed that AMF inoculation increased the relative abundance of Actinobacteria in the rhizosphere of rice plants, a bacteria group containing species that absorb Cd(II) from aqueous solutions. Thus, it is likely that co-cultivation may indirectly modulate Cd uptake and tolerance in plants under CMNs.

Compared to one single mycorrhizal individual, multiple plants connected via CMNs may have differential metal accumulation, tolerance, and competition dynamics in contaminated soils (Wang, 2017). Here, we investigated the effects of AMF (single cultures) and CMNs (co-cultures) in providing Cd tolerance and improving root Cd immobilisation in two co-cultivated species: *Populus trichocarpa* (Salicaceae, poplar), a tree species that is known to tolerate Cd(II) excess and is often considered for phytoremediation schemes (De Oliveira and Tibbett, 2018), and *Allium porrum* (Amaryllidaceae, leeks), a food crop that is able to accumulate high concentrations of metals, including Cd (Soudek et al., 2009).

Our general hypothesis is that there is a beneficial effect of AMF to plants growing under Cd(II) contamination, with Cd accumulation occurring in the following order: CMN (mycorrhizal co-cultures) < AM (mycorrhizal single cultures) < NM (non-mycorrhizal cultures). The specific hypotheses that we tested were: i) mycorrhizal plants present overall higher biomass than non-mycorrhizal plants under Cd exposure; ii) poplars under CMN with leeks have lower Cd uptake compared to either single mycorrhizal or non-mycorrhizal poplars; iii) due to being connected to multiple plant hosts (i.e., carbon sources), CMN release more GRSP than single AMF cultures, which possibly decreases Cd uptake; and iv) Cd(II) contamination decreases the activity of soil enzymes, an effect alleviated by mycorrhization.

2. Materials and methods

2.1. Experimental design, plant growth and inoculation

The growth substrate used in the experiment was comprised of washed sand and TerraGreen® clay (American Granules Plain, UK) (4:1 w/w) with a total of 3 kg per pot, all autoclaved twice (121 °C, 1 h). The experiment was conducted in glasshouse conditions using two plant species: *Populus trichocarpa* v. Trichobel (black cottonwood poplar) and *Allium porrum* (leek), Musselburgh variety. *P. trichocarpa* cuttings were acquired from Nicky's Nursery, England, UK and were chosen for being a well-known species tolerant to high Cd concentrations (De Oliveira and Tibbett, 2018). *A. porrum* is a crop species that is very responsive to

mycorrhization (Eason et al., 1999), with a unique root structure, facilitating the discrimination between the root systems of the two plant species during harvest. The *P. trichocarpa* cuttings (15 cm, two nodes) were rooted in water for a week before transplanting into the pots, while leeks were germinated directly from seeds.

The experiment was set up in a factorial design, with three factors: i) Cd (2-levels: contamination vs control); ii) inoculation (2-levels: non-mycorrhizal (NM) vs arbuscular mycorrhizal (AM); and iii) cultivation (3-levels: single poplars, single leeks, and co-cultures. This provided 12 different treatments, each with five replicates (Fig. 1). All AM co-culture conditions were considered to have formed CMNs. To inoculate AM treatments, we added 300 g of substrate from a pot culture of mycorrhizal *Plantago lanceolata* colonised only by the AM fungus *Rhizophagus irregularis*. Inoculum contained fungal propagules, spores and root fragments, while NM treatments received the same amount of sterile (autoclaved) inoculum. Single poplar cultures had one cutting per pot, and single leek cultures had 10 seeds sown per pot (as we anticipated lower biomass from leeks); co-cultures contained one poplar cutting in the centre, with 10 leek seeds sown around it. Cadmium (Cd(II)) was applied gradually via $CdCl_2$ solution (10 mL) over three consecutive days, each application was made to reach 9 mg kg^{-1} Cd, totalling 27 mg kg^{-1} Cd (De Oliveira et al., 2020); control plants received only deionised water.

During plant growth, 10 mL of a modified Long Ashton's solution were applied once a month, comprising of: $(NH_4)_2SO_4$ (4 mM), K_2SO_4 (2 mM), $CaCl_2{\cdot}2H_2O$ (3 mM), $MgSO_4{\cdot}7H_2O$ (1.5 mM), $NaNO_3$ (8 mM), FeEDTA (0.1 mM), H_3BO_3 (2.86 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (1.81 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg L^{-1}), $NaMoO_4{\cdot}2H_2O$ (0.025 mg L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg L^{-1})), according to Kariman et al. (2014). Phosphate was not added to avoid hindering AMF colonisation levels (De Oliveira et al., 2023). Plants were watered three days a week to reach 70 % of soil water capacity. The average temperature during this period was 26 °C.

After three months, plants were harvested, and poplars were split into leaves, stems, and roots, while due to their morphology, leeks were split into shoots and roots. For the co-culture treatments, leeks were too small to split meaningfully, therefore dry weight of the entire seedling was measured. Roots were rinsed with distilled water and immersed a solution of 0.05 mM $CaCl_2$ for 30 min to remove surface adhering metals (De Oliveira and Tibbett, 2018). A small sample of all roots was taken for mycorrhizal scoring (3 cm fragments of secondary roots), enough to fill half of an Eppendorf tube (1.5 mL). All plant material was then dried in an oven at 70 °C for five days before weighing (dry weight).

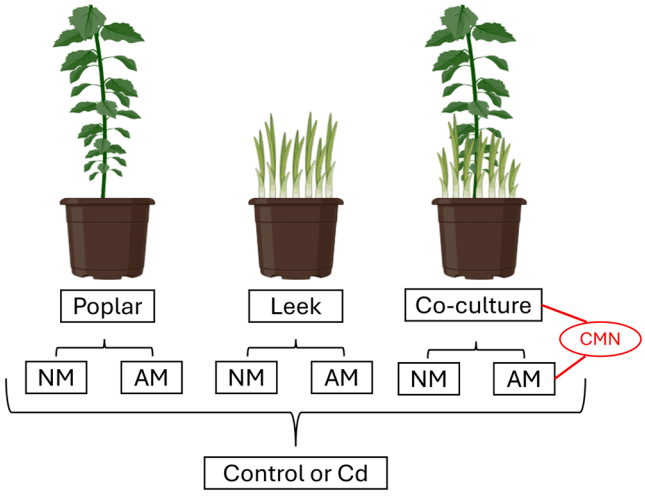

Fig. 1. Schematic representation of the factorial design with three factors; i) Cultivation: single poplars, single leeks or co-cultures; ii) Inoculation: non-mycorrhizal (NM) or mycorrhizal (AM) plants; iii) Cd(II): control or with Cd addition. The "Co-culture" + "AM" condition is referred to as "CMN" (common mycorrhizal network) throughout the text.

2.2. Plant digestion and elemental determination

The dried plant biomass was ground into a fine powder (Cyclone Mill, 1.0-mm sieve) and 50 mg of each sample was digested in 5 mL of 70 % HNO_3 (≥ 69 % TraceSELECT® for trace analysis) in capped glass tubes in a heating block at 110 °C for 8 h (De Oliveira and Tibbett, 2018), with two technical replicates. For quality control, a blank sample and a plant certified reference material were included (IAEA-359 cabbage leaves). Digested extracts were diluted with 2 % HNO_3 (+ 5 ppb Rh), and filtered in ashless filter paper. The Cd and other element concentrations were determined by ICP-MS (Thermo Scientific™ iCAP™ Q ICP-MS). Due to very low biomass production, we were not able to determine Cd concentrations in leek plants grown in co-cultures.

Fresh samples were dried at 75 °C for 10 h in an oven and then ground with a Cyclone Mill to pass through a 1.0-mm sieve. One gram of ground sample was ashed in a CMF (conventional muffle furnace) for 16 h at 450 °C. The same sample was digested in a CEM MAS-300 microwave for 20 and 40 min 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.

2.3. Mycorrhizal colonisation

Root sub-samples were cleared in KOH solution (10 % w/v) at room temperature for 10 days, and stained with a 5 % (v/v) black ink vinegar solution (Vierheilig et al., 1998) for 1 h before being washed and transferred to a solution of lactoglycerol for long term storage. Colonisation scoring was done by the line intercept method, in which the presence of either hyphae, arbuscules, or vesicles was considered as evidence of mycorrhiza colonisation (Giovannetti and Mosse, 1980).

2.4. Activity of hydrolytic (C-cycling) enzymes

The activity of four soil enzymes involved in C-cycling (organic matter degradation) were determined using different fluorogenically labelled substrates, based on 4-methylumbelliferone (MUB) (Courty et al., 2005). They were: MUB-β-D-cellobioside for β-D-cellobiosidase (CB, at 100 mg L^{-1}), MUB-β-D-xylopyranoside for β-xylosidase (BX, at 61.7 mg L^{-1}), MUB-N-acetyl-β-D-glucosaminide for N-acetyl-β-D-glucosaminidase (NAG, at 75.9 mg L^{-1}), MUB-β-D glucopyranoside for β-glucosidase (BG, at 67.7 mg L^{-1}). MUB solutions were adjusted to pH 5.5 using 1.0 M HCl and 1.0 M NaOH. Table S1 provides information on enzyme nomenclature and their functions. A modified universal buffer solution was prepared according to Turner (2010).

Liquid suspensions were prepared by mixing 2.75 g of plant growth substrate with 92 mL of buffer solution and pipetted (100 μL) into a 96-well microplate containing 100 μL of the respective enzyme substrate (Silva et al., 2021). Plates were incubated in the dark for 1.5 h at 35 °C, and fluorescence was measured immediately after on a multi-detection plate reader SpetraMax i3x (Molecular Devices, Sunnyvale, CA, USA), with excitation wavelength of 360 nm and an emission wavelength of 450 nm (Courty et al., 2005). All enzyme activities were expressed as μmol activity per gram of dry fungi per 1.5 h (nmol 1.5 h^{-1} g DW^{-1})

2.5. Glomalin-related soil proteins (GRSP)

GRSPs (Rillig, 2004), were extracted from substrate subsamples (four per pot) as easily extractable GRSP (EEG) 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. After each

autoclaving cycle the supernatant was removed by centrifugation at 500 rpm for 20 min and stored. Extracts centrifuged at 10,000 rpm for 10 min to remove soil particles and then analysed. The protein content in the supernatant was determined by the Bradford assay with bovine serum albumin as the standard. The concentration of GRSP was extrapolated to μg g^{-1} by correcting for dry weight of coarse fragments included in the soil extraction.

2.6. Statistical analyses

Analyses were performed using R software version 4.3.1 (R Core Team, 2023). When all assumptions were met, ANOVA was performed (three-way or two-way, depending on the factors included), followed by a post-hoc Tukey test to assess the significance of pairwise differences ($p < 0.05$). If data had homogeneous variances (Levene test, $p > 0.05$), but residuals were not normally distributed (Shapiro-Wilk test, $p < 0.05$), variables were transformed by log(x) or sqrt(x). Principal component analysis (PCA) was performed with Primer-e v7 software (with PERMANOVA+ add-on), and Three-way PERMANOVA ($p < 0.05$) was carried out using 26 variables (Table S2) to detect multivariate distances between treatments (12,000 permutations for main and pair-wise effects; Euclidean distances); variables were transformed by sqrt(x) and z-normalised prior to analyses (Anderson et al., 2008; Dago et al., 2021). However, the PCA figure was created using only seven variables, as they were the ones that most contributed to explaining the overall variability, allowing better visualisation of the data and removal of strongly correlated vectors.

Results are presented as two separate sets of analyses, addressing the effects of different treatments on plants and soils (substrate) (Fig. S1).

3. Results

3.1. Plant responses

3.1.1. Colonisation and biomass production

In both species, colonisation by *R. irregularis* was consistently between 40 % and 60 %, with no significant effects from being in single or co-cultures, or from Cd exposure (Fig. S2). No colonisation was detected in roots from non-inoculated pots (NM), and this treatment was not included in the ANOVA.

Leaf dry weight was unaffected by Cd exposure, mycorrhization, or culture type in poplars (Fig. 2a), as was the case for stem or total dry weight (Fig. S3). Poplar roots, however, exhibited overall 27 % lower biomass when under AM symbiosis, regardless of contamination or culture type (Fig. 2b).

In leeks, AM plants were significantly smaller than NM, but only in non-contaminated soils (Fig. 2c). However, the strongest effect was from the co-culture system. When leek seedlings were grown surrounding a poplar plant, their biomass was substantially lower, from an average of 1.5 g in single cultures (no poplars), to only 0.2 g in co-cultures, an 97 % reduction in total dry weight. Even though there were 10 leek seedlings in each pot, the total biomass was too low for Cd determination. Due to the very small size of co-cultured leeks (Fig. 2c), we were unable to split shoots from roots accurately, therefore we opted to present and analyse these results as Total Dry Weight (shoots + roots), to allow direct comparisons with single leeks.

3.1.2. Cadmium accumulation

Foliar Cd concentration was generally below 1 mg kg^{-1} in poplars from non-contaminated pots, but significantly higher under Cd(II) contamination, ranging from 3.5 to 5.7 mg kg^{-1} (Fig. 3a), representing an increase of 350 % in NM poplar shoots and 295 % in AM poplar shoots. There was a significant effect of mycorrhization in restricting foliar Cd accumulation, since AM poplars exhibited on average 33 % lower Cd than NM poplars (Fig. 3a). In roots of poplars growing in single culture contaminated pots, no difference was found between NM and mycorrhizal plants, with Cd concentrations averaging between 30 and 34 mg kg^{-1}. In co-culture with leeks, however, NM poplar roots had significantly higher Cd accumulation (102 mg kg^{-1}; Fig. 3b), a 200 % increase compared to single NM poplar roots. However, this effect was greatly attenuated when these co-cultures were under CMN, and Cd concentrations in poplar roots were of 37 mg kg^{-1} (Fig. 3b), representing only a 9 % increase compared to single AM poplars. Total Cd contents in poplars (in μg per plant) followed the same pattern (Fig. S4).

In leeks growing as single cultures, Cd concentrations were around 1 mg kg^{-1} in both shoots and roots under non-contaminated conditions (Figs. 3c and 3d), similar to the concentrations found in non-contaminated poplars (Figs. 3a, 3b). Under Cd exposure, however, Cd concentrations in shoots increased by 2300 % in AM leeks (18 mg kg^{-1}) and by 770 % in NM leeks (10 mg kg^{-1}), however root Cd remained similar (Figs. 3c, 3d); overall, these results suggest that higher Cd translocation occurred AM leeks compared to NM.

3.2. Soil responses

3.2.1. GRSP

The concentration of GRSP was assessed in all pots, but because they were not detected in NM treatments, we chose to perform the analyses only in the mycorrhizal pots, to better detect significant effects from culture types and Cd(II) contamination. Indeed, it was clear that Cd(II)

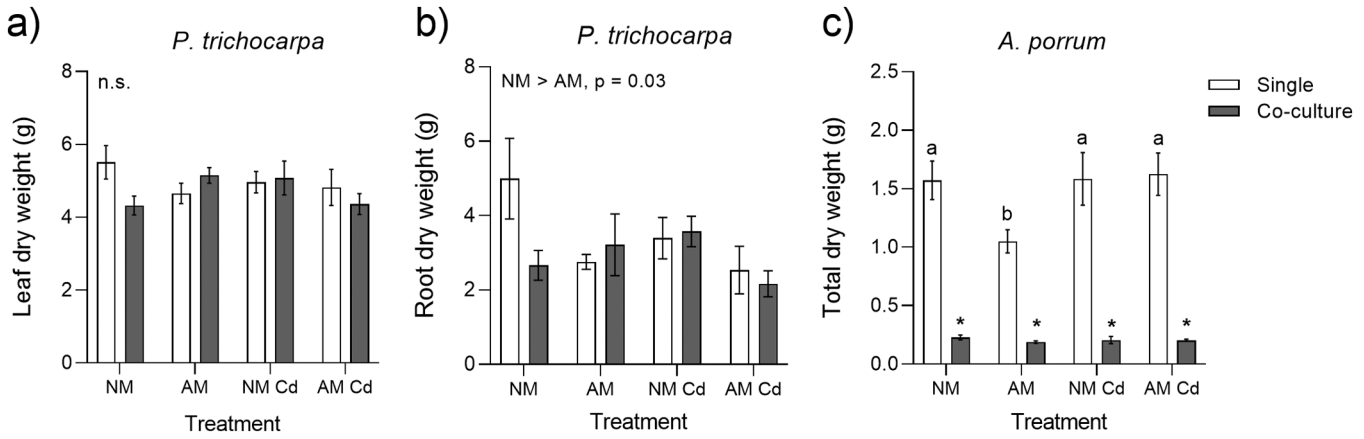

Fig. 2. Dry biomass of poplars – leaves (a) and roots (b) – and leek plants – total dry weight (c), in single or co-cultures, with (AM) and without (NM) mycorrhizal symbiosis or Cd(II) contamination. Bars represent the mean (n = 4) and standard error. Different letters represent significant differences between treatments and asterisks represent differences between culture types. n.s.: no significant differences.

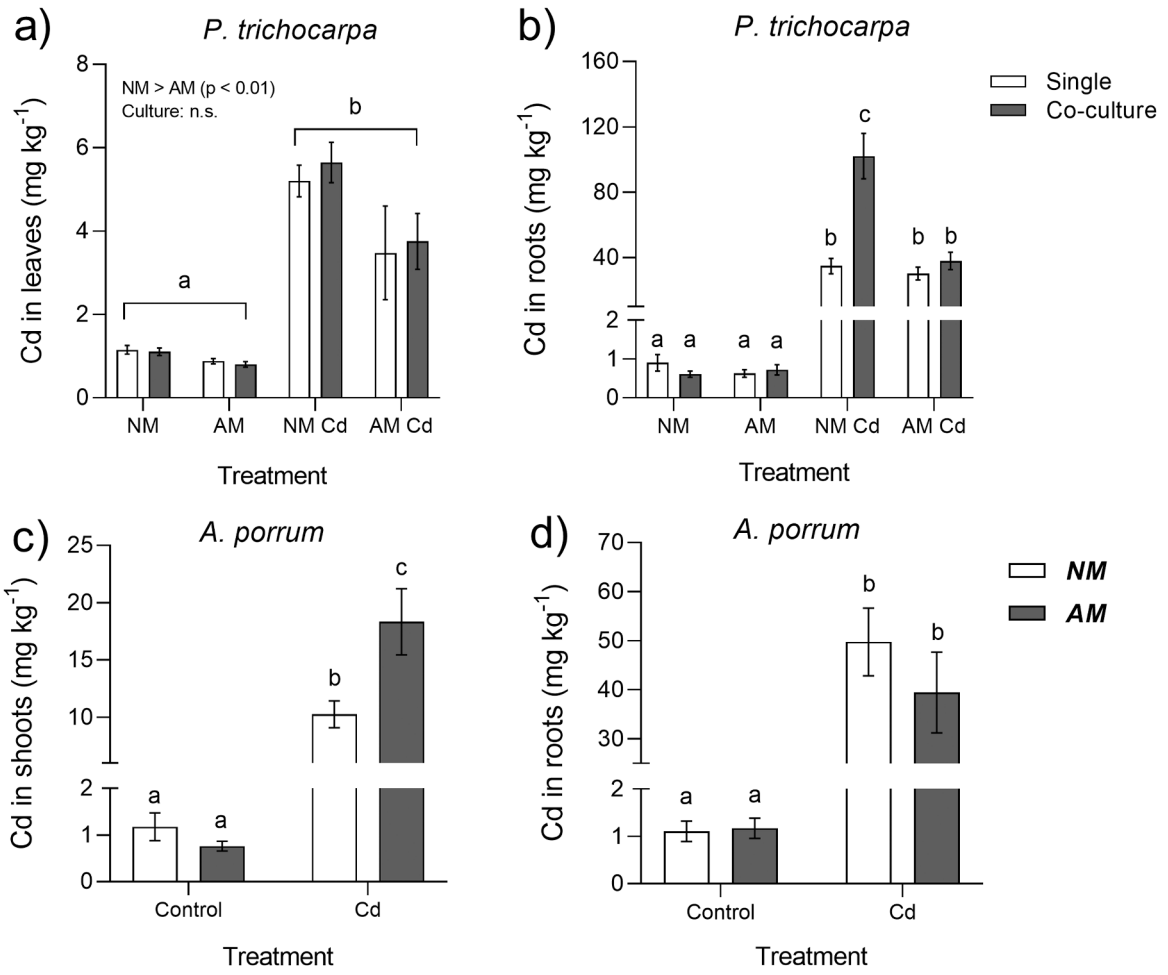

Fig. 3. Cadmium concentration in leaves (a) and roots (b) of poplar plants in single or co-culture with leeks; with (AM) and without (NM) mycorrhizal symbiosis or Cd(II) contamination. Figures (c) and (d) show Cd concentration in shoots and roots of leeks in single cultures. Bars represent the mean (n = 5) and standard error. Different letters represent significant differences between treatments (ANOVA; Tukey test, $p < 0.05$).

exposure had a significant effect in enhancing GRSP production (Fig. 4). Moreover, different cultures produced different amounts of GRSP: single AM leeks produced the highest amount of GRSP (70 μg g^{-1}), almost triple that of single AM poplars (25 μg g^{-1}). In co-culture pots (1 poplar + 10 leeks), GRSP was 36 % higher than in single poplars (34 μg g^{-1}), but still half of that in single leeks. It is worth mentioning that in co-cultures, leek roots comprised less than 7 % of the combined root biomass (poplar + leeks); yet they still significantly enhanced the GRSP pool in those pots by 36 % compared to poplar alone.

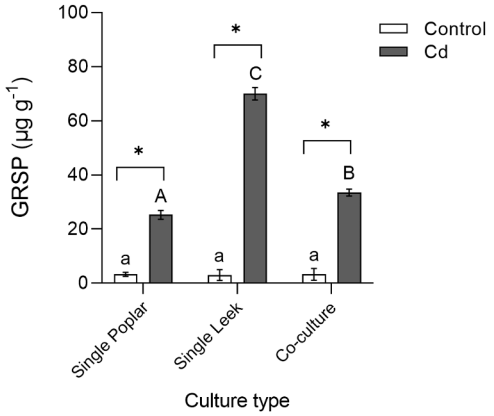

Fig. 4. Concentration of easily extractable glomalin-related soil proteins (GRSP) from mycorrhizal pots, with or without Cd(II) contamination, under different culture types: single poplars, single leeks or co-cultures. Bars represent the mean (n = 4) and standard error. Different lowercase letters represent significant differences between culture types in Control pots and uppercase between Cd pots. Asterisks denote Cd effects within each culture type (ANOVA and Tukey test, $p < 0.05$).

3.2.2. C-cycling enzyme activities

Overall, the activity of all enzymes assessed here were mostly altered in pots from single AM leeks under no contamination (Fig. 5), where activities of BG, CB and BX were the highest; BG activity was also higher in CMN pots, but only under Cd exposure. Overall, BX was the only enzyme to be generally impacted by Cd(II) contamination (main effect) (Fig. S5). Enzyme activities from pots containing poplars were little affected by Cd or any other treatments.

3.3. Multivariate analysis

The PCA containing seven variables from both plant and soil parameters was able to explain 71.8 % of the data variability (Fig. 6). A clear separation between inoculation and Cd treatments is observed, with plants under contamination placed on the right, associated with high Leaf and Root Cd concentrations, and control plants on the left, associated with higher enzymatic activities, represented by BG and XYL (Fig. 6). A negative relationship is clear between plant Cd concentrations and enzyme activities, as well as between root colonisation and leaf P

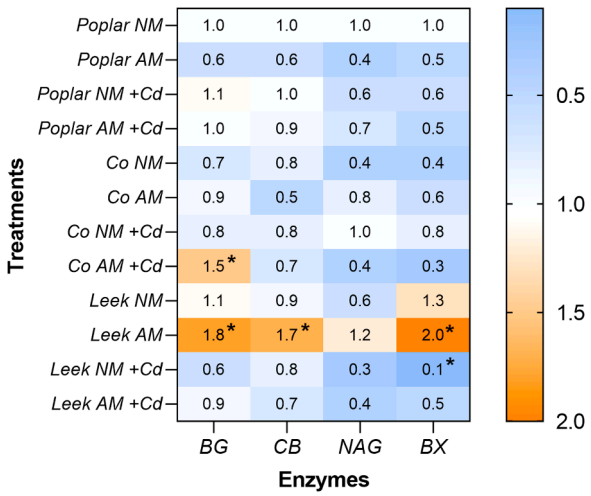

Fig. 5. Heatmap representing the variation in activities of four C-cycling enzymes isolated from poplar and leek pots, in single or co-cultures, with (AM) or without (NM) mycorrhizal symbiosis, and under control or Cd(II) contamination; Values are fold-changes considering Poplar NM pots as the baseline (1.0). BG: β-glucosidase, CB: β-D-cellobiosidase, NAG: N-acetyl-β-D-glucosaminidase, BX: β-xylosidase. Asterisks represent significant fold-changes (ANOVA and Tukey test, $p < 0.05$).

concentrations. Colonisation rates and glomalin concentrations are also associated.

PERMANOVA pairwise comparisons showed that the response patterns between single NM poplars and co-cultured NM poplars were significantly different ($p < 0.05$); however poplars under CMNs (co-culture) had similar responses to single AM poplars. It is worth noting that the PCA for PC1 and PC3 (58.5 %) shows a positive relationship between GRSP and Leaf Cd (Fig. S6).

4. Discussion

4.1. Plant responses

4.1.1. AMF and Co-culture conditions affect plant biomass but not Cd

All inoculated plants were successfully colonised by *R. irregularis* at rates between 40 % and 60 % on average, regardless of treatments or plant species. These figures are consistent with results reported for *P. trichocarpa* plants under Cd stress (at 81 mg kg^{-1}), with on average of 50 % colonisation by *R. irregularis*, unaffected by Cd (De Oliveira et al., 2020). Colonisation percentages of leek roots were also similar to the 50 % found by Mozafar et al (Mozafar et al., 2002)., in plants growing under metal polluted soils. However, colonisation response to Cd depends on multiple factors such as concentration, exposure time and species involved, for instance in *Cajanus cajan* (Fabaceae, pigeonpea) had a 10 % decrease in *R. irregularis* colonisation at 25 mg kg^{-1} Cd (Bisht and Garg, 2022).

Leaf biomass of poplar plants was unaffected by this level of Cd(II) contamination, or by any other applied treatments. This reflects the tolerance of *P. trichocarpa* to Cd in soils, which has been associated with modulation of Cd uptake and translocation by the plant via the *HMA4* transporter (De Oliveira and Tibbett, 2018). Therefore, in terms of biomass, there were no positive effects from AMF inoculation, or CMN conditions. An overall decrease in root biomass, however, was observed in inoculated plants compared to NM plants. Changes in biomass allocation between roots and shoots are often observed in AMF-inoculated plants, by relying on extra-radical hyphae for nutrient acquisition, C investment into photosynthesising tissues in detriment to root growth is not uncommon (Veresoglou et al., 2012). Moreover, it is important to mention that despite no plants being P deficient, mycorrhizal poplars, in general, presented lower P concentrations in leaves than NM poplars (Fig. S7a). This suggests that colonisation by *R. irregularis* may have hindered P uptake in poplars; when soil P is not at an optimum level - such as in the present work - AMF will still down-regulate the plant's

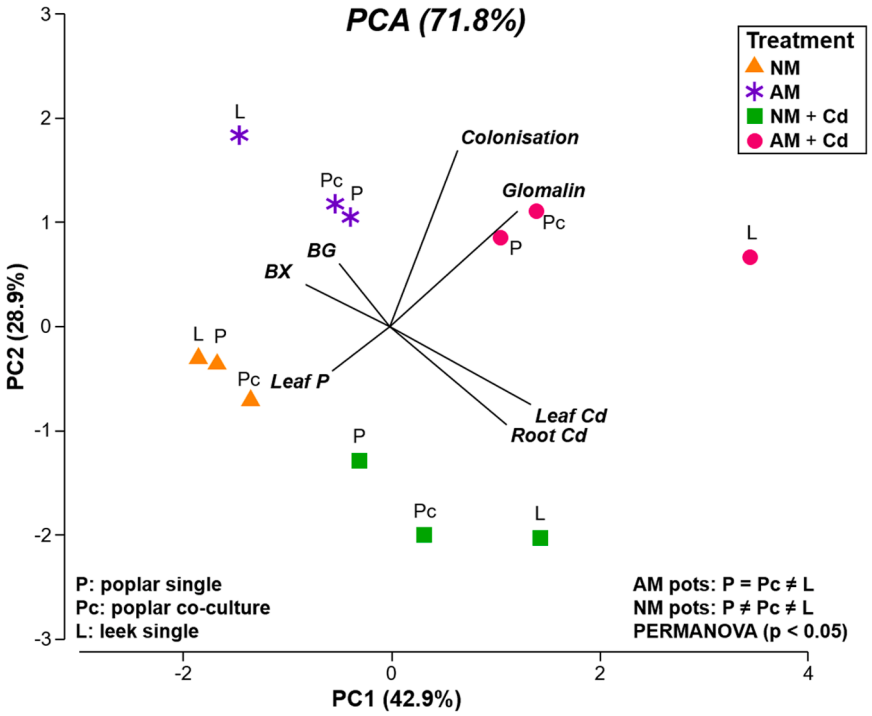

Fig. 6. Principal component analysis (PCA) using seven variables (plant and soil responses). Icons represent the averages of three replicates. NM: non-mycorrhizal plants, AM: mycorrhizal plants, NM + Cd: non-mycorrhizal under Cd(II) exposure, AM + Cd: mycorrhizal plants under Cd(II) exposure. L: single leeks, P: single poplars, Pc: poplars in co-cultures. Variables: Leaf P, Cd and Root Cd: element concentration in leaves/shoots and roots; BG: β-glucosidase activity, BX: β-xylosidase activity; Glomalin: GRSP concentration in substrates. PERMANOVA ($p < 0.05$).

direct P uptake pathway (root P transporters) in favour of the mycorrhizal pathway (hyphal uptake and delivery), but will be unable to deliver adequate nutrition (Bulgarelli et al., 2020). Low nutrient uptake in mycorrhizal plans, may explain the lack of growth promotion in these treatments.

Similarly, single mycorrhizal leeks had an overall lower biomass compared to NM plants, which may also be attributed to the factors mentioned above. Like poplars, leeks were not affected by Cd exposure, exhibiting remarkable tolerance to this metal (Soudek et al., 2009), but seedlings did suffer when in co-cultures with poplars, exhibiting an 86 % decrease in total biomass. *P. trichocarpa* trees are fast-growing pioneer species with strong competitive traits (De Oliveira et al., 2020), therefore its development likely hindered leek growth due to competition for water and nutrient resources. Co-cultivation of the tree *Salix viminalis* (Salicaceae, willow) with the herbaceous *Trifolium repens* (Fabaceae, clover) in contaminated soil, showed the same pattern as we observed here, no effects on the tree species, but a significant decreased in *T. repens* biomass (Nandillon et al., 2022). Moreover, as in Nandillon et al. (2022), the tree species was grown from cuttings, while the co-cultured species grew from seeds, probably giving an advantage towards the tree species. Therefore, the CMN condition did not ease plant-plant competition, as is sometimes described (Wagg et al., 2015), and poplars entirely dominated in co-culture pots, especially considering the confined space of 3 kg of substrate.

Interestingly, single AM leeks had larger biomass under Cd(II) contamination, than in controls; this suggests a positive influence of Cd exposure in plant development, perhaps via a hormesis effect (Silva et al., 2021), or by enhanced nitrogen nutrition in leeks (Fig. S8). There is a clear relationship between Cd and N uptake in plants, in which N fertilisers are known to often increase Cd accumulation by upregulating low-affinity NO_3 transporters (Yang et al., 2020). Here we see that the opposite may also occur, with Cd enhancing enhance N nutrition. Although seldom reported, higher N accumulation in rice shoots has been observed under 11 mg L^{-1} Cd (Yang et al., 2016).

4.1.2. CMN restricts Cd accumulation in poplars

In poplar leaves, Cd concentration was significantly lower in AM plants than NM plants as we initially hypothesised, however, poplars under CMN with leeks did not accumulate less Cd compared to single AM poplars. Although not consistent, restriction in plant Cd uptake due to mycorrhizal fungi has been observed (De Oliveira et al., 2020). This response has been attributed to several mechanisms, such as: i) Cd binding into fungal structures (e.g. vesicles, hyphae, spores), ii) enhanced host synthesis of chelating molecules (e.g. phytochelatins, metallothioneins), and iii) release of GRSP proteins into the soil (De Oliveira et al., 2020; Gao et al., 2023; Bisht and Garg, 2022). However, it seems unlikely that GRSP played a key role in Cd immobilisation here, since single AM leeks had significantly higher Cd in their shoots compared to NM leeks, despite presenting the highest levels of GRSP in their pots (Fig. 4). These results exemplify how mycorrhizal influence on Cd uptake is variable, depending on different factors such as metal availability in soils, plant transpiration rates (De Oliveira et al., 2020), and host-AMF pairings. In *Zea mays*, for instance, Aghababaei et al. (2014) reported a decrease in shoot Cd when plants were inoculated by *Funneliformis mossae*, but an increase when colonised by *R. irregularis*.

In roots, much higher Cd was found in NM poplars exposed to Cd, but only when grown in co-cultures with leeks. This is possibly a result of having multiple individuals (10 leeks and 1 poplar) developing in the same pot. Plant roots constantly release various organic compounds into the rhizosphere, including organic acids and proton ions (H^+) (Ma et al., 2022); a process that will eventually acidify the rhizosphere, promoting Cd(II) uptake, as its availability is greatly influenced by soil pH (De Oliveira et al., 2016). Indeed, Nandillon et al. (2022) showed that co-cultures between willows and clover plants significantly decreased soil pH and enhanced lead (Pb) accumulation in willow leaves.

Under CMNs, however, Cd uptake was not enhanced in poplar roots, highlighting a marked difference between co-cultures with or without AMF. In this case, we hypothesise that lower exudation of organic acids under CMNs could have attenuated the rhizosphere acidification process, as AMF symbiosis can often greatly reduce root acid exudation, sometimes by 50 % (Hajiboland et al., 2020; Nazeri et al., 2014). Moreover, foliar manganese (Mn) concentrations have been considered a proxy for organic acid exudation in plants, often increasing as more acids are released (Pang et al., 2018). Here, all AM poplars had significantly lower foliar Mn than NM plants (Fig. S7c), which strengthen our hypothesis that AM may have decreased exudation. Thus, further investigation is needed, as other possible mechanisms for lower root Cd under CMN could also be: i) transfer of Cd from poplars towards leeks via CMN (Ding et al., 2022), ii) higher GRSP levels present in CMN pots sequestering more Cd, decreasing its availability to poplars (Kaur et al., 2023), or even iii) higher mycelial biomass in CMN pots - due to the fungus having multiple hosts - leading to enhanced Cd binding onto fungal cell walls, spores, vacuoles and intraradical vesicles (Kaur et al., 2023; Nayuki et al., 2014).

4.2. Soil responses

4.2.1. Cd contamination induces GRSP release

A clear effect from Cd(II) contamination was observed on GRSP concentrations in all mycorrhizal pots when compared to non-contaminated controls. Due to variable responses across the literature (Bisht and Garg, 2022; Babadi et al., 2019), the effect of Cd on enhancing GRSP was not initially hypothesised, however these results are undeniable, especially for single AM leeks, which presented 25-fold higher GRSP under Cd than in control. Similarly, Babadi et al. (2019) observed a 1.2-fold increase in GRSP due to Cd contamination in *Sorghum bicolor* under symbiosis with AMF *Claroideoglomus etunicatum*, while Malekzadeh et al. (2016) reported a 10-fold increase for clover seedlings in vitro with *R. irregularis*. Due to ample evidence of GRSP being induced under stress conditions, including heavy metals (Holatko et al., 2021), and the role of GRSP in Cd immobilisation and binding (Kaur et al., 2023; González-Chávez et al., 2004), it is likely that what we have observed here is a protective response of mycorrhizal plants to mitigate Cd uptake and stress.

We initially expected that GRSP concentrations would be higher in co-cultures under CMN, assuming that fungal presence would be greater when connected to multiple plant hosts (i.e. C sources) as intercropping can enhance GRSP in soils compared to monocultures (Agnihotri et al., 2022). Indeed, a small but significant increase of GRSP was observed in CMN pots in comparison to single AM poplars (Fig. 4). This shows that despite the low biomass of leeks in the CMN pots, it was enough to improve GRSP release by *R. irregularis*. Pots with single AM leeks had the highest GRSP concentrations, supporting the idea that the presence of leeks was the reason for higher GRSP in co-culture pots. Soil GRSP can vary greatly depending on plant host species, for instance Hou et al. (2022) have observed higher concentrations of GRSP under grassland vegetation in comparison to shrubs. It is worth mentioning that single AM leeks had the highest shoot Cd concentrations out of all treatments (12 mg kg^{-1}), and a positive relationship was found between leaf Cd concentrations and GRSP (Fig. S4), suggesting that perhaps the internal Cd levels in plants, and not soil availability, signals for more GRSP release.

4.2.2. Higher C-cycling enzyme activities only in pots with AM leeks

Contrary to what we expected, Cd(II) exposure did not significantly decrease the activity of C-cycling enzymes except for BX (β-xylosidase), which was overall lower in contaminated pots (Fig. S3d), perhaps due to a decrease in microbial populations (Wu et al., 2018). Although the PCA indicated a negative relationship between plant Cd and enzyme activities, their large variability did not allow for the ANOVA to detect significant differences among treatments. It is important to consider that the initial microbial communities in our pot conditions were probably

very low in the growth substrate due to previous autoclaving procedures. Therefore, any enzyme activity detected here were from the communities that developed during only three months of plant growth, and perhaps more time was needed to observe any significant Cd effects.

Even though AMF lack exoenzymes to degrade organic matter, its presence can increase decomposition in soils, an effect associated with root and hyphal exudation and modulation of microbial communities (Xu et al., 2018; Kuyper and Jansa, 2023). However, we observed that most mycorrhizal conditions did not significantly affect the activities of these C-cycling enzymes, the exception being pots with single AM leeks, where much higher BG, CB and BX activities were observed. The fact these single AM leeks also presented lower biomass when compared to single leeks of any other treatment, suggests a higher drain of C from the plant towards the roots, which may have stimulated microbial growth. As demonstrated by Paterson et al. (2016), C flux from plants through AMF hyphae can have a priming effect, leading to more decomposition. Since the microbiome composition is highly influenced by both root and hyphae exudations (Kuyper and Jansa, 2023), it is possible that the community that developed under single AM leeks is particularly associated with organic matter decomposition, reflecting in higher C-cycling enzyme activities.

We initially hypothesised that CMN conditions would lead to higher soil enzyme activities due to multiple plant species and their AMF partner generating a C sink towards soils, stimulating microbial growth (Xu et al., 2018; Agnihotri et al., 2022). However, as observed with GRSP, single AM leeks were in fact a stronger C sink than co-cultures under CMN, promoting higher C-cycling enzyme activities. The main reasons could be that: 1) leek exudates are more efficient in recruiting C-degrading microorganisms than poplar's, as different exudation profiles between plant genotypes lead to different microbial assembly in the rhizosphere (Anderson et al., 2024); and 2) as the competition between poplars and leeks largely favoured the growth of the former, leeks grew poorly and were unable to effectively release exudates and modulate soil microbial activity under CMN.

The overall results reported here, and their variability were captured by a principal component analysis (Fig. 6), where PERMANOVA showed that single NM poplars had divergent multivariate responses to Cd(II) contamination in comparison to NM co-cultures. However, in inoculated poplars, single cultures and co-cultures (CMN) retained similar patterns ($p > 0.05$), demonstrating that CMN may buffer the overall poplar responses to Cd exposure. We hypothesise that plants under CMN may be more resilient to Cd contamination, but perhaps less efficient for metal removal strategies such as phytoremediation.

5. Conclusions

We evaluated poplar and leek responses to Cd(II) contamination (21 mg kg^{-1}), and effects from *R. irregularis* symbiosis in interspecific co-cultivation systems. Our results show that mycorrhizal symbiosis differentially affects Cd accumulation, depending on the host plant species, increasing it in leeks but decreasing it in poplars. Both species displayed tolerance to Cd exposure and growth was not impaired. Cd had little effect on C-cycling enzyme activities, but enhanced GRSP levels by 8- to 25-fold compared to the control. Mycorrhizal co-cultures between poplars and leeks - representing the CMN condition - did not maintain soil enzyme activities as initially hypothesised, but did decrease Cd concentrations in poplar roots when compared to NM co-cultures, from 102 to 37 mg kg^{-1}. The co-culture condition had a significant multivariate effect (e.g. mineral nutrients, Cd, glomalin) in poplars, an effect also buffered by CMNs, with poplars presenting similar patterns to single AM cultures. The mechanism(s) behind this response deserves further investigation, particularly the potential for hyphal binding, the possible transfer (offloading) of Cd from poplars towards leeks under CMN, and perhaps most importantly the differential organic acid exudation patterns that may have occurred in CMN conditions. Overall, these results suggest that co-cultivation of mycorrhizal host plants might mitigate Cd accumulation and stress in contaminated soils.

CRediT authorship contribution statement

Gilka Rocha Vasconcelos da Silva: Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **De Oliveira Vinicius Henrique:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mark Tibbett:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Brian J Pickles:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119141.

Data availability

Data will be made available on request.

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