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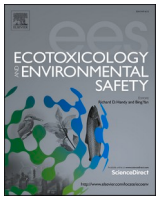

Species-specific effects of mycorrhizal symbiosis on *Populus trichocarpa* after a lethal dose of copper

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ABSTRACT

Poplars have been identified as heavy metals hyperaccumulators and can be used for phytoremediation. We have previously established that their symbiosis with arbuscular mycorrhizal fungi (AMF) may alter their uptake, tolerance and distribution to excess concentrations of heavy metals in soils. In this study we hypothesised that mycorrhizal symbiosis improves the tolerance of poplars to lethal copper (Cu) concentrations, but this influence may vary among different AMF species. We conducted an experiment in a growth chamber with three Cu application levels of control (0 mg kg^{-1}), threshold-lethal (729 mg kg^{-1}) and supra-lethal (6561 mg kg^{-1}), and three mycorrhizal treatments (non-mycorrhizal, *Rhizophagus irregularis*, and *Paraglomus laccatum*) in a completely randomized design with six replications. The poplars did not grow after application of 729 mg Cu kg^{-1} substrate, and mycorrhizal symbiosis did not help plants to tolerate this level of Cu. This can be explained by the toxicity suffered by mycorrhizal fungi. Translocation of Cu from roots to shoots increased when plants were colonised with *R. irregularis* and *P. laccatum* under threshold-lethal and supra-lethal applications of Cu, respectively. This result shows that mycorrhizal mediation of Cu partitioning in poplars depends on the fungal species and substrate Cu concentration. Multi-model inference analysis within each mycorrhizal treatment showed that in plants colonised with *R. irregularis*, a higher level of mycorrhizal colonisation may prevent Cu transfer to the shoots. We did not observe this effect in *P. laccatum* plants probably due to the relatively low colonisation rate (14%). Nutrient concentrations in roots and shoots were impacted by applied substrate Cu levels, but not by mycorrhizas. Magnesium (Mg), potassium (K), and manganese (Mn) concentrations in roots reduced with enhancing applied substrate Cu due to their similar ionic radii with Cu and having common transport mechanism. Synergistic effect on shoot concentration between applied substrate Cu levels and Mg, K, calcium, iron (Fe), and zinc was observed. Root Cu concentration was inversely related with root K and Mn concentrations, and shoot Cu concentration had a positive correlation with shoot Fe and K concentrations. Overall, mycorrhizal symbiosis has the potential to enhance plant health and their resilience to Cu toxicity in contamination events. However, it is important to note that the effectiveness of this symbiotic relationship varies among different mycorrhizal species and is influenced by the level of contamination.

1. Introduction

Copper is an essential micronutrient for plant growth and its deficiency results in losses in crop yield (Marschner, 1995), while elevated soil Cu concentrations have an adverse effect on plant growth (Alloway, 2012; Lepp et al., 1997). The average natural abundance of Cu in the earth's crust is 60 mg Cu kg^{-1} (Lide, 2009) and concentrations in soil typically vary between 2 and 50 mg kg^{-1} (Alloway, 2012). In soils affected by mining and smelting, Cu concentration may reach more than 9000 mg kg^{-1} (Dudka et al., 1995; Loredo et al., 2008; Stuckey et al., 2008; Degani et al., 2022). Degani et al. (2022) measured 3000 mg kg^{-1} of soil Cu in the Copperbelt of Zambia. Loredo et al. (2008) recorded maximum soil Cu concentrations of up to 9921 mg kg^{-1} around the old metallurgical plant in Texeo mine in Spain. Alloway (2012) investigated a number of monocotyledon and dicotyledon plant species and suggested a range of 18–698 mg Cu kg^{-1} soil as the chronic soil Cu toxicity

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threshold concentrations for plants. Cu toxicity in plants starts with rhizotoxicity, resulting in reduced root length and stunting, and thickening and darkening of the roots (Kopittke and Menzies, 2006; Marschner, 1995; Sheldon and Menzies, 2005). Roots act as a strong translocation barrier to excess Cu seen in root to shoot translocation data (Duddigan et al., 2023). Excessive Cu is accumulated and adsorbed mainly by the fine roots, and its translocation towards upper plant parts is restricted until the plant is no longer able to maintain this regulation (Pedersen et al., 2000). Critical root Cu concentration is in the range of 100–400 mg kg^{-1}, while it is in the range of 5–40 mg kg^{-1} in shoots (Degryse et al., 2008; Pedersen et al., 2000; Sheldon and Menzies, 2005). When this threshold is reached, direct toxicity of Cu damages cell structure, interferes with metabolic processes, and inhibits a number of cytoplasmic enzymes, which may lead to plant death (Van Assche and Clijsters, 1990).

Some higher plants can accumulate high concentrations of heavy metals in their tissues without showing toxicity symptoms (Bennett et al., 2003; Klassen et al., 2000). Phytoremediation is an in-situ soil remediation technique which uses these plants and associated microbiota for the clean-up of contaminated soils (Allen, 2019; Gomes et al., 2016; Pilon-Smits, 2005). *Populus* genus (poplars) have been identified as trees which can hyperaccumulate heavy metals (Guerra et al., 2011; Luo et al., 2016; Redovniković et al., 2017), and can be used for phytoremediation since they produce a high quantity of plant biomass, form deep root systems, and develop specific mechanisms for Cu accumulation (Bhargava et al., 2012; De Oliveira and Tibbett, 2018; Robinson et al., 2009). In natural ecosystems, poplar roots associate with arbuscular mycorrhizal fungi (AMF), forming a mutualistic symbiosis. The plant provides the fungi, which are obligate symbionts, with carbohydrates. In return, the fungi may improve plant nutrient uptake and significantly increase their biomass (Kariman et al., 2016). In addition, AMF may enhance plant tolerance to heavy metals through phytostabilisation by restricting their mobility and bioavailability in roots (Tibbett et al., 2021). In the root, Cu is mainly accumulated in the inner root parenchyma cells where AM fungal structures (intraradical hyphae, arbuscules and vesicles) reside. A number of mechanisms like extracellular binding of heavy metals, metal efflux, and intracellular chelation or compartmentation in the vacuoles by polyphosphate granules may be involved depending on the mycorrhizal and plant species (Chen et al., 2016; Ciadamidaro et al., 2017; Tibbett et al., 2021). Mycorrhizas also improve plant metal tolerance through changes in plant gene expression patterns like metallothionein genes which can confer tolerance to heavy metals (Cicatelli et al., 2010, 2014; Kumar et al., 2015; Rivera-Becerril et al., 2005). However, the effect of this symbiosis on heavy metal tolerance of plants may vary with AMF species and plant cultivars (Bissonnette et al., 2010; Sun et al., 2018). We have previously shown that root colonisation with AM can rearrange the epidermal and *in planta* transport and distribution of nutrients, and potentially toxic, elements. We observed that this effect also depends on the metal, whether it is a plant nutrient or a non-essential element (De Oliveira et al., 2020; Kariman et al., 2016; Tibbett et al., 2022).

In recent years, the extensive use of Cu in a series of industrial, agricultural, domestic, and technological applications have resulted in increased environmental Cu concentrations and soil pollution, but also enhanced the risk of contamination events (Hong et al., 1996; Richardson, 1997). In the case of a sudden contamination event, plants are exposed to a near instantaneous and acute lethal Cu toxicity. It is not known whether AM symbiosis can alleviate plant toxicity, at least transiently, under such immediate exposure conditions, and if they do, which are the mechanisms involved? To address these issues, we subjected poplar trees to threshold-lethal and supra-lethal doses of Cu in a pulse (mirroring a contamination event) and test whether rapid changes can occur under mycorrhizal compared to non-mycorrhizal conditions within 48 hours. We also used two different species of AMF to ascertain whether there is a fungal species effect, given the known functional diversity of AMF for heavy metal tolerance (Bissonnette et al., 2010; Sun et al., 2018).

Specifically, we tested the hypotheses that under lethal substrate Cu concentrations, 1) mycorrhizal plants survive for longer and show less damage than non-mycorrhizal plants, and 2) mycorrhizal fungal species differentially influence Cu translocation from root to shoot. Additionally, we hypothesised that 3) threshold-lethal and supra-lethal pulses of Cu on poplar affect other nutrient uptake and distribution within the plant.

2. Materials and methods

2.1. Growth substrate preparation, plant material and AMF inoculation

Hybrid poplar cuttings (known as B2, or Beaupre, *Populus trichocarpa* × *Populus deltoides*) were obtained from Bowhayes Trees Ltd, Devon, UK. *Rhizophagus irregularis* and *Paraglomus laccatum* inoculua were obtained from the University of Reading mycorrhizal collection, which is cultured using *Plantago lanceolata* as the host plant.

Growth substrate was made up from a mixture of TerraGreen® clay (American Granules Plain, OIL-DRI, UK) and sand (1:5 w/w) (Sibelco, UK) and autoclaved twice (105 °C for 1 h + 24 h rest + 105 °C for 1 h). Plastic pots (1 kg, 13 cm diameter) were prepared with 1 kg of the substrate and 100 g of the mycorrhizal inoculum (sand mix containing colonised root fragments, hyphae and fungal spores). Non-mycorrhizal treatments received 100 g of autoclaved inoculum. Fifty mg P kg^{-1} substrate was added in the form of calcium phosphate to each pot. The substrate surface in all pots was covered with a layer (0.5 cm) of plastic pellets to avoid possible cross contamination among treatments.

One poplar cutting (15 cm, two nodes) was planted in the centre of each pot to grow for twelve weeks in a growth chamber (23 °C; light per day, 16 h; photosynthetic photon flux, 100 μmolm^{-2} s^{-1} -Philips MCFE 40 W/840) and all plants were fertilised weekly for the first four weeks with 10 mL of a modified Long Ashton's solution (macronutrients: $(NH_4)_2SO_4$ (4 mM), K_2SO_4 (2 mM), $CaCl_2{\cdot}2\ H_2O$ (3 mM), $MgSO_4{\cdot}7\ H_2O$ (1.5 mM), $NaNO_3$ (8 mM), FeEDTA (0.1 mM); micronutrients: H_3BO_3 (2.86 mg L^{-1}), $MnCl_2{\cdot}4\ H_2O$ (1.81 mg L^{-1}), $CuSO_4{\cdot}5\ H_2O$ (0.08 mg L^{-1}), $NaMoO_4{\cdot}2\ H_2O$ (0.025 mg L^{-1}), $ZnSO_4{\cdot}7\ H_2O$ (0.22 mg L^{-1})), according to Kariman et al. (2014). Water holding capacity was maintained at 70% (330 mL of distilled water pot^{-1}) with a two-day gap between each occurrence.

2.2. Contamination and experimental design

The experiment was conducted with three levels of Cu application including control (0 mg kg^{-1} of Cu (Cu_0)), threshold-lethal (729 mg kg^{-1} of Cu (Cu_{729})) and supra-lethal (6561 mg kg^{-1} of Cu (Cu_{6561})) concentrations and three mycorrhizal treatments including non-mycorrhizal, *R. irregularis*, and *P. laccatum* in a completely randomized design with six replications. For Cu treatments, a 1.58 M solution of copper (II) sulphate (anhydrous) was prepared, then 8 and 72 mL of this solution were added to Cu_{729} and Cu_{6561} pots, respectively. To compensate for the amount of the solution added to each pot, 64 and 72 mL of deionised water were further supplemented to threshold lethal (Cu_{729}) and control pots concurrently.

The chlorophyll concentration (SPAD-value using Minolta SPAD 502 Plus Chlorophyll Meter), plant height and number of leaves were determined an hour before Cu application and at harvest.

2.3. Harvest, mycorrhizal colonisation, acid digestion and determination of the elements

Forty-eight hours after contamination, plants were harvested and separated into shoots (leaves and stems) and roots (original cutting was discarded). Roots were washed thoroughly with deionised water and random sections of 2 cm from the root tips were sampled for determination of mycorrhizal colonisation. All plant parts were dried in an oven

at 70 °C for seven days before dry weight (DW) was determined.

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

Total elemental concentrations were measured by ICP-MS after trace element grade concentrated nitric acid (8 mL) and ultra-pure water (2 mL) microwave digestion (Ethos Easy Digestion System) on 0.5 g of shoot and root dried samples. Nutrient contents were calculated by multiplying the determined elemental concentration by their respective biomass weight, for roots and shoots, separately, and then for the entire plant.

The translocation factor (T*f* %) is an index used to assess the plant's capacity to translocate total elements from roots to aboveground parts (Rafati et al., 2011), and is the ratio between the element concentrations in shoots and roots (Saraswat and Rai, 2009; Zacchini et al., 2009). We used the Cu contents (mg per plant part) instead of the concentrations, as it takes into account the biomass of each plant part, and provides a more accurate representation of the proportion of Cu that can be translocated and stored in shoots and roots:

$$Tf = \frac{Shoot\ \ Cu\ \ content}{Root\ \ Cu\ \ content} \times 100$$

2.4. Statistical analysis

All statistical analyses were performed using R software (version 4.2.2). Data were tested for normality (Shapiro-Wilks), homogeneity of variance, and transformed prior to analysis whenever necessary, using either a natural log or box-cox transformation. Two-way ANOVA was used to test the effect of mycorrhizas, applied Cu concentrations and their interaction on different evaluated parameters at 5% significance level. The Tukey test ($p < 0.05$) was applied for the comparison of means.

To understand the contribution of distinct plant parameters, which may represent a proxy of plant vigour, subsequently resulting in a better performance to Cu translocation from roots to shoots, we conducted a multi-model inference by modelling all possible combination of predictors to identify the most important plant predictors affecting shoot Cu concentration within each mycorrhizal treatment based on the sum of Akaike weights (AICc). The predictors included for shoot Cu concentration were plant height, number of leaves, chlorophyll concentration, and root colonisation rate. Data met the assumption that the independent predictor variables were not highly correlated with each other (Zuur et al., 2010). The full model had the following form: shoot Cu concentration ~ plant height + number of leaves + chlorophyll concentration + colonisation rate. We used the packages *AICcmodavg* for model selection and *MuMIn* for multi-model inference.

Plant macronutrients of calcium (Ca), magnesium (Mg), and potassium (K) and micronutrients of iron (Fe), zinc (Zn), manganese (Mn), and Cu in roots and shoots were submitted to principal component analysis (PCA) using the 'prcomp' function in R.

3. Results

3.1. The effect of threshold-lethal and supra-lethal pulses of Cu on mycorrhizal and non-mycorrhizal poplar plants

In inoculated treatments, the percentage of colonisation was 44% and 14% for *R. irregularis* and *P. laccatum*, respectively. No colonisation was detected in non-inoculated poplars. All measurements before pulse application showed no differences in plant height, number of leaves, or leaf chlorophyll concentrations among treatments. These data are presented in supplementary material.

At 48 h after pulse application, all plants showed an advanced stage of leaf epinasty and chlorosis, indicating a rapid plant death (Fig. 1). Based on our observation, the symptoms started to appear two hours after Cu application and intensified with time.

Shoot dry weight was not influenced by either mycorrhizas or applied substrate Cu but root dry weight was affected by applied substrate Cu (Table 1). The interaction of Cu levels with mycorrhizal treatments did not have a significant effect on root and shoot dry weights. Root dry weight decreased by 44% with Cu application to the substrate. Root dry weight was not changed with increasing applied substrate Cu from threshold-lethal to supra-lethal levels.

3.2. Copper content and distribution within the plant

Root, shoot, and total Cu content by poplars was strongly affected by Cu application to the substrate and increased in line with application rate, with a maximum total content of 75.5 mg plant^{-1} in supra-lethal level (Table 2). The Cu contents were not influenced by mycorrhizas or by the interaction of Cu levels with mycorrhizal treatments. Translocation factor increased with increasing applied substrate Cu under all mycorrhizal treatments (Table 2). When Cu was applied at threshold-lethal concentrations, non-mycorrhizal plants and plants inoculated with *P. laccatum* had the same T*f*, while T*f* in plants inoculated with *R. irregularis* increased by 7%. In the supra-lethal concentration treatment, T*f* was 52% and 51% in non-mycorrhizal treatment and under *R. irregularis*, respectively. The highest T*f* was recorded in *P. laccatum* treatment, where 74% of the Cu taken up by the roots was translocated to the shoots, in only 48 hours after Cu application.

Root and shoot Cu concentrations followed the same trend as the content. They increased with increasing applied substrate Cu levels under all mycorrhizal treatments, but mycorrhizal treatments and the interaction of Cu levels with mycorrhizal treatments did not change root and shoot Cu concentrations (Table 3).

The multi-model inference analysis resulted in a significant model explaining shoot Cu concentration relative to plant parameters, only in the plants colonised by *R. irregularis* ($p < 0.001$, R = 0.86, Table 4). The modelling did not show this effect on plants colonised by *P. laccatum*. The best model was selected based on the AICc with the following form: shoot Cu concentration ~ number of leaves + chlorophyll concentration + colonisation rate. These plant parameters explained 86% of the variation in shoot Cu concentration in plants colonised with *R. irregularis* and they had relatively strong negative effect for shoot Cu concentration (Table 4).

3.3. The effect of lethal dose of Cu addition on plant nutrient contents

The first two principal components of PCA explained 80.8% of the total variation in measured plant nutrients in roots (Fig. 2A) and 74.4% of that variation in shoots (Fig. 2B) among all applied Cu levels. Factor 1 in roots and shoots explained 49.2% and 62.3% of the variation in nutrients data, respectively, and separated different applied Cu levels from each other. Root Cu concentration was inversely related with root K and Mn concentration. Shoot Cu concentration had a positive correlation with shoot Fe and K concentration.

The concentrations of the investigated plant nutrients were not influenced by mycorrhizas or by the interaction of Cu levels with mycorrhizal treatments, hence we only presented the effect of Cu levels on the concentration of those elements in roots and shoots in Table 5. We observed significant changes in the concentrations of six key elements in the plant biomass (Table 5). Magnesium concentration in roots did not alter with Cu application from control to the threshold-lethal level, but it was decreased by 29% with increasing Cu application from threshold-lethal to supra-lethal concentrations (Table 5). Root K and Mn concentrations decreased with increasing substrate Cu (Table 5). Concentration of these elements decreased by 92% and 75%, respectively, when Cu was

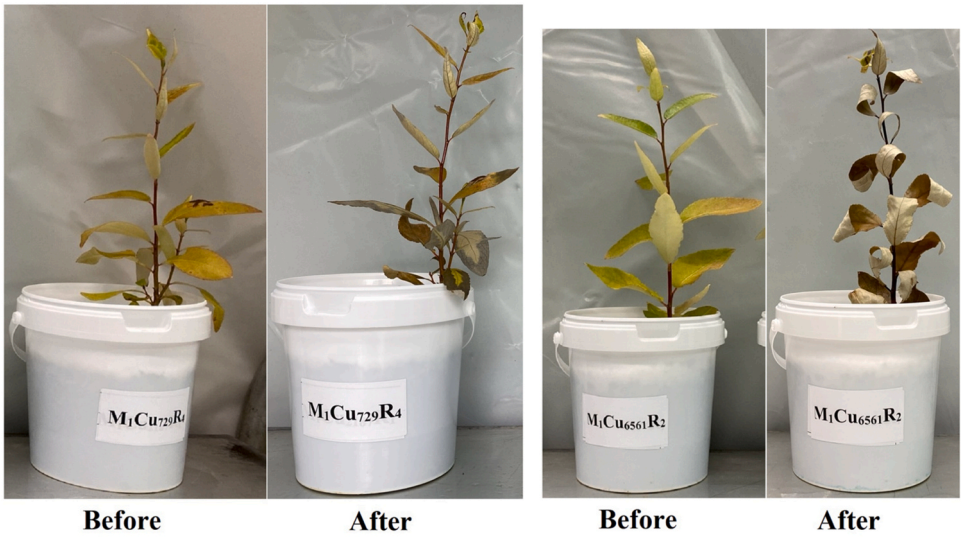

Fig. 1. Phytotoxic effects of threshold-lethal (729 mg kg^{-1}) and super-lethal (6561 mg kg^{-1}) Cu concentrations in *Populus trichocarpa* inoculated with *Rhizophagus irregularis*, after 48 hours of exposure.

Table 1
Root and shoot dry weight of mycorrhizal *Populus trichocarpa* under different levels of applied. Values represent mean ± standard error, n = 6. Different lowercase letters represent significant differences between applied substrate Cu levels regardless of mycorrhizal treatment by ANOVA and LSD test ($p < 0.05$). ns: non-significant.

	Applied substrate Cu			
	Control	Threshold-lethal	Supra-lethal	Mean
	Root dry weight (g)			
Non mycorrhizal	2.18±0.25	0.99±0.28	0.65±0.28	1.34^{ns}
Rhizophagus irregularis	1.17±0.25	0.90±0.25	1.15±0.28	1.07
Paraglomus laccatum	1.75±0.23	0.97±0.21	1.00±0.25	1.24
Mean	1.71^{a}	0.95^{b}	0.94^{b}	
	Shoot dry weight (g)			
Non mycorrhizal	2.37±0.32	1.80±0.36	1.81±0.41	2.04^{ns}
Rhizophagus irregularis	2.36±0.32	1.69±0.32	2.11±0.32	2.06
Paraglomus laccatum	2.38±0.29	1.29±0.29	1.78±0.32	1.82
Mean	2.37^{ns}	1.56	1.91	

Table 2
Cu content (mg $plant^{-1}$) and translocation factor (T*f*) in mycorrhizal *Populus trichocarpa* under different levels of applied Cu. Values are the means ± standard errors. Different lowercase letters represent significant differences between applied substrate Cu levels regardless of mycorrhizal treatment, by ANOVA and LSD test ($p < 0.05$). ns: non-significant. T*f* = (shoot content / root content) × 100.

	Applied substrate Cu			
	Control	Threshold-lethal	Supra-lethal	Mean
	Root Cu content (mg $plant^{-1}$)			
Non mycorrhizal	1.7±0.5	16.4±9.3	43.4±8.1	19.3^{ns}
Rhizophagus irregularis	0.5±0.5	22.8±8.0	61.3±8.1	28.2
Paraglomus laccatum	0.6±0.5	24.2±6.1	40.9±6.6	23.2
Mean	1.0^{c}	22.1^{b}	47.5^{a}	
	Shoot Cu content (mg $plant^{-1}$)			
Non mycorrhizal	0.012 ±0.005	2.3±0.3	22.5±3.0	8.3^{ns}
Rhizophagus irregularis	0.007 ±0.005	4.8±2.6	31.4±3.0	12.1
Paraglomus laccatum	0.010 ±0.003	3.4±2.6	30.3±2.1	11.2
Mean	0.010^{c}	3.5^{b}	28.1^{a}	
	Total plant Cu content (mg $plant^{-1}$)			
Non mycorrhizal	1.7±0.2	18.7±11.0	65.9±11.0	28.8^{ns}
Rhizophagus irregularis	0.5±0.2	27.6±9.5	91.6±11.0	39.9
Paraglomus laccatum	0.6±0.2	27.6±9.5	69.0±11.0	32.4
Mean	0.9^{c}	24.6^{b}	75.5^{a}	
	Translocation factor (%)			
Non mycorrhizal	0.7	14	52	
Rhizophagus irregularis	1.4	21	51	
Paraglomus laccatum	1.7	14	74	

applied at the supra-lethal level compared to control. Iron and Zn concentrations in roots were increased with increasing substrate Cu from control to the threshold-lethal concentrations, but were not changed with further application of Cu (Table 5).

Shoot Mg, K, and Zn concentrations were decreased by Cu application from control to threshold-lethal concentrations, while they did not change after further addition of Cu, from threshold-lethal to supra-lethal concentrations. Calcium and Fe concentrations in shoots were increased with increasing applied substrate Cu.

4. Discussion

4.1. Mycorrhizal symbiosis did not protect poplars against threshold-lethal and supra-lethal pulses of copper

We assessed the role of mycorrhizal fungi in the first 48 h of plant response to lethal and supra-lethal pulses of Cu, simulating a contamination event. Contrary to our first hypothesis, mycorrhizal symbiosis did not ameliorate Cu toxicity in young poplars. A dose of 729 mg Cu kg^{-1} substrate was lethal for young poplars. All plants, independent of mycorrhization showed dramatic leaf epinasty and chlorosis in the first 24 h. Commonly, soil concentrations of 20–100 mg Cu kg^{-1} can have toxic effects on soil microorganisms and plant roots (Guerra et al., 2011; Mir et al., 2021). However, the level of toxicity that results in plant death varies with plant species. For example, Wallace et al. (1977) observed that bush bean plants failed to grow under 500 mg Cu kg^{-1} soil, while herbaceous plants from Linaceae or Malvaceae families cope with concentrations above 2000 mg Cu kg^{-1} soil (Mir et al., 2021). However, poplar hybrids are considered heavy metal hyperaccumulators able to grow under exceptionally high Cu concentrations without negative effects in their performance. Vamerali et al. (2009) reported a reduction in biomass but not the death of one-year-old rooted

Table 3
Cu concentration (mg g^{-1}) in roots and shoots of mycorrhizal *Populus trichocarpa* under different levels of applied substrate Cu. Values are the means ± standard errors. Different lowercase letters represent significant differences between applied substrate Cu levels regardless of mycorrhizal treatment, by ANOVA and LSD test ($p < 0.05$). ns: non-significant.

	Applied substrate Cu			
	Control	Threshold-lethal	Supra-lethal	Mean
	Root Cu concentration (mg g^{-1})			
Non mycorrhizal	0.78±0.12	24.7±4.3	68.2±4.3	25.7^{ns}
Rhizophagus irregularis	0.44±0.14	30.6±3.3	66.1±3.3	34.6
Paraglomus laccatum	0.53±0.11	20.5±3.1	46.0±3.1	22.3
Mean	0.6^{c}	25.0^{b}	57.9^{a}	
	Shoot Cu concentration (mg g^{-1})			
Non mycorrhizal	0.005 ±0.002	1.16±0.21	15.8±0.21	5.1^{ns}
Rhizophagus irregularis	0.003 ±0.001	2.79±0.16	12.4±0.21	4.6
Paraglomus laccatum	0.006 ±0.002	2.36±0.18	18.9±0.15	7.7
Mean	0.005^{c}	2.24^{b}	16.5^{a}	

Table 4
The optimal model for shoot Cu concentration (mg kg^{-1}) in poplars colonised with *Rhizophagus irregularis*[a].

Model	Estimate	Standard error	*p*-value[b]	Adjusted R^2
Shoot Cu concentration[c]			***	0.86
Intercept	15.39	2.06	***	
Number of leaves	-0.50	0.12	**	
chlorophyll concentration (SPAD-value)	-0.17	0.06	*	
Colonisation rate	-0.05	0.01	**	

[a] The full model had the following form: shoot Cu concentration ~ plant height + number of leaves + chlorophyll concentration + colonisation rate, while the best model had the following form: shoot Cu concentration ~ number of leaves + chlorophyll concentration + colonisation rate;
[b] Significance of effects is indicated by *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$;
[c] Shoot Cu concentration was log transformed.

poplar cuttings under 1735 mg Cu kg^{-1} soil. Surprisingly, we found no differences in the shoot biomass among treatments despite the occurrence of leaf epinasty due to Cu toxicity. However, in line with previous findings, Cu toxicity caused reduced root biomass. Autooxidation of redox-active metals such as Cu^{+} generates reactive oxygen species that result in oxidative stress with damage to the cell membranes and eventually root necrosis (Garcia et al., 2006; Jouili and El Ferjani, 2004; Krzesłowska, 2011; Schützendübel and Polle, 2002). Although high levels of Cu inhibit shoot and root growth through interfering with important cellular processes such as photosynthesis and respiration (Marschner, 1995; Prasad and Strzalka, 1999; Yruela, 2005), given the immediate effect of toxicity, this process would not apply.

The lack of alleviation of Cu toxicity in plants with mycorrhizal colonisation under extremely high Cu levels can be explained by the toxicity suffered by mycorrhizal fungi. This has been shown when AMF extramatrical mycelial growth was stopped by application of 20 μM Cu in the growth medium (Hildebrandt et al., 2007). Oxidative stress is a major cause of Cu toxicity not only in plants but also in AM fungi

Table 5
Mg, K, Ca, Mn, Fe, and Zn concentrations (mg kg^{-1}) in *Populus trichocarpa* under different levels of applied substrate Cu. Values are the means ± standard deviations. Different lowercase letters represent significant differences between applied substrate Cu levels within the same element (column), by ANOVA and LSD test ($p < 0.05$). ns: non-significant.

	Mg	K	Ca	Mn	Fe	Zn
	mg kg^{-1}					
	Roots					
Control	6953 ±354^{a}	23970 ±1130^{a}	975 ±31^{ns}	406 ±37^{a}	3968 ±331^{b}	150 ±21^{b}
Threshold-lethal	6448 ±547^{a}	13326 ±1700^{b}	1460 ±134	212 ±24^{b}	6621 ±708^{a}	524 ±79^{a}
Supra-lethal	4551 ±206^{b}	1934 ±195^{c}	975 ±82	101 ±4^{c}	5847 ±491^{a}	511 ±47^{a}
	Shoots					
Control	5482 ±185^{b}	11105 ±818^{b}	1401 ±46^{c}	74 ±5^{ns}	244±7^{c}	127 ±38^{b}
Threshold-lethal	7012 ±311^{a}	18199 ±1260^{a}	1692 ±60^{b}	85±3	427 ±22^{b}	478 ±151^{a}
Supra-lethal	7101 ±352^{a}	17422 ±820^{a}	1909 ±54^{a}	86±3	905 ±28^{a}	446 ±134^{a}

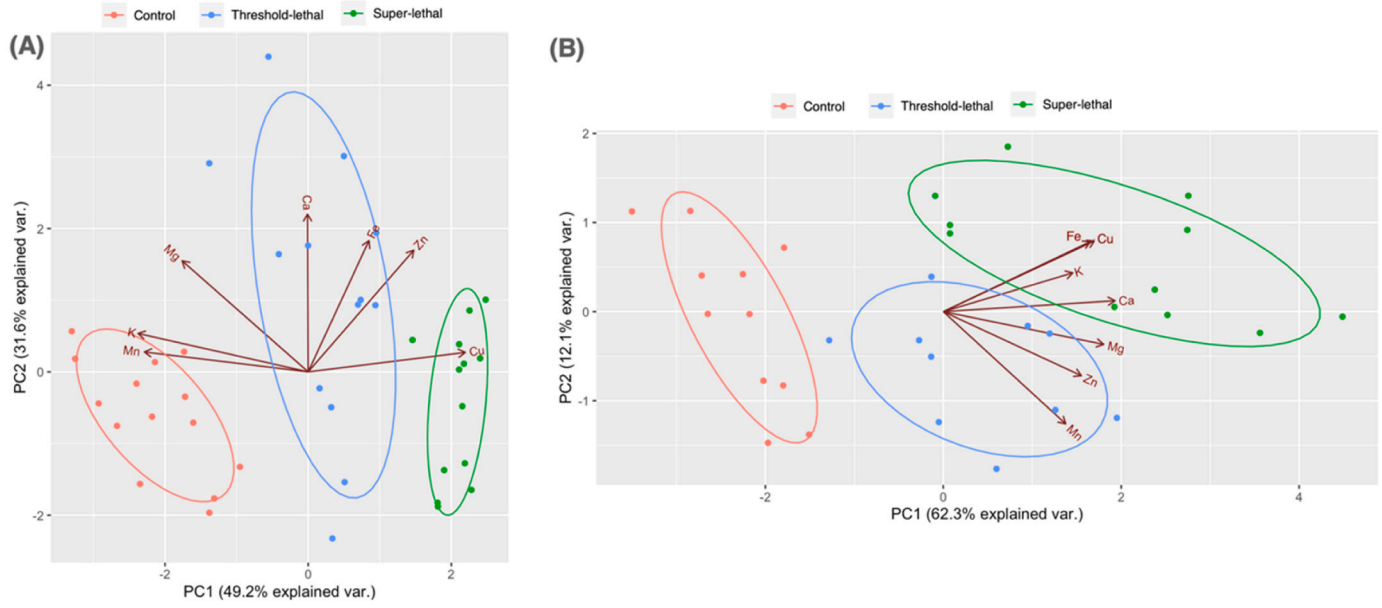

Fig. 2. Principal component analysis of plant elements (Ca, Mg, K, Cu, Zn, Fe, Mn) in: (A) roots and (B) shoots of *Populus trichocarpa* , under different concentrations of applied substrate Cu.

(Hildebrandt et al., 2007), giving rise to major cellular alterations and, ultimately fungal cell death. Functional gene analysis of *R. irregularis* has shown that genes encoding Cu transporters of the CTR family may contribute to oxidative stress protection. However, their up-regulation is effective at much lower concentrations of Cu toxicity than in our experiment (application of 500 μM $CuSO_4$, Gómez-Gallego et al., 2019).

4.2. Mycorrhizal fungal species differentially influence Cu translocation from root to shoot

In the control treatment, T*f* in non-mycorrhizal was lower than one, indicating that Cu transfer from root to shoot was not optimal (Yoon et al., 2006). In accordance with other reports, we found a positive influence of AMF on Cu translocation under non-toxic conditions (Lee and George, 2005). Under supra-lethal Cu concentrations, T*f* was in the excessive range of 50–70%, with higher values in *P. laccatum* than non-mycorrhizal and *R. irregularis* plants. We assume that where the transfer between root and shoot was so excessive, any potential mycorrhizal effect was overwhelmed.

We obtained a significant model in predicting shoot Cu concentration by plant parameters in the plants colonised with *R. irregularis*, but not in *P. laccatum* colonised plants. The plants which had more leaves, higher leaf chlorophyll concentration, and higher mycorrhizal colonization transferred less Cu to the shoots. One of the primary strategies of plants to cope with Cu-toxicity is preventing Cu transfer from entering the sensitive shoots by depositing the free Cu ions and ion-ligand complexes into vacuoles or cell walls (Printz et al., 2016). In mycorrhizal roots, Cu is additionally sequestrated in the intraradical fungal structures, most probably co-localised with polyphosphate in vacuoles. Since free Cu^{+} ions are redox-active and prone to induce oxidative stress, in the cytosol, they are sequestered and delivered in a bound form by Cu-chaperones (Ferrol et al., 2016). The genome of *R. irregularis* fungus contains three genes encoding putative chaperons. Therefore, a higher *R. irregularis* colonisation may increase Cu sequestration into the roots.

The variance of Cu concentration in the shoot was also explained by leaf chlorophyll concentration and the number of leaves, where more vigorous plants, with higher capacity to supply photosynthates, have lower Cu transfer to the leaves, diminishing toxicity for the most sensitive sites. These plant traits may lead to a larger root system which may retain more Cu and a higher efficiency of AMF due to the higher supply of carbohydrates. In contrast with *R. irregularis* plants, the *P. laccatum* plants showed no relationships between shoot Cu concentration and AMF colonisation or different plant traits. This shows how distinct AMF can have highly contrasting effects on the content and distribution of heavy metals. It has been previously shown that distinct AMF may differentially affect Zn translocation within young poplar trees (Lingua et al., 2008). An alternative explanation could be the relatively low colonisation (14%) of *P. laccatum* plants.

4.3. Lethal Cu concentrations change the contents and concentrations of other nutrients in poplars

Unexpectedly, elements other than Cu also significantly changed their concentrations in the plant under lethal Cu application within 48 h. In the root, Cu uptake follows two pathways, the passive uptake, driven by the concentration gradient across the membrane and the substrate-specific and energy-dependent uptake. Under the extreme stress of lethal Cu toxicity, the plant would not have the capacity to use an energy-dependent transport pathway. We presume that the changes we observed were driven by an extreme concentration gradient. Ionic radii of Cu (140 pm) is similar to K (133 pm) and Mn (137 pm), hence they may have common transport mechanism based on Marschner (1995) and Fageria (2001), which may result in competing with their uptake pathways, and this competition reduced K and Mn concentrations in roots under Cu application. Our findings also support the results of Lidon and Henriques (1993) who reported decreased root K concentrations under high Cu concentrations in a hydroponic experiment with rice. We should emphasise that root damages caused by Cu toxicity may reduce water content and disturb the balance of nutrient contents (Costa and Morel, 1994).

While we observed synergistic effect on both root and shoot concentration between Cu and Fe in poplars, contrary to our findings, Rivelli et al. (2012) observed that Fe concentrations increased in roots and decreased in shoots of *Helianthus annuus* L. with Cu application at the rate of 400 mg kg^{-1}, and they concluded that high Cu concentrations could negatively impact Fe translocation. Similar results were also observed by Madejón et al. (2003) and Garcia et al. (2006). Pätsikkä et al. (2002) observed that excess Cu caused Fe deficiency in soybean, Chen et al. (2004) stated that Fe deficiency resulted in high Cu accumulation in *Commelina communis* L. plants, and Rombolà et al. (2005) found that Fe deficiency increased Cu concentrations in sugar beet leaves. Some other studies observed that leaf Fe content was not influenced by high leaf Cu concentrations under soil Cu toxicity conditions in different plant species (Kitagishi and Yamane, 1981; Lanaras et al., 1993; Panou-Filotheou and Bosabalidis, 2004). We did not find any data in the literature confirming our results. These differences are maybe due to different Cu tolerance strategies adopted by different plant species.

In line with the relationships between applied substrate Cu levels and nutrient concentrations in roots and shoots, PCA showed that root Cu concentration was inversely related with root K and Mn concentrations, and shoot Cu concentration had a positive correlation with shoot Fe and K concentrations. Factor 1 in roots and shoots separated different applied Cu levels from each other, showing that the nutrient concentrations in roots and shoots were mostly impacted by applied substrate Cu levels, what we did observe in the same analysis under different mycorrhizas (figure not shown).

5. Conclusion

In this study, we subjected poplar trees to threshold-lethal and supra-lethal doses of copper in a pulse to mirror a contamination event and tested whether rapid changes can occur under mycorrhizal compared to non-mycorrhizal conditions within 48 hours. We also used two different species of arbuscular mycorrhizal fungi to ascertain whether there is a fungal species effect, given the known functional diversity of arbuscular mycorrhizal fungi for heavy metal tolerance. We concluded that 729 mg Cu kg^{-1} substrate was a lethal dose for young poplars and further research should investigate the highest levels of substrate Cu which these plants can tolerate. Cu toxicity caused reduced root biomass. *R. irregularis* and *P. laccatum* resulted in higher translocation of Cu from roots to shoots under threshold-lethal and supra-lethal applications of Cu, respectively. Based on this, we state that mycorrhizal mediation of Cu partitioning in poplars is specific to fungal species and substrate Cu levels. We also observed that in plants colonised with *R. irregularis*, a higher mycorrhizal colonisation may prevent Cu transfer to the shoot. We probably did not observe this effect in *P. laccatum* plants due to the relatively low colonisation rate (14%). Magnesium, K, and Mn concentrations in roots decreased with increasing applied substrate Cu due to their similar ionic radii with Cu and having common transport mechanism. We also observed synergistic effect on shoot concentration between applied substrate Cu levels and Mg, K, Ca, Fe, and Zn. Root Cu concentration was inversely related with root K and Mn concentrations, and shoot Cu concentration had a positive correlation with shoot Fe and K concentrations. Nutrient concentrations in roots and shoots were impacted by applied substrate Cu levels, but not by mycorrhizas.

CRediT authorship contribution statement

Tibbett Mark: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Hales-Henao Aysha:** Investigation,

Methodology. **Pena Rodica:** Conceptualization, Data curation, Methodology, Writing – review & editing. **Soltangheisi Amin:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mark Tibbett reports financial support was provided by UK Research and Innovation.

Data Availability

Data will be made available on request.

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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.2024.116112.

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