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Short communication

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

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ABSTRACT

We hypothesised that cadmium exposure would hinder growth and secretion of carbon-degrading enzymes by mycorrhizal fungi, and that this would vary according to their tolerance to cadmium stress. The enzymes measured were β -Glucosidase, β -Xylosidase, β -D-cellulobiosidase, N-acetyl- β -Glucosaminidase in three strains of ectomycorrhizal fungi *Hebeloma subsaponaceum*, *Scleroderma* sp., *Hebeloma* sp. and a feremycorrhizal fungus *Austroboletus occidentalis*. Fungi were subjected to cadmium stress for 28 d (in modified Melin-Norkrans liquid medium). The results showed unanticipated differential response of enzyme activities among the fungal species, including potential hormesis effects. *Austroboletus occidentalis* showed an increase in enzyme activity under cadmium stress.

Keywords: ecotoxicity, ectomycorrhiza, enzyme activity, heavy metal.

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Secretion of enzymes by soil borne mycota are 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, & Bremen, 2001). This mycorrhizal symbiosis is based on a reciprocal exchange of solutes; the fungus providing nutrients and water to the plant in return for sugars from the phytobiont (Smith & Read, 2008). A newly described non-colonising symbiosis feremycorrhiza (FM) in which the fungal hyphae inhabits rhizospheric soil but does not penetrate the plant roots (Kariman, Barker, Finnegan, & Tibbett, 2013; Kariman, Barker, & Tibbett, 2018), presents a remarkable capacity for biological dissolution of soil minerals and nutrient mobilization (Kariman, Barker, Finnegan, & Tibbett, 2012; Kariman et al., 2013). 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 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, Bréda, & 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 facultatively 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 saprotrophic role in ECMF can be affected by heavy metal contamination in soils (Bellion, Courbot, Jacob, Blaudez, & Chalot, 2006), which is a pressing concern due 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 a 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 nutritional status and growth (Krznicic et al.,

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2009). Direct and indirect effects on the fungal performance are expected in polluted soils, as studies have shown that Cd affects soil microbiota (Landi, Renella, Moreno, Falchini, & Nannipieri, 2000; Chen et al., 2014) 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, Fransson, Finlay, & Hees, 2008). There is a need for a better understanding the influence of Cd on 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 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.

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: *H. subsaponaceum*, *Hebeloma* sp., *Scleroderma* sp. and the FM species *Austroboletus occidentalis* (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 $\pm 22^{\circ}\text{C}$ 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, Darmstadt, Germany) to separate the mycelium from the liquid culture. The culture filtrate was collected for determination of enzyme activities.

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 μL of culture filtrate, 100 μL of the respective substrate and 50 μL 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).

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 methylumbelliferon (MU), and MMN medium without Cd addition. Microplates were covered with aluminium foil and incubated for 1.5 h at 26°C, the fluorescence was determined immediately on a multidetection plate reader SpectraMax i3x (Molecular Devices, LLC. Sao Jose, United States), with excitation at 360 nm and emission at 460 nm. All enzyme activities were expressed as μmol activity per gram of dry weight of fungi (mycelium) per 1.5 h ($\mu\text{mol}/1.5 \text{ h/g DW}$).

Statistical analyses were performed on the dry weight of fungi's mycelium (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. 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.

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. 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 increases 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, Castro, & Azevedo, 2020). In an experiment, Baldrian and Gabriel (2002) also found that under Cd exposure, a wood-rotting fungus *Piptoporus betulinus* form a dense layer of hyphae, which does not happen without Cd. In contrast, in the work from De 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* 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*.

Table 1. Strains of mycorrhizal basidiomycetes selected for this study

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

Table 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-Cellulobiosidase (CB) EC 3.2.1.91	Hydrolysis of cellulose

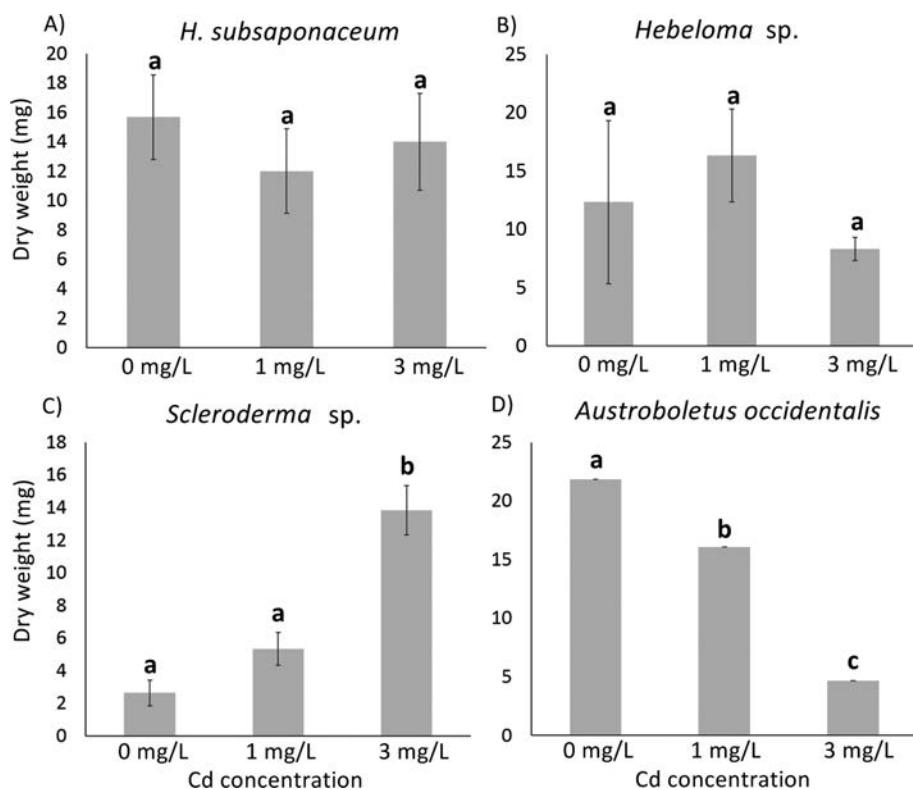


Fig. 1 - Dry weight of three ectomycorrhizal fungi species and one feremycorrhiza species after growing under Cd exposure for 28 d. A: *Hebeloma subsaponaceum*, (W), B: *Hebeloma* sp. (D), C: *Scleroderma* sp. (Sc Hu) and D: *Austroboletus occidentalis* (AB). Different letters represent significant differences by Dunn test ($p < 0.05$).

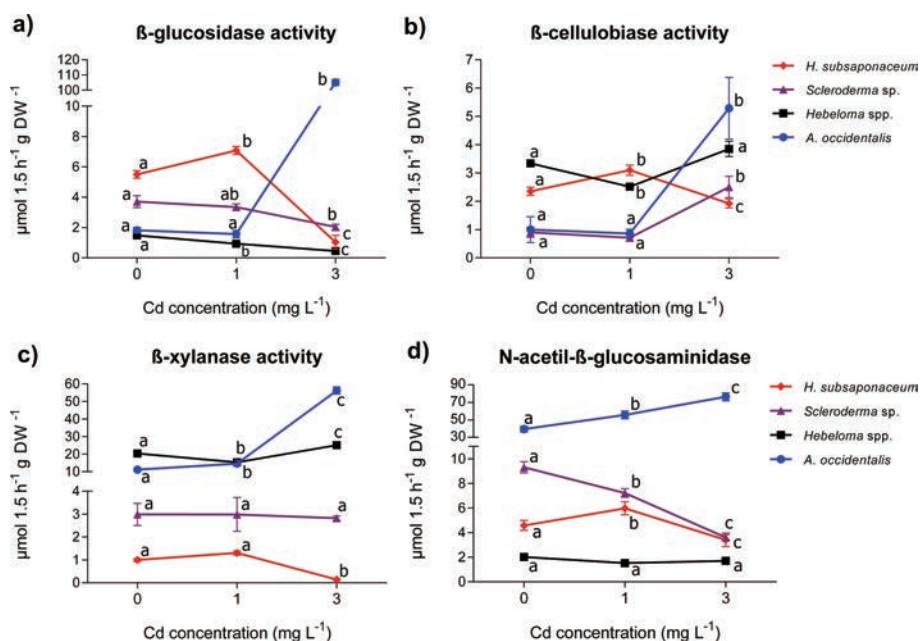


Fig. 2 - Activities of four extracellular enzymes produced by *Hebeloma subsaponaceum* (W), *Scleroderma* sp. (Sc Hu), *Hebeloma* sp. (D) and *Austroboletus occidentalis* (AB) grown under three different Cd concentrations for 28 d. A: β-glucosidase, B: N-acetyl-β-glucosaminidase, C: β-D-cellubiosidase, D: β-xylanase. Different letters represent significant differences between Cd treatments within the same species (Dunn test, $p < 0.05$).

dentalis, while *H. subsaponaceum* had a decrease in activity under the same Cd concentration (Fig. 2A). In contrast to our hypothesis, 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, Young, Stephenson, & Wei, 2012). For instance, Wang et al. (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 & Scherer, 1992). Tibbett, Sanders, Grantham and Cairney (2000) also emphasized 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 -xytanase was higher in *Hebeloma* sp. under 3 mg/L of Cd (Fig. 2B, C). Similar increase was also observed in an ericoid mycorrhizal fungi (*O. 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, increase in the release of extracellular enzymes has been reported in the bacteria *Erwinia* spp. due to general DNA damage by stress factors (Barras, van Gijsegem, & 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 Shah et al. (2016) 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 the environment (Shah et al., 2016; Zak et al., 2019). 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 decrease 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.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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