Copper and zinc affect the activity of plasma membrane H\(^{+}\)-ATPase and thiol content in aquatic fungi

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Aquatic hyphomycetes are the major microbial decomposers of plant litter in streams. We selected three aquatic hyphomycete species with different abilities to tolerate, adsorb and accumulate copper and zinc, and we investigated the effects of these metals on H\(^{+}\)-ATPase activity as well as on the levels of thiol (SH)-containing compounds. Before metal exposure, the species isolated from a metal-polluted stream (Heliscus submersus and Flagellospora curta) had higher levels of thiol compounds than the species isolated from a clean stream (Varicosporium elodeae). However, V. elodeae rapidly increased the levels of thiols after metal exposure, emphasizing the importance of these compounds in fungal survival under metal stress. The highest amounts of metals adsorbed to fungal mycelia were found in the most tolerant species to each metal, i.e. in H. submersus exposed to copper and in V. elodeae exposed to zinc. Short-term (10 min) exposure to copper completely inhibited the activity of H\(^{+}\)-ATPase of H. submersus and V. elodeae, whilst zinc only led to a similar effect on H. submersus. However, at longer exposure times (8 days) the most metal-tolerant species exhibited increased H\(^{+}\)-ATPase activities, suggesting that the plasma membrane proton pump may be involved in the acclimation of aquatic hyphomycetes to metals.

INTRODUCTION

There is a growing interest in understanding the ecological, physiological and biochemical properties that allow some fungi to colonize and live in metal-polluted habitats. Fungi play a critical role in organic matter turnover in streams. Amongst fungi, aquatic hyphomycetes appear to have the greatest ecological role as decomposers of plant detritus in streams (Baldy et al., 2002; Pascoal & Cássio, 2004). Even though metal pollution lowers the biodiversity and activity of aquatic hyphomycetes, the occurrence of these groups of fungi has been consistently reported in metal-polluted streams (Pascoal et al., 2005a; Sridhar et al., 2005). This means that fungi, similar to other living organisms, have to tightly regulate the intracellular metal concentration in such a way that safe uptake of the required metal ions in the cytosol and organelles can occur without cellular damage due to metal toxicity (Kneer et al., 1992). Metal tolerance in fungi can be achieved by several complex mechanisms, including extracellular precipitation, biosorption, controlled uptake and intracellular sequestration and/or compartmentalization, whose relative contributions to metal detoxification can vary with metal type and fungal species (Krauss et al., 2011). Therefore, we are still far from fully understanding the mechanisms underlying metal tolerance/resistance in fungi, despite the large amount of information on the effects of metals in living organisms.

Metals toxicity in fungi may result from direct interaction between metal ions and biomolecules or from mechanisms related to the ability of metals to generate reactive oxygen species (ROS) (Stohs & Bagchi, 1995; Azevedo et al., 2007). Transition metals, such as copper and iron, greatly increase ROS production through the Fenton reaction (Bai et al., 2003). However, even non-redox-active metals, such as zinc, can lead to ROS production by depleting free radical scavengers, such as thiol (SH) compounds.

Abbreviations: DTNB, 5, 5\'-dithio-bis(2-nitrobenzoic acid); NP-SH, non-protein thiol; PB-SH, protein-bound thiol; ROS, reactive oxygen species; T-SH, total thiol.
(Dietz et al., 1999). Amongst the cellular macromolecules, the polyunsaturated fatty acids of biological membranes are preferential targets for ROS attack (Howlett & Avery, 1997). In fungi, lipid peroxidation induced by copper leads to a decline in plasma membrane lipid order (Howlett & Avery, 1997; Fernandes et al., 1998), subsequently increasing the non-specific permeability of the membrane (Ohsumi et al., 1988). Therefore, copper readily permeates the plasma membrane, acting as a depolarizer of cell electrical potential (Kennedy & Gonsalves, 1987). In fungi, the proton pump ATPase ($H^+\text{-ATPase}$) is the major protein component of the plasma membrane, accounting for 15–20 % of the total plasma membrane proteins (Ambesi et al., 2000). It couples ATP hydrolysis to the extrusion of protons, generating an electrochemical gradient (Serrano, 1988). This proton pump plays a key role in cell physiology by controlling essential cellular functions such as nutrient uptake and intracellular pH regulation (Serrano, 1988; Portillo, 2000). In addition, fungal responses to several environmental stressors, including heat (Piper, 1993), ethanol (Rosa & Sá-Correia, 1992), weak acids (Holyoak et al., 1996; Viegas et al., 1998) and metals (Karamushka & Gadd, 1994; Fernandes et al., 1998), have been associated with changes in $H^+\text{-ATPase}$ activity. In Saccharomyces cerevisiae, mild copper stress stimulates $H^+\text{-ATPase}$ probably for re-establishing the cellular electrochemical gradient, but the activity of this pump declines at maximal copper concentration that allows yeast growth (Fernandes et al., 1998). Specific Cys residues of the $H^+\text{-ATPase}$ are targets for iron and copper Fenton reagents, leading to enzyme inactivation (Stadler et al., 2003). Consistently, reduced-thiol groups were shown to be essential for maintaining $H^+\text{-ATPase}$ activity under iron stress in wheat root plasma membranes (Yang et al., 2003).

We previously reported that exposure of aquatic hyphomycetes to copper and zinc led to intracellular ROS accumulation and plasma membrane disruption (Azevedo et al., 2007). In the hyphomycetes, metal tolerance was associated with increased levels of thiol compounds in cells (Miersch et al., 2001; Braha et al., 2007; Guimaraes-Soares et al., 2007), and there is evidence pointing to glutathione, phytochelatins and metallothioneins as putative metal sequesters or ROS scavengers (Jaeckel et al., 2005; Guimaraes-Soares et al., 2006).

We selected three aquatic hyphomycete species with different sensitivities to copper and zinc, and examined their ability to adsorb and accumulate these metals. Subsequently, we assessed the effect of copper and zinc on $H^+\text{-ATPase}$ activity and on the levels of thiol-containing compounds. We expected that species more tolerant to metals would have a higher ability to adsorb metal ions, to minimize their uptake and/or have higher intracellular levels of thiol-containing compounds to deal with metal accumulation within cells. In addition, we expected an increased activity of the plasma membrane proton pump to counteract metal-induced dissipation of the electrochemical gradient, which is essential for fungal survival.

**METHODS**

**Fungi and culture maintenance.** The aquatic hyphomycetes *Helioclis submersus* H.J. Huds. (UMB-135.01), *Flagellospora curta* J. Webster (UMB-39.01) and *Varicosporium elodeae* W. Kegel (UMB-142.01) were isolated from single spores collected in streams in northwest Portugal. The former two species were isolated from leaves collected in the Este River, downstream from the industrial park of the city of Braga, at a site with high nutrient loading (Pascoal et al., 2005b) and metals in the stream water (Soares et al., 1999). The latter species was isolated from foams collected in a clean stream of the Peneda-Gerês National Park (Pascoal et al., 2005b).

In the laboratory, fungi were maintained on 2 % (w/v) malt extract and 1.5 % (w/v) agar at 18 °C under artificial light.

**Growth conditions and metal exposure.** Conidial suspensions (six conidia ml$^{-1}$, final concentration) of each aquatic hyphomycete species were placed in Erlenmeyer flasks containing mineral medium with vitamins and 2 % (w/v) glucose (van Uden, 1967) at pH 5.0, with or without addition of copper (CuCl$_2$) or zinc (ZnCl$_2$). Metals were added to the culture medium in final concentrations ranging from 50 to 2000 μM for copper and from 250 to 10 000 μM for zinc. The flasks were kept for 8 days at 18 °C, 160 r.p.m. under artificial light (Certomat BS 3; Braun Biotech International). At the harvest time, fungal cultures were at the end of exponential growth phase (not shown).

To assess short-term effects, fungal mycelia grown for 8 days without metal addition were transferred to fresh medium and exposed to copper or zinc for 10 min, in the case of $H^+\text{-ATPase}$ assays, or for 14 and 62 h to estimate the concentration of thiol compounds in mycelia (see Preparation of cell-free extracts and quantification of thiol compounds).

To quantify fungal biomass, mycelia were harvested by filtration, washed twice with deionized water and dried at 85 °C to constant mass before being weighed (0.001 g).

**Plasma membrane ATPase assay.** The $H^+\text{-ATPase}$ activity was evaluated by measuring the rate of proton efflux after addition of 0.2 % glucose to suspensions of fungal mycelium. Fungal suspensions were prepared in deionized water with a Dounce homogenizer. Rate of proton efflux was measured by recording proton movements with a standard pH meter (PHM 92 Lab pH Meter) connected to a recorder (Kipp and Zonen 024). The pH electrode was immersed in a water-jacketed chamber that was magnetically stirred. An aliquot of 1 ml mycelium suspension was diluted in water to a final volume of 5 ml and the pH of the mixture was adjusted to 5.0 with NaOH (1 M to 10 mM) or HCl (1 M to 10 mM) prior to the addition of glucose. The slope at the initial part of the acidification curve allowed the determination of initial proton movements. Changes in pH were converted in nmol H$^+\text{m}^{-2}$ (mg dry mass)$^{-1}$ by comparing the acidification curve after glucose addition to cell suspensions with that of the addition of known amounts of 10 mM HCl.

**Preparation of cell-free extracts and quantification of thiol compounds.** Fungal mycelia were harvested by filtration, washed twice with deionized water and pressed between two layers of filter paper to remove surplus water. The mycelia were then mixed with purified sea sand [2 g (g mycelium wet mass)$^{-1}$] and ground in liquid nitrogen in a cooled mortar for 4 min. The mixture was suspended in 20 mM Tris/1 mM EDTA, pH 7.5, and cell-free extracts were obtained by two sequential centrifugation steps (6200 for 10 min; 18 000 for 50 min) at 4 °C.

The concentrations of total thiols (T-SH) and non-protein thiols (NP-SH) were determined according to Sedlak & Lindsay (1968) with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). To quantify T-SH...
compounds, 50 μl cell-free extract was mixed with 150 μl 0.2 M Tris, pH 8.2 and 10 μl 0.01 M DTNB in a final volume of 1.0 ml absolute methanol. After 15 min, the mixtures were centrifuged (3000 g, 15 min) and the A412 measured (Perkin Elmer Lambda 2 UV/Vis spectrometer) against a blank (without sample) using reduced glutathione (Sigma) as standard.

To determine the concentration of NP-SH, 50 μl cell-free extract was mixed with 400 μl MilliQ water and 100 μl 50 % trichloroacetic acid. The mixtures were shaken for 12 min before being centrifuged (3000 g, 15 min). Then, 200 μl supernatant was mixed with 400 μl 0.4 M Tris, pH 8.9 and 10 μl 0.01 M DTNB, and the A412 measured within 3 min. The concentration of protein-bound thiols (PB-SH) was calculated by subtracting the NP-SH concentration from the T-SH concentration. All buffers and solutions were previously gassed with nitrogen stream.

Metal adsorption and accumulation. Samples of 50 mg fungal mycelia were washed with 100 ml deionized water, then with 100 ml 20 mM NiCl$_2$ three times and finally with deionized water (100 ml) to remove metals adsorbed to fungi. The mycelia were then digested with 4 ml 65 % (v/v) HNO$_3$ and 2 ml 30 % (v/v) H$_2$O$_2$ in a water bath at 100 °C for 40 min to further quantify metal accumulation. The concentration of copper or zinc in the NiCl$_2$ washings and in the digested mycelia was determined by inductively coupled plasma atomic emission spectrometry (PU 7000 ICP; Philips).

Data analysis. Data on the effects of copper and zinc on the H$^+$-ATPase activity and production of thiol compounds were expressed as percentage of control. Values were divided by 1000 and arcsine-square-root-transformed to achieve normal distribution and homoscedasticity (Zar, 2010). For each metal and fungal species, H$^+$-ATPase activity and production of thiol compounds were compared by one-way ANOVA, followed by Dunnett’s tests to identify treatments that differed significantly from control (Zar, 2010).

The effective metal concentrations inhibiting fungal biomass production by 25 % (EC$_{25}$) and 50 % (EC$_{50}$) were estimated by the Probit method (Warldaw, 1985) and compared by one-way ANOVA, followed by Tukey’s tests to identify significant differences (Zar, 2010).

Statistic analysis was performed with Prism 4 for Windows (GraphPad).

RESULTS

Metal toxicity, adsorption and accumulation

The sensitivity of the aquatic hyphomycetes to copper and zinc was assessed by EC$_{25}$ and EC$_{50}$ after 8 days of growth (Table 1). H. submersus was the most resistant species to copper, followed by V. elodeae and F. curta. The most resistant species to zinc was V. elodeae, whilst H. submersus was the most sensitive species.

The mycelium of H. submersus showed a higher ability to adsorb copper (70.5 μmol g$^{-1}$) than the other fungal species and accumulated 2.6 times more copper [13.5 μmol (g dry mass)$^{-1}$] than the less accumulative species (V. elodeae) (Table 2). Differences in the amounts of metal adsorbed and accumulated between fungal species were more pronounced for zinc than for copper (Table 2). Mycelium of V. elodeae had the highest amounts of adsorbed zinc [354 μmol (g dry mass)$^{-1}$]; zinc adsorption in this species was 8.5 and 32 times higher than in F. curta and H. submersus, respectively. Zinc accumulation in F. curta mycelium [175.1 μmol (g dry mass)$^{-1}$] was five and 159 times higher than in the mycelium of V. elodeae and H. submersus, respectively (Table 2).

Table 1. EC$_{25}$ and EC$_{50}$ of copper and zinc inhibiting biomass production by aquatic hyphomycetes

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Copper (μM)</th>
<th>Zinc (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{25}$</td>
<td>EC$_{50}$</td>
</tr>
<tr>
<td>H. submersus</td>
<td>1013 ± 12$^a$</td>
<td>1510 ± 19$^a$</td>
</tr>
<tr>
<td>V. elodeae</td>
<td>323 ± 38$^b$</td>
<td>457 ± 48$^b$</td>
</tr>
<tr>
<td>F. curta</td>
<td>61 ± 2$^c$</td>
<td>183 ± 7$^c$</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM n=3. In each column, different letters indicate significant differences (Tukey’s tests, P<0.05).

Effects of copper and zinc on H$^+$-ATPase activity

The H$^+$-ATPase activity was evaluated by measuring the rate of H$^+$ efflux after addition of glucose to suspensions of fungal mycelium. In the absence of metals, H$^+$ efflux varied between 0.023 and 0.061 nmol H$^+$ s$^{-1}$ (mg dry mass)$^{-1}$, with the lowest value found in V. elodeae and the highest in F. curta (Table 3). Long-term exposure (8 days) of fungal mycelium to the EC$_{50}$ of copper significantly increased the H$^+$ efflux associated with the addition of glucose in H. submersus (2.3 times) and in F. curta (1.7 times), but did not affect the activity of H$^+$-ATPase in V. elodeae (Table 3). Long-term exposure to the EC$_{50}$ of zinc led to a 2.7-fold increase in H$^+$-ATPase activity in V. elodeae (Table 3). On the contrary, zinc had no significant effect on H$^+$ efflux in H. submersus and F. curta (Table 3).

Short-term exposure (10 min) to the EC$_{25}$ or EC$_{50}$ of copper led to a total inhibition of H$^+$ efflux in H. submersus and V. elodeae (Table 4). The H$^+$-ATPase activity of F. curta was not significantly affected by exposure to the EC$_{25}$ of copper, but its activity increased 1.5 times after exposure to the EC$_{50}$ (Table 4). The H$^+$-ATPase activity was not affected by short-term exposure to the EC$_{25}$ of zinc in any fungal species. Exposure to the EC$_{50}$ of zinc totally inhibited the proton pump of H. submersus, had no effect on that of V. elodeae and stimulated that of F. curta (Table 4).

Effects of copper and zinc on the production of thiol compounds

The levels of thiol compounds were evaluated in 8-day-old mycelia of aquatic hyphomycetes exposed or not to the EC$_{50}$ of copper or zinc (for metal concentrations, see Table 1).
Values are presented as mean ± SEM, n=3; –, total inhibition.
*Significant differences (Dunnett’s tests, P<0.05).

Table 4. Effects of short-term exposure (10 min) to copper or zinc on the H⁺-ATPase activity in aquatic hyphomycetes

Responses of aquatic fungi to metal stress
Table 5. Concentration of T-SH, NP-SH and PB-SH in the mycelia of aquatic hyphomycetes grown for 8 days with no addition of metals and transferred to fresh medium for 14 or 62 h

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Incubation period (h)</th>
<th>T-SH</th>
<th>NP-SH</th>
<th>PB-SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. submersus</td>
<td>0</td>
<td>6.32 ± 1.22</td>
<td>5.06 ± 0.91</td>
<td>1.25 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4.93 ± 0.60</td>
<td>2.33 ± 1.67</td>
<td>2.60 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>4.36 ± 0.62</td>
<td>2.03 ± 0.76</td>
<td>2.33 ± 1.22</td>
</tr>
<tr>
<td>V. elodeae</td>
<td>0</td>
<td>2.59 ± 0.32</td>
<td>1.28 ± 0.18</td>
<td>1.3 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.55 ± 0.12</td>
<td>1.06 ± 0.10</td>
<td>1.49 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>5.35 ± 0.34</td>
<td>2.9 ± 0.18</td>
<td>2.44 ± 0.5</td>
</tr>
<tr>
<td>F. curta</td>
<td>0</td>
<td>5.41 ± 1.11</td>
<td>3.51 ± 0.14</td>
<td>1.89 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5.19 ± 2.07</td>
<td>2.25 ± 0.78</td>
<td>2.95 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>7.54 ± 1.67</td>
<td>4.75 ± 0.24</td>
<td>2.79 ± 1.44</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM of at least three independent experiments.

i.e. when H. submersus was exposed to copper and V. elodeae was exposed to zinc. The stimulation of H⁺-ATPase by metals at longer exposure times may help to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane (Serrano, 1988; Fernandes et al., 1998), suggesting that H⁺-ATPase may be involved in aquatic hyphomycete acclimation to metals. Moreover, the acidification of the extracellular medium by proton pump activation may lead to an increased competition of cations for cellular binding sites, reducing potential interactions between metals and cells (Gadd, 1993), even when the decrease in pH increases metal bioavailability (Spry & Wiener, 1991). Accordingly, metal uptake in fungi is often reduced by a decrease in the extracellular pH (copper, Gadd & White (1985); zinc, Ross (1994)). In plant roots, copper inhibited the hydrolytic activity of H⁺-ATPase (Buzynski & Kolano, 2003; Janicka-Russak et al., 2008). Protein phosphorylation depends on the energetic status of the cell, and metal exposure decreased the amount of the ATP in root tissues (Janicka-Russak et al., 2008). This suggests that, in addition to the dephosphorylation intensity of the proton pump, the ATP level is an important factor in enzyme deactivation under metal stress. In fact, bearing this in mind we can speculate that metals affect the respiration and ATP content in aquatic hyphomycetes.

In this study, the highest amounts of copper and zinc adsorbed to fungal mycelia were found in H. submersus and V. elodeae, respectively. As these fungi were the most tolerant species to each metal, biosorption appears to be a relevant mechanism to avoid unstrained uptake of metals, minimizing their deleterious effects. Nevertheless, these fungal species accumulated metals in their mycelia, although in much lower amounts compared with those adsorbed. The ability of aquatic hyphomycetes to take up and store large quantities of metals makes them potential candidates for bioremediation. In this context, V. elodeae and F. curta showed a remarkable ability to adsorb and accumulate zinc [390 and 217 μmol (g dry mycelium)⁻¹ during only 14 h metal exposure, respectively] compared with values reported for other aquatic fungi (Jaekel et al., 2005). Also, H. submersus was able to retain around seven times more copper than metal-tolerant strains of Helicus lugdunensis (Braithwaite et al., 2007). However, adsorption of metals to filamentous fungi depends on the environmental context, including pH, initial metal concentration and medium composition (Gardea-Torresdey et al., 1997; Lo et al., 1999), probably explaining why no noticeable metal adsorption was previously found in V. elodeae and H. submersus (Azevedo et al., 2007). The environment is also expected to alter the sensitivity of fungi to metals. Indeed, the sensitivity of these species varies when cultivated in different carbon sources (Azevedo et al., 2007; Azevedo & Cašio, 2010; present work). Although the background of the fungal species (or strain) may influence their responses to metal stress (Guimarães-Soares et al., 2007; Fernandes et al., 2011), this is not always the case. In our study, the species more tolerant to zinc (V. elodeae) was isolated from a clean stream, whilst the species more tolerant to copper (H. submersus) was isolated from a metal-polluted stream. To clarify these aspects, further studies with more fungal species and strains would be helpful, but unfortunately fungal populations with different backgrounds are not easily available.

In aquatic fungi, metal tolerance has been also associated with the synthesis of thiol-enriched compounds, which are able to bind metals within cells (Miersch et al., 1997, 2001; Guimarães-Soares et al., 2006, 2007), and/or scavenging ROS (Bai et al., 2003). In the present work, H. submersus and F. curta, the two species isolated from a metal-polluted stream, had higher levels of NP-SH and PB-SH compounds before metal exposure compared with V. elodeae, the species isolated from a clean stream. Short-term exposure to copper led to a decrease in both types of thiol compounds in the most resistant species (H. submersus), but not in the most sensitive species (F. curta). The decrease in reduced thiol...
compounds might be due to their oxidation during metal sequestration (Cobbett & Goldsbrough, 2002; Guimarães-Soares et al., 2006) or ROS scavenging (Bai et al., 2003), suggesting that the high constitutive levels of thiols might have helped H. submersus to deal with copper stress. In addition, the decrease in NP-SH compounds in all fungal species after long-term exposure to copper is consistent with the ability of peptides with very low molecular mass, most likely glutathione and phytochelatins, to bind copper in aquatic hyphomycetes (Guimarães-Soares et al., 2006).

Fig. 1. Concentration of T-SH (diagonal lines), NP-SH (chequer-board) and PB-SH (horizontal lines) after short-term (14 and 62 h) and long-term (8 days) exposure to the EC_{50} of copper in (a) H. submersus, (b) V. elodeae and (c) F. curta. Values are percentage of control (control is 100 %) and are presented as mean ± SEM, n=3. * Dunnett’s test, P<0.05. Actual values of controls are given in Table 5.

V. elodeae, which had the lowest constitutive thiol levels, responded to metal exposure with a rapid increase in the levels of NP-SH and PB-SH. These findings reinforce previous observations that high constitutive levels of thiols or the rapid increase in their production may help aquatic hyphomycetes to deal with metal stress (Guimarães-Soares et al., 2006, 2007).

ACKNOWLEDGEMENTS

The European Regional Development Fund – Operational Competitiveness Programme (FEDER-POFC-COMPETE) and the Portuguese Foundation for Science and Technology supported this study (PEst-C/BIA/UI4050/2014).
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Edited by: V. Cid