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Responses of antioxidant defenses to Cu and Zn stress in two aquatic fungi

Maria-Manuel Azevedo [a], Agostinho Carvalho [b], Cláudia Pascoal [a], Fernando Rodrigues [b], Fernanda Cássio [a,*]

[a] *Centre of Biology, Department of Biology, University of Minho, 4710-057 Braga, Portugal*
[b] *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal*

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Abstract

Aquatic hyphomycetes are fungi that play a key role in plant litter decomposition in streams. Even though these fungi occur in metal-polluted streams, the mechanisms underlying their tolerance to metals are poorly documented. We addressed the effects of Zn and Cu in *Varicosporium elodeae* and *Heliscus submersus* by examining metal adsorption to cell walls, plasma membrane integrity and production of reactive oxygen species at metal concentrations inhibiting biomass production in 50% or 80%. The activity of the enzymes catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase was measured to elucidate their role in coping with oxidative stress induced by metals at short- (14 h) and long- (8 days) term exposure. Results show that *V. elodeae* was more susceptible to the toxic effects induced by Cu and Zn than *H. submersus*, as indicated by more extensive inhibition of biomass production. Both metals, particularly Cu, induced oxidative stress in the two fungal species, as shown by the noticeable recovery of biomass production in the presence of an antioxidant agent. In both fungi, Cu induced a more severe disruption of plasma membrane integrity than Zn. Our studies on antioxidant defenses showed that catalase had a greater role alleviating stress induced by Zn and Cu than superoxide dismutase. Chronic metal stress also stimulated the production of NADPH, via the pentose phosphate pathway by increasing the activity of glucose-6-phosphate dehydrogenase. Our results suggest that the tolerance of aquatic hyphomycetes to Cu and Zn is associated with the ability of these fungi to initiate an efficient antioxidant defense system.

Keywords: Aquatic fungi; Metal stress; ROS; Antioxidant enzymes

1. Introduction

Freshwater pollution by heavy metals is a worldwide problem with serious environmental consequences. Heavy metals can be introduced into ecosystems through industrial effluents and wastes, agricultural fungicide runoff, domestic garbage dumps and mining activities (Merian, 1991). The non-degradability of metals, their accumulation in biota, and biomagnification along aquatic food chains (Spacie et al., 1995) contribute to the importance of studying metal effects in biological systems.

Metals, such as Cu and Zn, are essential for living organisms, including fungi, although elevated concentrations of metals can result in growth inhibition and toxicity. The ability of organisms to survive in environments with high levels of metals depends on their capacity to regulate

* Corresponding author. Tel.: +351 253604045; fax: +351 253678980.
E-mail address: fcassio@bio.uminho.pt (F. Cássio).

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intracellular concentration of metal ions. In fungi, metal tolerance has been attributed to several mechanisms, including trapping of metal by cell-wall components, altered metal uptake, extracellular chelation or precipitation by secreted metabolites, and intracellular complexation by metallothioneins (Gadd, 1993).

The toxicity of metals can be the result of the generation of reactive oxygen species (ROS) that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Moradas-Ferreira et al., 1996; Bai et al., 2003). In *Saccharomyces cerevisiae*, the primary mechanism of Cu toxicity is the disruption of cell and organelle membranes, resulting in a loss of membrane integrity and impairment of membrane function (Ohsumi et al., 1988). This effect has been attributed to the redox active nature of Cu and its ability to generate free radicals that promote lipid peroxidation (Stohs and Bagchi, 1995). On the other hand, non-redox active metals like Zn can deplete free-radical scavengers, such as thiol-containing compounds, resulting in ROS production (Dietz et al., 1999).

Tolerance of the yeast *Candida intermedia* to different metals has been associated with its ability to deal with ROS generation (Fujs et al., 2005). Fungi display several antioxidant enzymes against ROS, including catalase (CAT), superoxide dismutases (SOD), glutathione peroxidase and glutathione reductase, capable of removing oxygen radicals and their products and/or repairing oxidative damage (Jamieson, 1998; Bai et al., 2003). In addition, molecules such as glutathione, besides playing an important role in cellular protection during oxidative stress, may complex metals in cells (Pócsi et al., 2004). Glutathione recycling is dependent on the maintenance of an intracellular pool of NADPH mainly via the pentose phosphate pathway, in which the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting step (Jamieson, 1998).

Aquatic hyphomycetes are a phylogenetically heterogenous group of fungi that play a crucial role in plant litter decomposition in streams, mediating carbon and energy transfer to higher trophic levels (Bärlocher, 1992). Metals, such as Cd, Cu and Zn, are known to inhibit the growth and reproduction of aquatic hyphomycetes in both axenic cultures (Abel and Barlocher, 1984; Miersch et al., 1997) and natural mixed assemblages (Sridhar et al., 2001; Duarte et al., 2004). Metals are also reported to decrease fungal diversity in freshwaters (Sridhar et al., 2000). However, several aquatic hyphomycete species have been found in severely metal-polluted streams (Sridhar et al., 2000; Krauss et al., 2001), increasing the interest of elucidating the mechanisms underlying the resistance/tolerance of these fungi to metal stress.

In this study, we investigated the response mechanisms to Cu and Zn exposure in two aquatic hyphomycete species, *Varicosporium elodeae* and *Heliscus submersus*. In a first approach, biochemical responses associated with cellular barriers against metal stress, like metal ion adsorption to cell walls and plasma membrane integrity, were evaluated. Since cell damages by metals may occur through the generation of ROS, we can expect changes in the enzymatic antioxidant defenses to deal with metal-induced oxidative stress. Therefore, the activities of CAT, SOD and G6PDH were examined under acute and chronic stress induced by Cu and/or Zn.

2. Materials and methods

2.1. Fungal species, growth conditions and metal exposure

The aquatic hyphomycetes were isolated from single spores collected in streams in the Northwest of Portugal. *V. elodeae* W. Kegel (UMB-142.01) was isolated from foam sampled in a clean stream at the Peneda-Gerês National Park, while *H. submersus* H. J. Huds. (UMB-135.01) was isolated from leaves retrieved in the Este River at the industrial park of the town of Braga, where Zn and Cu concentrations in the water column attained 14.8 μM and 34.4 μM, respectively. Details on fungal species and characterization of water chemistry of their origin sites are in Pascoal et al. (2005).

The fungi were grown in 1% malt extract (pH 5.0), with or without addition of Cu or Zn, with shaking (160 rpm; Certomat BS 3, B. Braun Biotech International) at 18 °C under permanent artificial light, using spores as inoculum (final concentration, 6 conidia ml^{-1}). Growth medium was autoclaved and solutions of copper ($CuCl_2$) and zinc ($ZnCl_2$) were sterilized by filtration (0.22 μm pore size membrane), before aseptic addition to the medium. Final metal concentrations ranged from 10 to 150 μM for Cu and from 50 to 200 μM for Zn. For long-term exposure, fungi were grown in media with or without metal addition for 8 days. At this time, cultures of both fungi were at the same growth phase, with fungal biomass increasing at constant rate (not shown). For short-term exposure, mycelia grown 8 days without metal were transferred to fresh media with or without added Cu, Zn, or a mixture of the two metals for periods from 30 min to 14 h. The pH of cultures was measured at the end of experiments.

To determine the contribution of metal-induced ROS to biomass inhibition, fungi were grown 8 days in the absence or presence of Cu or Zn, at concentrations inhibiting biomass production by 50% (EC_{50}) or 80%

(EC_{80}), with or without the antioxidant butylated hydroxytoluene (BHT; final concentration, 1.13 μM).

To quantify fungal biomass, mycelia were dried at 85 °C to constant mass and weighed to the nearest 0.001 g.

2.2. Scanning electron microscopy

Scanning electron microscopy was used to examine the surface of mycelia and to evaluate Cu and Zn adsorption to cell walls, after short- (14 h) and long-term (8 days) exposure to metals at EC_{50} and EC_{80}, under the conditions indicated above. Mycelia were harvested by filtration, washed twice with deionized water, dissociated into small pieces and fixed in 3% (v/v) glutaraldehyde for 22 h. Subsequently, mycelia were dehydrated in ethanol (v/v) as follows: 30%, 5 h; 60%, 2 h; and 100%, 1 h. Mycelia were then glued onto 20-mm diameter metal mounts, coated with gold under vacuum and scanned with scanning electron microscopy (Leica Cambridge S 360) coupled to an energy dispersive X-ray microanalysis setup (20 keV).

2.3. Plasma membrane integrity

Plasma membrane integrity was assessed by a membrane impermeable dye, propidium iodide (PI; Molecular Probes, Eugene, OR), which enters the cells and binds to nucleic acids when plasma membrane disruption occurs. Mycelia were dissociated into small pieces in phosphate buffer (1× PBS, pH 7.4) and incubated with PI (final concentration, 0.005 μg μl^{-1}) for 15 min at room temperature. Subsequently, mycelia were exposed to EC_{50} concentrations of Cu or Zn during 150 min and scanned each 30 min under an epifluorescence microscope (Zeiss Axioskop connected to an AxioCam HRc camera).

2.4. Reactive oxygen species production

ROS production was monitored with the Mito-Tracker Red CM-H_2XRos (Molecular Probes, Eugene, OR). The reduced form of this dye does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in mitochondria. Mycelium suspensions, prepared as above, were passed through a syringe, and incubated with CM-H_2XRos (final concentration, 3.3 μg ml^{-1}) for 15 min at room temperature. Mycelia were then exposed to EC_{50} and EC_{80} concentrations of Cu or Zn for 30 and 90 min and scanned under an epifluorescence microscope.

2.5. Preparation of cell-free extracts and determination of enzymatic activities

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

Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969). One unit of SOD is the amount of enzyme able to inhibit the reduction of cytochrome c by 50%. The reaction mixture consisted of: 800 μl 50 mM potassium phosphate, 0.1 mM EDTA (pH 7.8); 50 μl 0.2 mM cytochrome c; 50 μl 1 mM xanthine in 1 M sodium hydroxide; 50 μl xanthine oxidase (5 units); 45 μl buffer solution (20 mM Tris 1 mM EDTA; pH 7.5); and 5 μl sample.

Catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption according to Beers and Sizer (1952). The reaction mixture consisted of: 657 μl 50 mM phosphate buffer pH 7.0; 333 μl 30 mM H_2O_2; and 10 μl sample.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was based on the increase in absorbance at 340 nm, resulting from NADP reduction according to Postma et al. (1989). The reaction mixture consisted of: 870 μl H_2O deionized; 50 μl 1 M Tris–HCl pH 8.0; 10 μl 0.04 M $NADP^+$ (disodium); 10 μl 0.5 M $MgCl_2 \cdot 6H_2O$; 50 μl 0.1 M glucose-6-phosphate; and 10 μl sample.

Enzymatic activities were measured after short- (14 h) and long-term (8 days) exposure to metals, and were expressed as U mg^{-1} of total protein.

Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.6. Statistical analysis

Data of Cu and Zn effects on enzymatic activities were expressed as percentage of control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity (Zar, 1996). For each metal and fungal species, enzymatic activities were compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects ($p < 0.05$; Zar, 1996).

Metal concentrations corresponding to EC_{50} and EC_{80} of biomass inhibition were determined by non-

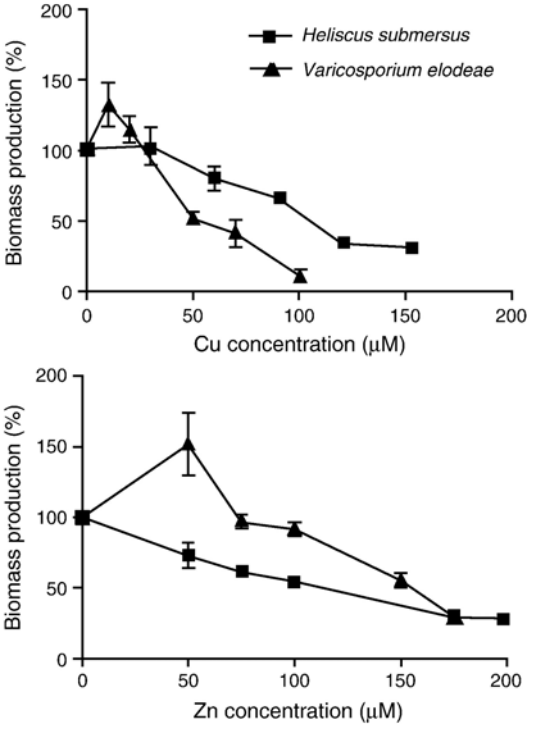

Fig. 1. Biomass production by the aquatic hyphomycetes *H. submersus* and *V. elodeae* exposed for 8 days to Cu and Zn. Mean±SEM, $n=3$.

linear regression. Inhibition rates of biomass production (k_i) were compared by an F test using non-transformed data (Motulsky and Christopoulos, 2003).

Statistical analysis was done using Prism 4 for Macintosh (GraphPad software Inc., San Diego).

3. Results

3.1. Comparison of metal sensitivity

To characterize the sensitivity of aquatic hyphomycetes to Cu and Zn, we determined the effects of these metals in biomass production after 8 days of growth on a concentration-dependent basis (Fig. 1). The analysis of growth inhibition parameters, namely metal concentration inhibiting biomass production by 50% (EC_{50}) and 80% (EC_{80}), and inhibition rate of biomass production (k_i), showed that the two species had different levels of resistance to metal stress (Table 1). *H. submersus* was more resistant to Cu than *V. elodeae*, even though the effect of Cu on the k_i did not differ significantly between species. The exposure to Zn did not inhibit biomass production in *V. elodeae* until 100 μM, a concentration corresponding to EC_{50} in *H. submersus* (Fig. 1). However, *H. submersus* had a significantly lower k_i than *V. elodeae* when exposed to Zn, suggesting higher tolerance of the former species to this metal (Table 1). Higher concentrations of Zn than Cu were necessary to promote identical toxicity effects in both fungi.

3.2. Biochemical responses associated with cellular barriers against metal stress

To assess the biochemical responses associated with cellular barriers against Cu and Zn, metal adsorption to cell walls and plasma membrane integrity were evaluated. In addition, we followed changes in the pH of the medium, because some fungi are able to release organic acids that can bind metal ions (Gadd, 1993). In both aquatic hyphomycete species metals did not elicit medium acidification (not shown).

Scanning electron microscopy of mycelia of *V. elodeae* and *H. submersus*, exposed to Cu or Zn for 14 h or 8 days at EC_{50} and EC_{80}, showed some morphological alterations such as cell shrinkage, particularly after short-term exposure to either metal or long-term exposure to the highest Cu concentration (see Fig. 2, for *H. submersus* exposed to Cu). However, metal adsorption onto cell walls was not detected under these conditions.

To elucidate whether plasma membrane could be a primary target of metal-induced stress, we assessed cell permeabilization to propidium iodide (PI) of mycelia exposed for short-term (30–150 min) to Cu or Zn. Increased red fluorescence is indicative of plasma membrane disruption. Exposure of *V. elodeae* or *H. submersus* to Cu resulted in severe disruption of plasma membrane integrity, particularly in the former species,

Table 1
Concentrations inhibiting biomass production in 50% (EC_{50}) and 80% (EC_{80}), and biomass production inhibition rate (k_i) for Cu and Zn in the aquatic hyphomycetes *Varicosporium elodeae* and *Heliscus submersus*

Fungal species	Cu			Zn		
	EC_{50} (μM)	EC_{80} (μM)	k_i (μM^{-1})	EC_{50} (μM)	EC_{80} (μM)	k_i (μM^{-1})
V. elodeae	54±4.7	85±2.6	3.3±0.8[a]	152±4.9	189±4.2	5.7±0.8[b]
H. submersus	102±1.7	174±3.6	2.8±0.5[a]	103±1.6	267±5.1	1.5±0.1[c]

Values are means±SE.
Similar letters indicate no significant differences ($p>0.05$) between k_i (F test).

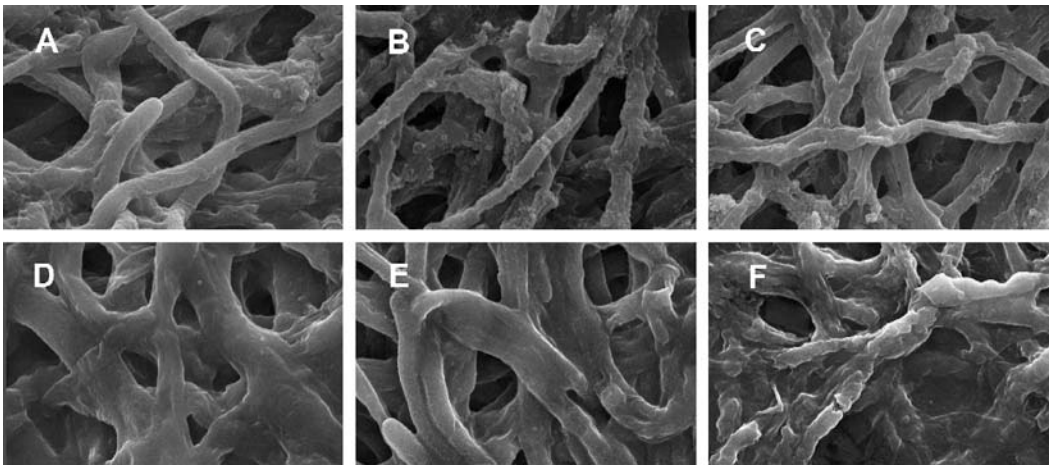

Fig. 2. Scanning electron microscopy of *H. submersus* mycelia exposed for short- (B, C) or long-term (E, F) to Cu at concentrations of EC_{50} (B, E) or EC_{80} (C, F). Control mycelia for short- (A) and long-term (D) experiments; magnification, ×5000.

while Zn elicited a much less pronounced response (see Fig. 3-I, for *V. elodeae*). A recovery of plasma membrane integrity was detected after 150 min of exposure to Cu in *H. submersus*, but not in *V. elodeae* (not shown).

3.3. Oxidative stress induced by Cu and Zn

The exposure of *H. submersus* and *V. elodeae* to Cu or Zn induced ROS generation, as shown by an increase in red fluorescence of mycelia stained with MitoTracker Red CM-H_2XRos (see Fig. 3-II, for *V. elodeae*). Higher levels of ROS were detected under Cu exposure in both fungal species. Moreover, these free radicals increased in *H. submersus* mycelia in a time- and concentration-dependent manner, while in *V. elodeae* only a concentration-dependent effect was observed (not shown).

To determine the effects of ROS induced by metals in the inhibition of biomass production, the antioxidant butylated hydroxytoluene (BHT) was included in the

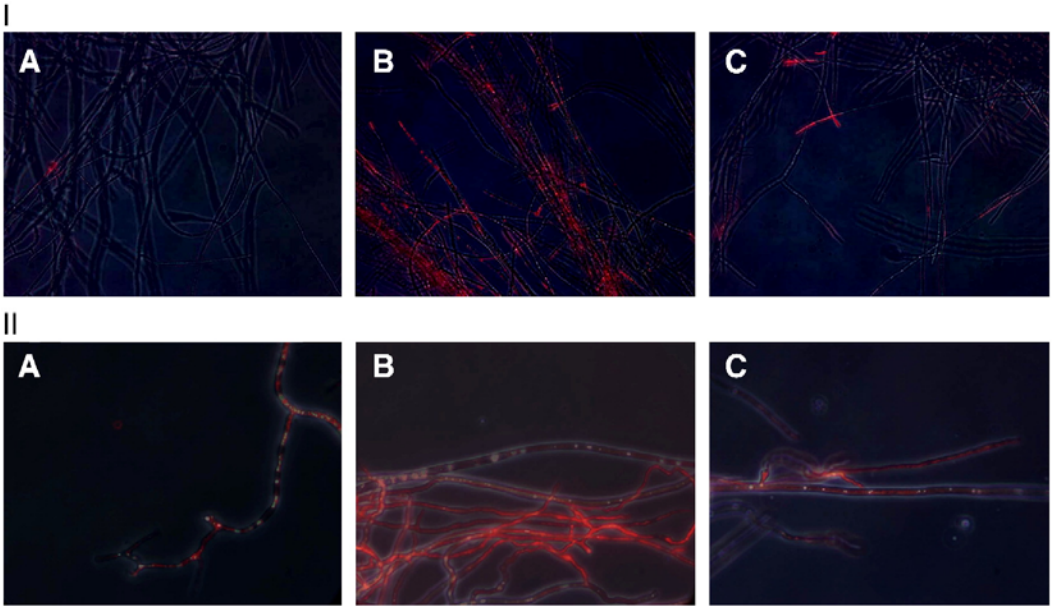

Fig. 3. Fluorescence microscopy images of *V. elodeae* mycelia. I, Plasma membrane integrity assessed by propidium iodide in mycelia unexposed (A) or exposed for 30 min to EC_{50} of Cu (B) or Zn (C); magnification, ×400. II, ROS production assessed by CM-H_2XRos in mycelia unexposed (A) or exposed for 30 min to EC_{50} of Cu (B) or Zn (C); magnification, ×1000.

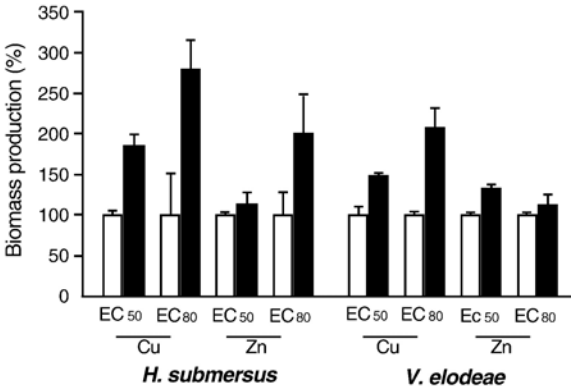

Fig. 4. Contribution of metal-induced ROS to biomass production by *H. submersus* and *V. elodeae* exposed to EC_{50} or EC_{80} of Zn or Cu in the absence (white) or presence of the antioxidant BHT (black). Biomass production in the absence of BHT was equaled to 100%. Mean±SEM, $n=3$.

culture medium. The presence of this antioxidant agent resulted in an increase in biomass production in both species (Fig. 4), particularly in the case of Cu. In cultures without metal addition, biomass production did not differ significantly in the presence or absence of BHT (not shown).

3.4. Antioxidant defenses triggered by Cu and Zn exposure

The specific activities of the enzymes CAT, SOD and G6PDH in mycelia of aquatic hyphomycetes grown in media without addition of metals were higher in *V. elodeae* than in *H. submersus* (Table 2). Short-term exposure (14 h) of *H. submersus* to Cu led to a general increase in the SOD and CAT activities (Fig. 5A, C). CAT activity was also increased after long-term exposure (8 days) to Cu, particularly at the highest concentration, in which CAT appeared to replace SOD as the major antioxidant defense (Fig. 5A, C). In the case of *V. elodeae*, SOD activity remained unaltered (Fig. 5B), while an increase in CAT activity was observed under short-term exposure to Cu (Fig. 5D). In addition, we found that the activity of G6PDH was stimulated after long-term exposure of *V. elodeae* to the lowest Cu concentration (Fig. 5F).

Long-term exposure to Zn enhanced the activity of G6PDH (Fig. 6E, F) and CAT (Fig. 6C, D) in *H. submersus* and *V. elodeae*. CAT also seemed to be an important antioxidant defense in *H. submersus* under acute Zn stress, because its activity increased at short exposure time (Fig. 6C). Conversely, SOD did not appear to be involved in Zn stress (Fig. 6A, B).

3.5. Effects of Cu and Zn in mixtures

The response pattern of the enzymes SOD, CAT and G6PDH in *H. submersus* and *V. elodeae* after short-term exposure (14 h) to equitoxic mixtures of metals (EC_{50} or EC_{80}) was similar to that of single Cu exposure (Figs. 5–7), except for CAT whose activity was severely inhibited in the former species (Fig. 7C). In addition, metal effects on enzymatic activities were generally more pronounced after exposure to mixtures than to Cu alone.

4. Discussion

Because of the crucial role of aquatic hyphomycetes in organic matter turnover in freshwater ecosystems (Bärlocher, 1992) and their ability to survive in metal-polluted environments (Krauss et al., 2001), it is clearly of interest to elucidate the cellular mechanisms underlying metal tolerance of this group of fungi. In this study, tested concentrations of metals (Zn up to 200 μM; Cu up to 150 μM) are environmentally realistic because they are within the range reported in metal-polluted streams (e.g., mining district in Central Germany: up to 19,076 μM Zn and 93.8 μM Cu, Krauss et al., 2001; Sridhar et al., 2000; industrial park of Braga, Northwest Portugal: 14.8 μM Zn and 34.4 μM Cu, Gonçalves, 2001; Pascoal et al., 2005).

Our study showed that Cu and Zn induced alterations in cell-wall morphology of *H. submersus* and *V. elodeae* as shown by scanning electron microscopy (Fig. 2) and previously found in *Mucor rouxii* (Gardea-Torresdey et al., 1997), but not in the aquatic hyphomycete *Heliscus lugdunensis* (Braha et al., 2007). Even so, in our study, no noticeable adsorption of these metals to cell walls was found, minimizing the protective role of this

Table 2
Specific activities of catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PDH) in *H. submersus* and *V. elodeae* grown 8 days without addition of metals

Fungal species	CAT (U mg^{-1} protein)	SOD (U mg^{-1} protein)	G6PDH (U mg^{-1} protein)
V. elodeae	$21\times10^{-6}\pm4.9\times10^{-6}$	0.10±0.036	$20\times10^{-5}\pm4.3\times10^{-5}$
H. submersus	$11\times10^{-6}\pm4.7\times10^{-6}$	0.08±0.009	$15\times10^{-5}\pm2.4\times10^{-5}$

Values are means±SEM, $n=3$.

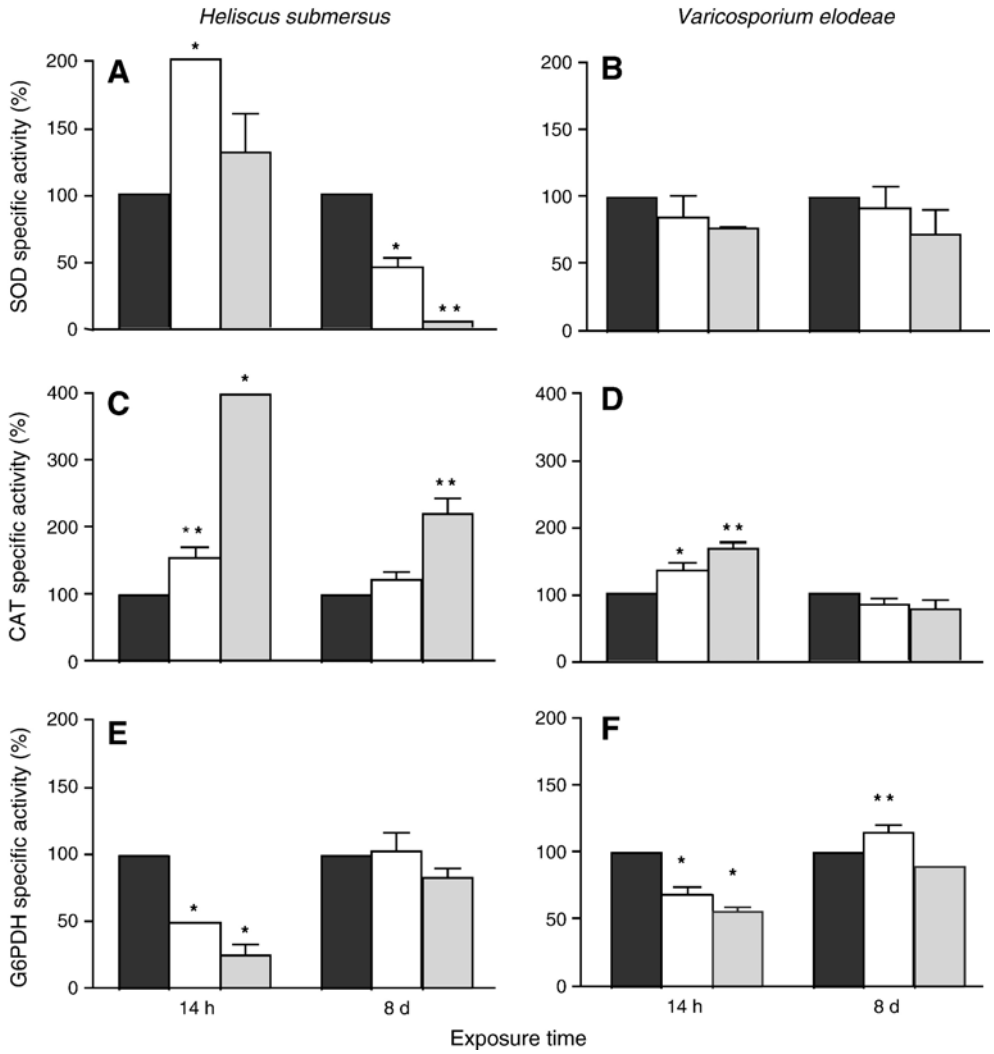

Fig. 5. Activity of the enzymes SOD (A, B), CAT (C, D), and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed (black) or exposed for short- (14 h) and long-term (8 days) to Cu at concentrations of EC_{50} (white) or EC_{80} (grey). Mean ± SEM, $n=3$. Significant differences: $^{*}p<0.05$ or $^{**}p<0.01$.

structure in the internalization of Cu or Zn ions. The adsorption of metals by filamentous fungi has been reported to be affected by pH, initial metal ion concentration, medium composition and exposure time (Gardea-Torresdey et al., 1997; Lo et al., 1999); therefore, the ability of the cell walls of these aquatic hyphomycetes to bind metals cannot be excluded in conditions differing from those of our study (see Braha et al., 2007).

Metal effects on biomass production by aquatic hyphomycetes indicated higher toxicity of Cu than Zn, which agrees with reports for several other organisms (e.g., microorganisms, Gadd, 1993; Miersch et al., 1997; algae, Collén et al., 2003; invertebrates, Kobayashi and Okamura, 2005). Moreover, the similarity in the magnitude of inhibition rates of biomass production after Cu exposure suggests that Cu may have identical cellular targets in *V. elodeae* and *H. submersus*. It has been reported that Cu induces plasma membrane disruption in fungi (Ohsumi et al., 1988; Stohs and Bagchi, 1995). In agreement, our work showed that plasma membrane integrity of *V. elodeae* and *H. submersus* was more affected by Cu than Zn, pointing to this cellular structure as a potentially vulnerable target of Cu. Loss of membrane integrity has been attributed to the formation of ROS (Stohs and Bagchi, 1995). We clearly demonstrated that generation of ROS contributed noticeably to metal toxicity, with a particularly strong

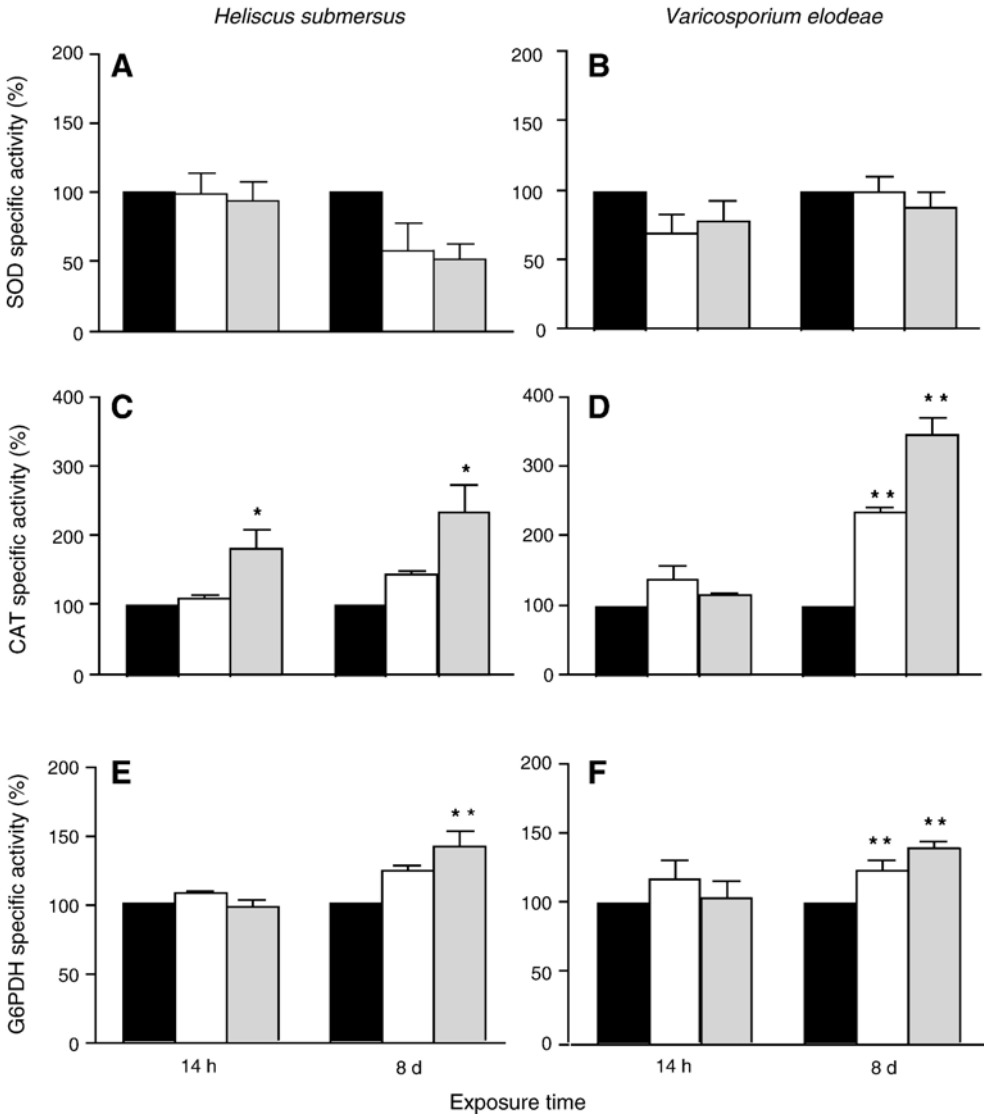

Fig. 6. Activity of the enzymes SOD (A, B), CAT (C, D) and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed (black) or exposed for short- (14 h) and long-term (8 days) to Zn at concentrations of EC_{50} (white) or EC_{80} (grey). Mean±SEM, $n=3$. Significant differences: $*p<0.05$ or $**p<0.01$.

effect under Cu stress, as indicated by the increase of biomass production in the presence of an antioxidant agent.

Fungi, like all aerobic organisms, have a set of defense mechanisms to deal with oxidative stress (Moradas-Ferreira et al., 1996; Bai et al., 2003). Enzymes, such as SOD, CAT and G6PDH, have been reported to be activated against ROS in several organisms under Cu and/or Zn stress (yeasts, Romandini et al., 1992; algae, Collén et al., 2003; Tripathi et al., 2006; mussels, Geret and Bebianno, 2004). The first two enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Bai et al., 2003; Pócsi et al., 2004); G6PDH is essential for the replenishment of NADPH intracellular pool to maintain cellular redox balance (Pócsi et al., 2004). In our study, control cultures of *V. elodeae* had higher activities of CAT, SOD and G6PDH than those of *H. submersus* (Table 2). Although *V. elodeae* had been isolated from a clean stream, it is distributed worldwide (e.g., Portugal, Pascoal et al., 2005; France, Chauvet, 1991; Canada, Nikolcheva et al., 2005) and it may have antioxidant defenses against environmental stressors, including metals. Consistently, metal exposure did not

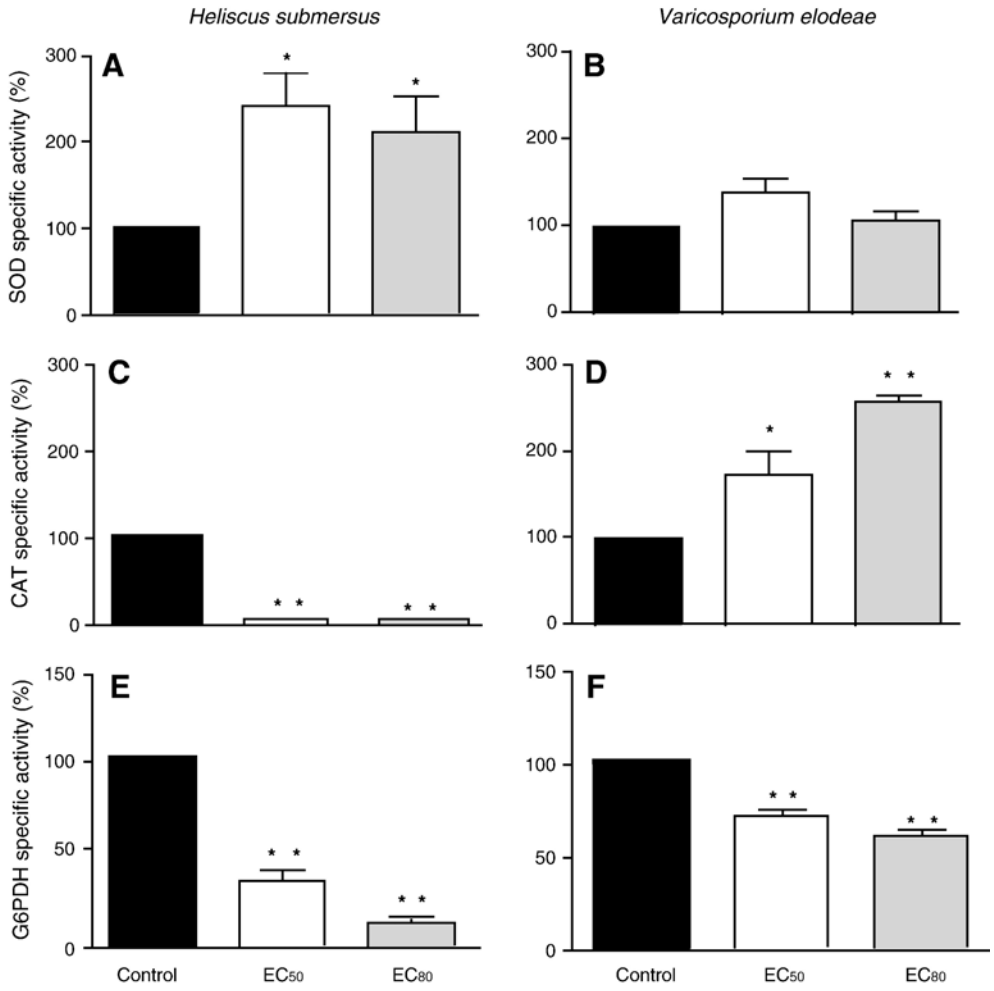

Fig. 7. Activity of the enzymes SOD (A, B), CAT (C, D), and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed or exposed for 14 h to equitoxic mixtures of Cu and Zn corresponding to EC_{50} or EC_{80}. Mean ± SEM, $n = 3$. Significant differences: $*p < 0.05$.

inhibit the growth of *V. elodeae* until a threshold concentration of 100 μM Zn or 20 μM Cu, above which the biomass production was inhibited. CAT appeared to have a primary defense role against acute Cu stress, but not under chronic stress. In addition, Cu seemed to change the cellular redox status in *V. elodeae*, as suggested by the inhibition of G6PDH activity under acute stress followed by its stimulation under chronic stress. Copper diminished the activity of glutathione reductase (GR) in *H. lugdunensis* (Braha et al., 2007). In *Scenedesmus* sp., both Cu and Zn affected GR activity (Nagalakshmi and Prasad, 2001; Tripathi et al., 2006) through metal binding to SH-groups at the active site of the enzyme (Nagalakshmi and Prasad, 2001). If inactivation of GR had also occurred in our study, it might explain the inhibition of G6PDH after short-term exposure to Cu in *V. elodeae* and *H. submersus*, avoiding a futile production of NADPH. Overall, our results point to a possible role of G6PDH in aquatic hyphomycete acclimation to Cu and Zn. In this connection, Izawa et al. (1998) reported that G6PDH-deficient cells of *S. cerevisiae* were more susceptible and unable to adapt to oxidative stress. Thus, in aquatic hyphomycetes, it is conceivable that NADPH could be used for glutathione recycling needed for metal detoxification during acclimation. This hypothesis is supported by previous observations pointing to a major role of glutathione and phytochelatins in Cu and Zn binding in aquatic hyphomycetes under chronic stress (Guimarães-Soares et al., 2006).

In *H. submersus* both SOD and CAT were stimulated under acute stress by Cu, and CAT activity increased with the increasing of Cu concentration. Also the magnitude of the increase in the CAT activity was

much more pronounced in *H. submersus* than in *V. elodeae*, probably contributing to the higher tolerance of *H. submersus* to Cu and to its ability to survive in metal-polluted streams as at the site from which the fungus was originally isolated (industrial park of Braga; metal concentrations are above). The activity of CAT remained high during chronic exposure to Cu, suggesting that CAT also plays an important role in the acclimation of *H. submersus* to Cu stress. In this fungus the inhibition of SOD activity under chronic stress could be related to its potential function as a metallothionein (Culotta et al., 1995), which is important for cellular detoxification either as metal-chelating agent or ROS scavenger (Kiningham and Kasarskis, 1998). Overall, our findings point to SOD and CAT enzymes as having an effective role in protecting *H. submersus* against ROS induced by Cu, which agrees with the less pronounced effects of this metal on the plasma membrane of *H. submersus* than that of *V. elodeae*.

An important role of Zn in living organisms is related to its antioxidant properties (Powell, 2000). However, in our study, excess of Zn caused severe effects on biomass production, and oxidative stress was evident although to a lesser extent than that induced by Cu. In contrast to reports on *Phaseolus vulgaris* (Weckx and Clijsters, 1997), CAT activity was stimulated in both *H. submersus* and *V. elodeae* highlighting the importance of this enzyme as a major antioxidant defense in aquatic hyphomycetes.

In metal-polluted streams aquatic hyphomycetes are commonly exposed to mixtures of metals, but so far no data are available on the effects of metal mixtures in this group of fungi. It is reported that *Pleurotus ostreatus* exposed to Cu and Zn mixtures, accumulates more Cu than Zn (Baldrian, 2003), probably causing an increased production of ROS. Similarly, Franklin et al. (2002) reported that in equitoxic mixtures, Cu reduced the binding and cellular uptake of Zn by *Chlorella* sp., but Zn had no appreciable effect on the uptake of Cu. This suggests that effects of Cu are dominant over Zn in eliciting cell responses when a mixture of Cu and Zn is applied. In our study, the antioxidant defenses displayed by the aquatic hyphomycetes exposed to Cu and Zn mixtures were similar to those of Cu. In addition, the responses of SOD in *H. submersus* and of CAT in *V. elodeae* were stronger under exposure to Cu plus Zn mixtures than to Cu alone, suggesting that metal mixtures induced higher oxidative stress than the individual metals.

In summary, both Zn and Cu induced oxidative stress in aquatic hyphomycetes. CAT appeared to play a greater role alleviating the stress induced by metals. In addition, the increased activity of G6PDH after long-term exposure to metals points to the involvement of the pentose phosphate pathway in metal acclimation. Our results suggest that the ability of aquatic hyphomycetes to cope with metal stress is related to their ability to mount an efficient defense against oxidative stress. These findings may contribute to a better understanding of the response mechanisms of aquatic hyphomycetes to metal stress and to gain insights into metal–microbe interactions in natural environments.

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