Maria Manuel da Silva Azevedo

Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms

December 2007
Universidade do Minho
Escola de Ciências

Maria Manuel da Silva Azevedo

Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms

Ph.D. Thesis in Sciences

Work Supervised By
Prof. Dr. Fernanda Cássio

December 2007
Acknowledgements

I compare this phase of my life to a big and agitated boat trip where a lot of things have happened, however this journey is over, I have resisted and the final balance is highly positive.

I started this “voyage” which was for me a small conquer with the support and incentive of some friends and I have finished with new friends, which make part of my life today. I am grateful to all of them.

I am also grateful:

To my Supervisor Prof. Fernanda Cássio for this opportunity, but mainly for believing in me. Thanks for all the scientific support and for the friendship specially in the difficult moments.

To Cláudia Pascoal for all her involvement in this work and friendship.

To the current and the former Head of the Biology Department, Prof. Margarida Casal and Prof. Helena Cardoso for receiving me at the Biology Department.

To Prof. Cecília Leão for giving me the opportunity to develop a considerable part of this work at the ICVS and for all her support over these years.

To Prof. Paula Ludovico and Prof. Fernando Rodrigues, for the many fruitful suggestions and all the scientific discussion and specially for their friendship.

To Paula Ludovico I am also grateful for the help in the bad days and for all the good moments, which I wish to continue in the future (I promise to buy a better instructions book).

To Prof. Fátima Baltazar which received me as a friend since the first day we met. Thank you for listening and helping me whenever I needed, for the opportune suggestions and mainly for the good relationship that I am sure will last.

To Prof. Sandra Paiva, I am grateful for the friendship, the great moments, etc (you known what “etc” means).

To my laboratory colleagues Amaro, Isabel, Luís and Sofia, I am grateful for their help whenever I needed and for their friendship. To my friend Sofia a special reference for listening to me whenever I need.

To all colleagues and friends from the Biology Department specially Isabel João, Herlânder, Patrícia, Paulo, Célia, Rita, Raul, Jorge for helping me in different ways during this work.
To the students of Prof. Paula Ludovico and Prof. Fernando Rodrigues for kindly receiving me at their laboratory and make me feel one of them. To Ana Mesquita one special reference since she is a very dear friend which is always present in the good and in the bad moments.

To the Staff of the School E.B. 2,3 D. Maria II V.N. Famalicão, specially Cândida Pinto and Amélia Granja and all the colleagues, thanks!

To my cousin Ana Paula Costa for her friendship and all the involvement during these years.

I am also very grateful to my family (my mother, my sister Cristina, my brother Tó and João Pedro), which encouraged and listened to me during this phase of my life. To my nephew, Xaninho, for the wonderful moments, which constitute important therapies. With you all the adversities were overtaken.

To finish I want to dedicate this work to the memory of my father which always encouraged me throughout all the challenges of my life, but much more for transmitting me his sensitivity, his faith and his strength to always fight for what I believe in. Although you are not physically present, your example of persistence will always be with me, in every day of my life. Thank you.

Part of this work was supported by the project grant POCTI/34024/BSE/2000.
Abstract

Human activities contribute to a high release of heavy metals to the environment at rates and concentrations sufficient to make them pollutants. Certain metals, such as Cu and Zn, are needed for the growth and metabolism of organisms, while others, as Cd, have no recognized biological functions. However, above critical levels, both essential and non-essential metals became toxic to living organisms.

Aquatic hyphomycetes are a polyphyletic group of fungi that play a key role in plant-litter decomposition in streams. They produce an array of exoenzymes able to degrade plant cell-wall polymers and increase plant-litter palatability for invertebrate detritivores. Even though these fungi occur in metal-polluted streams, the mechanisms underlying their resistance/tolerance to metals are poorly documented.

In this study, the exposure to metals inhibited reproduction, as sporulation rates, of the aquatic hyphomycetes. Moreover, fungal reproduction was more sensitive to metals than growth. The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% ($EC_{50}$), showed that *Ypsilina graminea* and *Varicosporium elodeae* were the most resistant species to Zn, while *Heliscus submersus* was the most resistant to Cu. The $EC_{50}$ values were about 20-times higher in solid medium than in liquid medium. However, the patterns of species resistance to metals in either liquid or solid medium, with similar composition, were identical. Generally, Ni or Cd were more toxic than Zn or Cu to fungi. *H. submersus* and *V. elodeae* had remarkable ability to adsorb Cu and Zn, respectively. Because these fungal species were highly tolerant to each metal, biosorption may be a relevant mechanism to avoid unrestrained uptake of metals.

We demonstrated that the generation/accumulation of reactive oxygen species (ROS) contributed noticeably to metal toxicity in aquatic hyphomycetes, particularly under Cu stress, as indicated by a recovery in biomass production by the presence of an antioxidant agent. Our results 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. At short-term (10 min), Cu completely inhibited the activity of the plasma membrane $H^+$-ATPase of *H. submersus* and *V. elodeae*, while Zn only led to a similar effect on that of *H. submersus*. However, a recovery of plasma membrane integrity was observed after 150 min of metal exposure. A strong stimulation
of the proton pump was found in the most tolerant species (i.e. when *H. submersus* was exposed to Cu and *V. elodeae* was exposed to Zn) at longer times (8 days). The activation of H⁺-ATPase may contribute to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane, suggesting that H⁺-ATPase may be involved in aquatic hyphomycete acclimation to metals.

Our studies on antioxidant defenses showed that catalase had a greater role in alleviating the stress induced by Cu and Zn than superoxide dismutase. In addition, the increased activity of glucose-6-phosphate dehydrogenase, after long-term exposure to metals (8 days), points to the involvement of the pentose phosphate pathway in metal acclimation. Before metal exposure, *H. submersus* and *Flagellospora curta* isolated from a metal-polluted stream had higher levels of thiol compounds than *V. elodeae*, isolated from a clean stream. However, the latter species rapidly increased the levels of thiols after metal exposure. These findings are in agreement with the recognized role of thiol compounds as metal sequesters and/or ROS scavengers.

Finally, we showed that Cu and Zn are able to induce programmed cell death (PCD) in aquatic hyphomycetes, a process in which cells actively participate in their own death. The exposure to Cu promoted ROS production and caspase activation in *H. submersus* and *F. curta*. Conversely, under Zn stress, aquatic hyphomycetes showed high number of cells with nuclear morphological alterations and/or DNA strand-breaks. The different pattern of PCD markers suggests that the triggering cell death signal is most probably related to different cellular targets for Cu and Zn in aquatic hyphomycetes.
Resumo

As actividades humanas contribuem para o aumento da libertação de metais pesados para o ambiente, a taxas e a concentrações que os tornam poluentes. Alguns metais, como o Cu e o Zn, são necessários para o crescimento e metabolismo dos organismos, enquanto que outros, como o Cd, não lhes é atribuída qualquer função biológica. Contudo, acima de certas concentrações, os metais, quer os essenciais quer os não essenciais, tornam-se tóxicos para os organismos vivos.

Os hifomicetos aquáticos são um grupo de fungos filogeneticamente heterogéneo que desempenham um papel chave na decomposição dos detritos vegetais nos rios. Estes fungos produzem um conjunto de enzimas extracelulares capazes de degradar os polímeros das paredes das células vegetais aumentando a palatabilidade dos detritos vegetais para os invertebrados detritívoros. Apesar dos hifomicetos aquáticos estarem presentes quer em rios de referência quer em rios poluídos com metais pesados, os mecanismos subjacentes à sua resistência/tolerância aos metais são pouco conhecidos.

Neste estudo, a reprodução dos fungos, avaliada pela taxa de esporulação, foi inibida pela exposição aos metais. Além disso, a reprodução dos fungos foi mais sensível aos efeitos negativos dos metais do que o seu crescimento. A sensibilidade dos hifomicetos aquáticos aos metais, avaliada pela concentração de metal capaz de inibir a produção de biomassa em 50% (EC\textsubscript{50}), mostrou que \textit{Ypsilina graminea} e \textit{Varicosporium elodeae} foram as espécies mais resistentes ao Zn, enquanto que \textit{Heliscus submersus} foi a mais resistente ao Cu. Os valores de EC\textsubscript{50} foram cerca de 20 vezes mais elevados em meio sólido do que em meio líquido. Porém, os padrões de resistência aos metais exibidos pelos hifomicetos aquáticos foram semelhantes em meio sólido ou em meio líquido com idêntica composição química. Geralmente, o Ni ou o Cd foram mais tóxicos do que o Zn ou o Cu. \textit{H. submersus} e \textit{V. elodeae} exibiram capacidade elevada para adsorver, respectivamente, Cu e Zn. Dado que estes fungos foram muito tolerantes a esses metais, a bioadsorção pode constituir um mecanismo relevante para controlar a entrada dos metais nas células.

Neste trabalho demonstrámos que a produção de espécies reactivas de oxigénio (ROS) contribuiu notavelmente para a toxicidade dos metais, sobretudo no caso do Cu, como indicado pela recuperação da biomassa produzida pelos fungos na presença de um agente antioxidante. A integridade da membrana plasmática de \textit{V. elodeae} e de \textit{H. submersus} foi significativamente reduzida quando os fungos foram tratados com diferentes concentrações de metais.
submersus foi mais afetada pelo Cu do que pelo Zn, sugerindo que esta estrutura celular pode ser um alvo potencial para o Cu. A tempos curtos de exposição (10 min), o Cu bloqueou a actividade da H⁺-ATPase da membrana plasmática de H. submersus e de V. elodeae, enquanto que o Zn só promoveu um efeito semelhante em H. submersus. Contudo, uma recuperação da integridade da membrana plasmática foi observada a tempos mais longos (150 min). Após 8 dias de exposição, um estímulo forte da bomba de protões foi encontrado nas espécies mais tolerantes, i.e. em H. submersus exposto a Cu e em V. elodeae exposto a Zn. A activação da H⁺-ATPase pode contribuir para contrabalançar a dissipação do gradiente electroquímico de protões induzida pelo metal, sugerindo o envolvimento desta bomba na aclimatação dos fungos ao stress metálico.

Os nossos estudos sobre as defesas antioxidantes mostraram que a catalase teve um papel mais importante do que a superóxido dismutase na mitigação do stress induzido pelo Cu e pelo Zn. Além disso, o estímulo da actividade da glucose-6-fosfato desidrogenase após 8 dias de exposição aos metais, sugere o envolvimento da via das pentoses na aclimatação dos fungos aos metais. As espécies H. submersus e Flagellospora curta, isoladas de um rio poluído com metais, tinham níveis mais elevados de compostos ricos em grupos tiol do que a espécie V. elodeae, isolada de um rio de referência. Contudo, esta última espécie aumentou rapidamente o seu conteúdo em compostos tiólicos após a exposição ao metal. Estes resultados estão de acordo com o reconhecido papel dos compostos tiólicos na sequestração de metal e/ou ROS nas células.

Finalmente, os nossos resultados mostraram que o Cu e o Zn foram capazes de induzir morte celular programada (PCD) em hifomicetos aquáticos, um processo de morte celular activa. O stress induzido pelo Cu estimulou sobretudo a produção de ROS e a actividade das caspases em H. submersus e em F. curta. Por outro lado, os hifomicetos aquáticos expostos a Zn mostraram um elevado número de células com alterações morfológicas no núcleo e/ou quebras na cadeia de DNA. O diferente padrão de resposta dos marcadores de PCD sugere que o sinal de morte celular pode estar relacionado com diferentes alvos celulares do Cu e do Zn em hifomicetos aquáticos.
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>General introduction</td>
<td>3</td>
</tr>
<tr>
<td>1.1. Aquatic hyphomycetes in streams</td>
<td>3</td>
</tr>
<tr>
<td>1.2. Metal pollution in aquatic life</td>
<td>4</td>
</tr>
<tr>
<td>1.3. Cellular mechanisms involved in metal detoxification and tolerance</td>
<td>4</td>
</tr>
<tr>
<td>1.3.1. Extracellular complexation</td>
<td>4</td>
</tr>
<tr>
<td>1.3.2. Cellular barriers against metal stress</td>
<td>5</td>
</tr>
<tr>
<td>1.3.2.1. Cell wall</td>
<td>5</td>
</tr>
<tr>
<td>1.3.2.2. Plasma membrane</td>
<td>6</td>
</tr>
<tr>
<td>1.3.2.3. The role of H⁺-ATPase</td>
<td>7</td>
</tr>
<tr>
<td>1.3.2.4. The role of efflux pumps and vacuole</td>
<td>9</td>
</tr>
<tr>
<td>1.3.2.5. Cu and Zn transport and its transcriptional regulation</td>
<td>9</td>
</tr>
<tr>
<td>1.3.3. Intracellular complexation of heavy metals: the role of thiol compounds</td>
<td>10</td>
</tr>
<tr>
<td>1.3.4. Oxidative stress induced by metals</td>
<td>12</td>
</tr>
<tr>
<td>1.3.4.1. Enzymatic and non-enzymatic defenses against oxidative stress</td>
<td>12</td>
</tr>
<tr>
<td>1.3.4.2. Gene expression in response to oxidative stress</td>
<td>14</td>
</tr>
<tr>
<td>1.3.4.3. DNA damage induced by ROS and programmed cell death</td>
<td>17</td>
</tr>
<tr>
<td>1.4. Aim and outline of the thesis</td>
<td>19</td>
</tr>
<tr>
<td>References</td>
<td>20</td>
</tr>
</tbody>
</table>

| Chapter 2 | Page |
| Effects of metals on growth and sporulation of aquatic hyphomycetes | 35 |
| Abstract | 35 |
| 2.1. Introduction | 36 |
| 2.2. Materials and methods | 37 |
| 2.2.1. Fungal species and culture maintenance | 37 |
| 2.2.2. Growth experiments | 37 |
| 2.2.2.1. Effects of metals on fungal growth in solid medium | 37 |
| 2.2.2.2. Effects of metals on fungal growth in liquid medium | 37 |
| 2.2.3. Effects of metals on fungal sporulation | 38 |
| 2.2.4. Data analysis | 39 |
| 2.3. Results | 40 |
| 2.3.1. Effects of metals on fungal growth | 40 |
| 2.3.1.1. Fungal growth in solid medium | 40 |
| 2.3.1.2. Fungal growth in liquid medium | 44 |
| 2.3.2. Effects of Zn and Cd on fungal sporulation | 47 |
| 2.4. Discussion | 49 |
| References | 52 |

| Chapter 3 | Page |
| Biochemical responses to Cu and Zn stress in aquatic fungi: the major role of antioxidant defenses | 57 |
| Abstract | 57 |
| 3.1. Introduction | 58 |
| 3.2. Materials and methods | 59 |
| 3.2.1. Fungal species, growth conditions and metal exposure | 59 |
| 3.2.2. Scanning electron microscopy | 60 |
| 3.2.3. Plasma membrane integrity | 60 |
| 3.2.4. Reactive oxygen species production | 61 |
| 3.2.5. Preparation of cell-free extracts and determination of enzymatic activities | 61 |
| 3.2.6. Statistical analysis | 62 |
Chapter 1

*General introduction*
General introduction

1.1. Aquatic hyphomycetes in streams

Aquatic hyphomycetes are a group of freshwater fungi, also named Ingoldian fungi in honour to Prof. Ingold the pioneer of the study of its taxonomy. Although the anamorphic stages of aquatic hyphomycetes, characterized by the production of asexual spores or conidia, have been extensively studied, less is known about their teleomorphic stage (Shearer et al., 2007). Until now, a small number of aquatic hyphomycetes are known to have sexual states, and the described anamorph/teleomorphic connections show links mainly to Ascomycota, lesser to Basidiomycota (Shearer et al., 2007). These results are in agreement with studies of phylogenetic relationships based on homologies in rDNA sequences (Campbell et al., 2002). The degree to which the sexual stage is relevant in the natural life cycle of this group of aquatic fungi remains largely unknown (Shearer et al., 2007). Conidia of these fungi are generally large and exhibit several distinctive shapes, such as sigmoid or tetraradiate, which allow their identification. These structures can be found in foams, dispersed in the water, floating on the water surface or associated with decomposing organic substrates as leaf litter and twigs (Suberkropp, 1998).

Nowadays, about 300 species of aquatic hyphomycetes are described with a worldwide distribution (Shearer et al., 2007). They can be found in relatively clean and well-aerated running waters (Bärlocher, 1992) and are the major fungal decomposers in either clean or polluted streams (Pascoal and Cássio, 2004; Pascoal et al., 2005a). Aquatic hyphomycetes play an important role as intermediaries between plant detritus and invertebrates in streams (Bärlocher, 1992), mainly because they are able to degrade the major polysaccharides of plant cell walls (Suberkropp, 1998). Morphological and physiological adaptations, such as the production of a variety of extracellular degradative enzymes, the capacity to grow at low temperatures and the efficient attachment of conidia to substrata, may be responsible for the success of aquatic hyphomycetes as decomposers in freshwaters (Suberkropp, 1998).

Several studies have demonstrated that the distribution and activity of aquatic hyphomycetes are affected by the physical and chemical characteristics of the stream water (Pascoal et al., 2005a,b) and the riparian vegetation (Graça et al., 2002).
1.2. Metal pollution in aquatic life

In recent years, toxic effects of heavy metals to living organisms, mainly as a result of their continuing anthropogenic mobilization in the environment, have attracted considerable worldwide attention. Metals are common in urban aquatic ecosystems and, in contrast to most pollutants, they are not biodegradable and thus persistent in the environment. Aquatic organisms can incorporate these elements directly or indirectly through the food chain. Metals like Fe, Cu, Zn and Ni are essential to the organism’s maintenance; many others have no apparent essential function such as Al, Cd, Hg and Pb (Gadd, 1993). However, both essential and non-essential metals can be toxic when present above certain threshold concentrations (Gadd, 1993). Metal toxicity varies among organisms, with the physico-chemical properties of each metal and environmental factores (Gadd, 1993). In aquatic environments, organisms may be exposed not only to a single chemical but also to a mixture of different substances at the same or nearly the same time, and this can affect biotic communities and ecological processes in a non-predictable way (Duarte et al., 2008).

Heavy metals are known to inhibit the growth (Miersch et al., 2001; Guimarães-Soares, 2005) and reproduction (Abel and Bärlocher, 1984; Rodrigues, 2002) of aquatic hyphomycetes. Additionally, metals can decrease aquatic hyphomycete diversity (Pascoal et al., 2005; Krauss et al., 2005). It has been shown that Zn and/or Cu slow down leaf decomposition due to alterations in the structure and activity of aquatic hyphomycete communities (Duarte et al., 2004, 2008). Furthermore, it has been observed that acute metal contamination has the greatest impact to aquatic hyphomycete communities during the initial stages of leaf colonization (Sridhar et al., 2005).

1.3. Cellular mechanisms involved in metal detoxification and tolerance

1.3.1. Extracellular complexation

Complexation of metals by organic molecules is a significant process in which organic acids play fundamental roles in the environmental mobilization of metals (Gadd, 1999). Wood-rotting fungi can overexcrete organic acids (oxalic and citric acids)
with strong metal-chelating properties, suggesting that a ligand-promoted mechanism is the main mechanism of metal dissolution (Jarosz-Wilkolazka and Gadd, 2003). Also, *Aspergillus Niger* produces metal oxalates in the presence of a wide range of metal compounds, including insoluble metal phosphates, for example of Co, Zn, Cu and Mn (Sayer and Gadd, 1997). On the contrary, organic acid production was not detected in aquatic hyphomycetes exposed to metals, despite the observed decrease in the pH of extracellular medium (Guimarães-Soares, 2005).

1.3.2. Cellular barriers against metal stress

1.3.2.1. Cell wall

Cell walls act as the first physical barrier restricting solute uptake. Chitin, chitosan, glycoproteins and melanins, among others, are the main components of fungal cell walls, and may confer some protection against metal ions (Gadd, 1993).

Metals can rapidly bind to fungal cell walls by non-metabolic processes such as ion exchange, adsorption, complexation, precipitation and cristalization (Gadd, 1993; Cervantes and Gutierrez-Corona, 1994; Blaudez *et al*., 2000). Surface biosorption may be the most significant mechanism in controlling metal uptake, being implicated in metal resistance of microrganisms (Podgorskii *et al*., 2004). Metal sequestration during biosorption occurs by means of complex mechanisms that include mainly ionic interactions and formation of complexes between metal cations and ligands in the structure of the cell wall, as well as precipitation on the cell wall matrix (Schiewer and Volesky, 1996).

It has been reported that metal-tolerant fungal species exhibit higher metal biosorption rates to cell walls than less tolerant ones (Gardea-Torresdey *et al*., 1997). A research with wood-rotting fungi exposed to Cu found that 38% to 77% of metal could be biosorbed (Gabriel *et al*., 2001). Studies on biosorption of Pb by filamentous fungi show that *Aspergillus niger* and *Mucor rouxii* had an exceptionally high Pb biosorption capacity, and curiously both contain chitin and chitosan in the cell wall, which appear to provide prominent metal adsorption ability. Biosorption of metals by filamentous fungal biomass is strongly affected by pH, initial metal ion concentration, medium composition and exposure time (Waihung *et al*., 1999). In *Mucor rouxii*, a decreased biosorption capacity with a simultaneous decrease in pH suggests that metal cations and protons compete for the same binding sites in the cell wall (Waihung *et al*., 1999).
In yeasts, the role of cell walls in conferring metal protection is controversial; *Saccharomyces cerevisiae* treated with Cu did not show metal bound to the cell wall, but localized intracellularly (Sarais *et al.*, 1994); however, Podgorskii and co-workers (2004) found that yeasts of the genera *Saccharomyces*, *Pichia* and *Candida* can efficiently promote biosorption of metals.

In aquatic hyphomycetes, the involvement of the cell wall in heavy-metal resistance is less documented. In *Heliscus lugdunensis*, Cd and Zn biosorption increased with the increase of metal concentration (Jaeckel *et al.*, 2005). However, Cd and Cu biosorption rates in two strains of *H. lugdunensis* isolated from sites with different degree of metal pollution were not correlated to metal tolerance (Braha *et al.*, 2007).

### 1.3.2.2. Plasma membrane

Toxic effects of metals include disruption of cellular membrane integrity. In *S. cerevisiae*, it has been extensively reported that Cd and Cu induce plasma membrane permeabilization with cellular K\(^+\) efflux (Gadd, 1993; Avery *et al.*, 1996). Similar effects were reported in higher organisms and have been attributed to the redox-active nature of Cu and its ability to catalyze the generation of free radicals, promoting lipid peroxidation (Stohs and Bachi, 1995). For *S. cerevisiae*, Cu was more toxic than Cd; however, Cu exposure resulted in less K\(^+\) release than the observed for Cd (Howlett and Avery, 1997). In this situation, toxicity of Cu was attributed to the direct interaction with nucleic acids or misincorporation into metallothioneins (Cervantes and Gutierrez-Corona, 1994).

Physical properties of cell membranes are largely determined by their lipid composition, especially the degree of fatty acid unsaturation, which may be an important characteristic determining the differential susceptibility of individual microorganisms to Cu toxicity (Avery *et al.*, 1996). Primary targets of free radicals in biological membranes are the polyunsaturated fatty acids (PUFA) and the enrichment of *S. cerevisiae* membranes with the PUFA linoleate markedly enhanced the susceptibility to Cu (Avery *et al.*, 1996). The increased susceptibility of PUFA-enriched *S. cerevisiae* to Cd and Cu induced plasma membrane perturbation, and toxicity was correlated with elevated lipid peroxidation (Howlett and Avery, 1997). During normal cellular metabolism, the formation of high levels of thiobarbituric acid reactive substances (TBARS) can be disallowed by glutathione peroxidase activity (GPx). Glutathione
peroxidase converts lipid hidroperoxides to their corresponding hidroxy fatty acids (Davies, 1995). Since reduced glutathione (GSH) is a principal cellular target or sequestration site of Cd (Stohs and Bachi, 1995), the higher levels of TBARS in Cd-exposed cells may reflect GSH depletion and a reduced capacity of cells to repair lipid peroxidation (Howlett and Avery, 1997).

In *Scenedesmus sp.* increased lipid peroxidation was found in the presence of both Cu and Zn, although more severely with Cu (Tripathi et al., 2006). Copper and Zn induced less oxidative stress in adapted than in non-adapted cells. In the latter situation, the ability of Cu or Zn to generate lipid peroxidation was significantly lower (Tripathi et al., 2006). However, lipid peroxidation was not found in aquatic hyphomycetes grown in the presence of Cu, Zn or Cd, but all metals induced loss of plasma membrane integrity (Guimarães-Soares, 2005).

### 1.3.2.3. The role of \(H^+\)-ATPase

The chemical and electrical gradients across membranes is one of the main requirements of living cells, and transport proteins embedded in plasma membrane are responsible for the maintenance of these gradients (Wolfgand, 1997). A key component of fungal plasma membrane responsible for the maintenance of the electrochemical gradient of protons is the plasma membrane \(H^+\)-ATPase (Serrano, 1988).

Proton ATPases belong to the P-type ATPase family, which are proton pumps driven by ATP hydrolysis. Proton extrusion provides energy for the transport of ions and nutrients in and out of cells and contributes for the maintenance of intracellular pH (Gancedo and Serrano, 1989). Specific conditions are required for the \(H^+\)-ATPase activity such as: i) stability of plasma membrane, ii) stability of \(H^+\)-ATPase enzyme and iii) presence of sufficient ATP (Karamushka and Gadd, 1994). This enzyme is the major protein in plasma membrane of *S. cerevisiae* and was estimated to consume between 10 and 15% of the total ATP during yeast growth (Gancedo and Serrano, 1989).

In yeasts, the major plasma membrane \(H^+\)-ATPase is encoded by *PMA1* (Ghislain and Goffeau, 1991). In addition, a second gene, *PMA2*, encoding \(H^+\)-ATPase and showing high homology with *PMA1* was found (Ghislain and Goffeau, 1991). Nevertheless, Pma2p is expressed at very low levels (Supply et al., 1993).

Various authors have shown that the \(H^+\)-ATPase activation in cells under stress constitutes a response that presumably helps the cells to counteract the stress-induced
dissipation of proton motive force across the plasma membrane and the decrease of intracellular pH (Alexandre et al., 1996; Fernandes and Sá-Correia, 2000). A more active plasma membrane H\(^+\)-ATPase was observed in *S. cerevisiae* in the presence of Cu rather than in cells grown the absence of this metal. This activation is not due to increased expression of *PMA1*, whose expression is normally low under Cu stress (Fernandes and Sá-Correia, 1999). Under these conditions, a slightly lower content of ATPase protein was detected in the plasma membrane of cells grown in the presence of Cu.

Copper is a potent depolarizer of cell electrical potential and can inhibit the H\(^+\)-ATPase at relatively low concentrations (Karamushka and Gadd, 1994). Tallineau and co-workers (1984) suggested that the formation of Cu-ATP complexes might directly inhibit H\(^+\)-ATPase. Moreover, the disruption of membrane permeability leads to a considerable reduction of ATP in cells limiting proton extrusion (Serrano, 1980). Cells of *S. cerevisiae* adapted to intermediate Cu stress exhibited a more active plasma membrane ATPase, which decreases at higher Cu concentrations. Under high Cu stress, the capacity of the yeast cells to cope with the deleterious effects of Cu was exceeded and plasma membrane H\(^+\)-ATPase activity drastically declined (Fernandes et al., 2000).

Copper induced drastic alterations in plasma membrane lipid organization, probably due to higher levels of lipid peroxidation, which may affect the performance of H\(^+\)-ATPase and plasma membrane function as a barrier. H\(^+\)-ATPase activation may be the result of differences in plasma membrane physical properties and/or lipid composition of cells growing under Cu-induced stress (Howlett and Avery, 1997). This kind of explanation was also suggested for ATPase activation by decanoic-acid (Alexandre et al., 1996).

The proton pump activity is also modulated by other metals. For instance, increasing Zn concentrations led to a decrease in H\(^+\) pumping activity in *S. cerevisiae* (Karamushka and Gadd, 1994), and Cd inhibited the activity of this enzyme in rice roots (Ros et al., 1992). Furthermore, a depolarization of transmembrane electrical potential after Cd and Al exposure was observed in roots of maize (Pavlovkin et al., 2006) and *Arabidopsis* (Illéš et al., 2006).
1.3.2.4. The role of efflux pumps and vacuole

The involvement of active efflux pumps in mechanisms of drug resistance has been described in different cell types (Hirata et al., 1994b; Ramage, et al., 2002). The human P-glycoprotein, an integral membrane protein that functions as an ATP-dependent efflux pump, has been described to be important to reduce intracellular drug accumulation in resistant cells (Scarborough, 1995). Gray and co-workers (2003) isolated a gene from the fungus *Paracoccidioides brasiliensis*, which encodes a half-ABC transporter, designated as *Pfr1*, which shares high identity with members of the ABC-superfamily involved in multidrug resistance.

In the freshwater protozoa, *Euglena gracilis*, an efflux pump similar to the multidrug resistance P-glycoprotein was found to be involved in Cd resistance (Einicker-Lamas et al, 2003). Also, a multixenobiotic resistance protein was induced by several metals in *Corbicula fluminea*, a freshwater clam (Achard et al., 2004).

The control of metal concentrations within cells may also depend on metal transport to organelles, such as vacuoles. In plants and fungi, metals are sequestered into the vacuole, and ABC-type transporters may play a major role in metal detoxification pathways (Ortiz et al., 1995). Several studies show that Zn accumulation in vacuoles of plants is decisive for Zn homeostasis (Küpper et al., 1999; Kobae et al., 2004). Vacuoles can store Zn for later use under deficient conditions, and acts as a buffer when rapid changes in intracellular Zn levels occur (MacDiarmid et al., 2002). Experiments in vacuolar-defective mutants of *S. cerevisiae* confirmed the essential role of the vacuole in Zn, Co, Mn and Ni detoxification, but not in compartmentalization of Cu and Cd (Ramsay and Gadd, 1997). However, the accumulation of Cd in the vacuole of *Paxillus involutus* appears to be essential for metal detoxification (Blaudez et al., 2000).

1.3.2.5. Cu and Zn transport and its transcriptional regulation

Copper uptake occurs by high- and low-affinity transport systems. This metal exists in two different valence states; the lower valence form is the substrate for both the high- and low-affinity transport systems (Hassett and Kosman, 1995). In *S. cerevisiae*, reduction of the most commonly occurring Cu$^{2+}$ is achieved by two plasma membrane reductases encoded by *FRE1* and *FRE2* genes (Georgatsou et al., 1997). Transcription of *FRE1* is regulated by intracellular Cu concentration through the copper-dependent
transcription factor Mac1p (Georgatsou et al., 1997; Hassett and Kosman, 1995). The high-affinity Cu uptake is mediated by two plasma membrane transporters encoded by 
CTR1 and CTR3 (Knight et al., 1996). Under Cu-limiting conditions, CTR1, CTR3 and FRE1 are highly expressed, whereas under Cu-replete conditions these genes are downregulated (Martins et al., 1998). Under high concentrations of Cu ions, the otherwise stably present Mac1p is rapidly degraded, preventing the expression of the Cu transport genes (Zhu et al., 1998).

In S. cerevisiae, Zn uptake is carried out by one of two transport systems: a high-affinity system, encoded by ZRT1 that is induced by low Zn concentrations (Zhao and Eide, 1996a) and a low-affinity system, encoded by ZRT2 that is active in Zn-replete cells (Zhao and Eide, 1996b). Expression studies show that the Zrt1p is specific for Zn and does not transport other metals (van Ho et al., 2002). However, uptake assays showed that Cu$^+$ and Fe$^+$ inhibited Zn uptake by the low affinity system, suggesting that they can be substrates for the transporter (van Ho et al., 2002). Evidence for transcriptional regulation is based on the fact that ZRT1 mRNA levels are regulated in response to cellular Zn levels; Zn-depleted cells had 10-times more ZRT1 mRNA than Zn-replete cells. Mutants of Neurospora crassa showed increased Zn resistance due to lower uptake of this metal, suggesting a partial block of Zn uptake (Rama Rao et al., 1997).

1.3.3. Intracellular complexation of heavy metals: the role of thiol compounds

Thiol compounds include nonproteinaceous glutathione (GSH), phytochelatins (PCs) and the metallothionein proteins of families 8-13 (fungi I-VI MTs), which can sequester metal ions (Cervantes and Gutierrez-Corona, 1994; Cobbett and Goldsborough, 2002). Biosynthesis of GSH occurs in two consecutive ATP-dependent steps; in the first step, γ-glutamylcysteine synthetase catalyzes the synthesis of γ-glutamylcysteine from L-glutamate and L-cysteine; in the second step, catalyzed by glutathione synthetase, glycine is added to the C-terminal site of γ-glutamylcysteine to yield the GSH tripeptide (Meister, 1988).

Metals, such as Zn and Cu, led to a decrease in GSH (Nagalakshmi and Prasad, 2001; Geret and Bebianno, 2004) Additionally, GSH play an important role in Cd detoxification in S. cerevisiae (Li et al., 1997). In S. pombe (Al-Lahham et al., 1999), N. crassa (Kneer et al., 1992), Mucor racemosus (Miersch et al., 2001) and Paxillus
involutus (Courbot et al., 2004), Cd exposure led to an increase in nonprotein thiol compounds. In aquatic hyphomycetes, a linear increase in GSH levels with increasing Cd concentrations was found (Miersch et al., 2001).

Phytochelatins are a family of small cysteine-rich peptides enzymatically synthesized from GSH. Its general structure is \((\gamma\text{-Glu Cys})_n\text{-Gly}\), where \(n=2-11\) (Cobbet and Goldsbrough, 2002). Zinc and Cu induce the production of phytochelatins and/or phytochelatin-related peptides in \(S.\ cerevisiae\) (Kneer et al., 1992) and \(Schizosaccharomyces pombe\) (Hayashi and Mutoh, 1994). In the latter species, detoxification of Cd can occur by synthesis of PCs, which mediate Cd sequestration into the vacuole (Vande Weghe and Ow, 2001). On the other hand, in the aquatic hyphomycete \(Articulospora tetracladia\) diminished levels of GSH after Cu-exposure were not accompanied by the synthesis of PCs (Miersch et al., 2001).

Metallothioneins are cysteine-rich molecules with low molecular weight (6.5 kDa) that in their reduced state provide thiols for sulphur-seeking metals (Gadd, 1993; Miersch et al., 2001). These compounds are important for metal detoxification either as metal-chelating agents or ROS scavengers (Kinningham and Kasarskis, 1998). Metallothioneins are produced after Zn exposure in \(S.\ pombe\) (Borrelly et al., 2002) and after Cu exposure in \(S.\ cerevisiae\) (Gadd, 1993; Macreadie et al., 1994), \(Candida glabrata\) (Mehra et al., 1989) and \(Neurospora crassa\) (Münger et al., 1987). In ectomycorrhizal fungi, tolerance to Cd was also associated with the presence of MTs which probably protect the host plant in metal-polluted sites (Courbot et al., 2004). Some strains of aquatic hyphomycetes are known to increase the production of MTs under metal stress (Miersch et al., 2001; Jaeckel et al., 2005; Guimarães-Soares et al., 2006).

Glutatione and MTs have cooperative protection role against Cd toxicity, as an initial defence for the former and a second-stage defence for the latter. In fact, although the main role in metal detoxification can be attributed to MTs, induction of MTs by metal cations is relatively slow, and considerable toxic effects can occur before the establishment of effective levels of MTs (Ochi et al., 1988).
1.3.4. Oxidative stress induced by heavy metals

1.3.4.1. Enzymatic and non-enzymatic defenses against oxidative stress

Molecular oxygen (O$_2$) is essential for aerobic organisms, as terminal electron acceptor in mitochondrial respiration, where it is ultimately reduced to water during oxidative phosphorylation. However, the reduction of O$_2$ to water requires four electrons and this reduction precedes sequentially through the one-, two-, and three-electron products, namely superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (·OH) respectively (Di Guilio et al., 1995). Superoxide anion and ·OH are potent oxidants and ·OH is extremely reactive, attacking non-specifically biomolecules, such as proteins and nucleic acids (Bai et al., 2003). Although not considered a free radical, H$_2$O$_2$ is also reactive, and via the Waber-Weiss reaction with O$_2^-$ serves as an important precursor to ·OH. Superoxide anion, O$_2^-$, is not a particularly damaging species, but it can generate H$_2$O$_2$ and ·OH. When transition metals are involved (Fe$^{2+}$ or Cu$^+$) a higher production of ·OH can occur via Fenton reaction (Bai et al., 2003).

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} (\text{Cu}^+) \rightarrow \text{Fe}^{3+} (\text{Cu}^{2+}) + \cdot \text{OH} + \cdot \text{OH}
\]

To keep O$_2^-$, H$_2$O$_2$ and transition metals, such as Cu and Fe, under control, cells developed sophisticated strategies (Bai et al., 2003). Under normal physiological conditions, antioxidant defense mechanisms are almost certainly adequate to maintain ROS at basal unharmed levels and to repair cellular damages.

Adaptative responses to oxidative stress include increased activities of antioxidant enzymes and/or concentrations of non-enzymatic antioxidant components (Gaetke and Chow, 2003; Fujs et al., 2005). Important antioxidant enzymes are: i) superoxide dismutases (SODs), which include a group of metalloproteins, namely Fe-SOD, Mn-SOD and Cu/Zn-SOD, with a major role in the O$_2^-$ detoxification; ii) catalase (CAT), an ubiquitous enzyme which detoxifies H$_2$O$_2$; iii) glutathione peroxidases (GPx), which catalyse the reduction of H$_2$O$_2$ and other peroxides, using GSH as the electron donor; iv) glutathione reductase (GR) responsible for the reduction of oxidized GSH and maintenance of the GSH:GSSH ratio in cells; and v) glucose-6-phosphate dehydrogenase (G6-PDH), the first and rate-limiting enzyme of the pentose phosphate pathway, important for the generation of NADPH essential to maintain the cellular redox balance (Pócsi et al., 2004).
In algae, an increase in CAT and SOD activities under Cu and Zn stress was observed (Tripathi et al., 2006). 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). Catalase activity was enhanced by Zn exposure in mussels (Geret and Bebianno, 2004). Different yeast strains under Cu and Zn stress also showed SOD and CAT activation (Lapinskas et al., 1993; Fujs et al., 2005). In aquatic hyphomycetes, it was found an increase in CAT and G6-PDH activities in presence of Cu, Zn and Cd in *Fontanospora fusiramosa* and an increase in CAT activity in *F. curta* after Cu exposure (Guimarães-Soares, 2005). Accordingly, G6PDH-deficient cells of *S. cerevisiae* are more susceptible and unable to adapt to oxidative stress (Izawa et al., 1998). In addition, an increase in peroxidase activity under Cd stress and a decrease in GR activity after Cd and Cu exposure were found in strains of the aquatic hyphomycete *H. lugdunensis* (Braha et al., 2007).

Non-enzymatic defense systems consist of small molecules present in aqueous or lipidic environments that remove oxidants from solution, acting as free radical scavengers (Jamieson, 1998). Important non-enzymatic antioxidants in microorganisms are GSH, trehalose, carotenoids, L-ascorbic acid and tocopherols (Bai et al., 2003). Gluthatione can directly scavenge radicals and/or provide reducing equivalents for the reduction of peroxides by GPx (Di Giulio et al., 1995). In *S. cerevisiae*, the γ-glutamylcysteine synthetase (*gsh1*) mutants, deficient in GSH synthesis, are hypersensitive to H$_2$O$_2$ and O$_2^-$ (Stephen and Jamieson, 1996). Under peroxide stress, imposed by H$_2$O$_2$ and tert-butyl hydroperoxide, *Penicillium chrysogenum* showed remarkable resistance to this hyperoxidant environment, and both GSH and GSH-dependent enzymes were involved in H$_2$O$_2$ elimination (Bai et al., 2003). In lichens, elevated Cu concentrations caused a significant decrease in GSH, possibly due to metal-induced oxidation of GSH to GSSH (Bačkor et al., 2006). In contrast, induction of GSH synthesis was detected in *Paxillus involutus* and in *H. lugdunensis* under Cd stress (Courbot et al., 2004; Jaeckel et al., 2005).

Trehalose, a non-reducing disaccharide, is found in a wide variety of microorganisms and its involvement in the resistance to heat-shock and oxidative stress has been reported (Fillinger et al., 2001; Bai et al., 2003). Several studies in yeasts
showed trehalose accumulation during exposure to H$_2$O$_2$, CuSO$_4$, or 4-hydroxy-2-nonenal (a product of lipid peroxidation) (Wonisch et al., 1997; Pedreno et al., 2002).

Ascorbic acid is important especially for higher eukaryotes and can complex Cd or redox metal ions displaced by Cd, preventing lipid peroxidation (Stohs and Bagchi, 1995). High intakes of ascorbic acid and Zn may provide protection against Cu toxicity preventing excess of Cu uptake (Gaetke and Chow, 2003). Tocopherols are also important for the inhibition of lipid peroxidation in membranes as demonstrated by the protective effect of vitamin E against lipid peroxidation induced by Cr (Valko et al., 2005).

1.3.4.2. Gene expression in response to oxidative stress

Several of the genes that participate in cellular defense against oxidative stress are known to display increased expression under oxidative stress conditions (Moradas-Ferreira et al., 1996). Much of the regulation of the antioxidant responses in S. cerevisiae is at the transcription level. Several transcription factors regulate gene expression in response to oxidants (Jamieson, 1998). From these, Yap1p, Yap2p and Gcn4p have been extensively studied and all play a crucial role protecting yeast cells against stress (Fernandes et al., 1997). The role of Yap1p in the regulation of antioxidant enzymes was first suggested when Yap1 mutants of S. cerevisiae were found to be hypersensitive to oxidants (Schnell al., 1992). These mutants showed reduced activities of SOD and G6-PDH, and their adaptive responses to H$_2$O$_2$ were severely affected, showing that Yap1p affects the transcription of genes involved in such responses (Stephen et al., 1995). However, the Yap1 mutant retained a small H$_2$O$_2$-adaptive stress response, implicating additional factors in this process. Expression of YAP1 in high copy number resulted in a modest increase of GSH levels and activity of SOD and G6-PDH. YAP2 was identified by its ability, when overexpressed, to confer resistance to Cd, and by the hypersensitivity of Yap2 null mutants to oxidants (Hirata et al., 1994a). Yap2p plays an important role in the regulation of the H$_2$O$_2$ adaptive stress response, since induction of this response was diminished in Yap2 null mutant (Stephen et al., 1995). Furthermore, YAP1 and YAP2 overexpression can both enhance Cd resistance (Hirata et al., 1994a).

Hap1p is responsible for the regulation of the expression of both CYC1 (iso-1-cytochrome) and CYC7 (iso-2-cytochrome) genes in response to oxygen and heme
General introduction

(Zitomer and Lowry, 1992). For this reason, Hap1p has consequently been involved in the regulation of a variety of genes encoding hemeo-proteins, such as CTT1 and CTA1 (the cytosolic and peroxisomal catalases) and components of the mitochondrial respiratory chain as SOD2 (mitochondrial manganese superoxide dismutase) (Gralla and Kosman, 1992). Cta1p and Ctt1p are hypersensitive to H₂O₂ and both single and double catalase yeast mutants are unable to display an adaptive stress response to H₂O₂ (Izawa et al., 1995).

Copper is an important co-factor for Cu/Zn-SOD, stimulating both SOD mRNA accumulation and enzyme activity in vitro. The cytoplasmatic SOD, which is coded by SOD1, appears to be a key enzyme involved in the regulation of intracellular levels of ROS, protecting cells from exogenous toxicity of oxidant agents (Jamieson, 1998). Previous studies demonstrated that Ace1p is the transcription factor responsible for Cu induction of SOD1 expression (Gralla et al., 1991). In S. cerevisiae, the integrity of only one SOD gene is enough to confer resistance to oxidative conditions originated by H₂O₂ (Pereira et al., 2001). However, the defense against oxygen toxicity involves both Cu/Zn-SOD and Mn-SOD (Longo et al., 1996). S. cerevisiae null mutants of SOD have several biochemical defects, indicating that SOD genes may protect numerous metabolic enzymes against oxygen-induced damage (Gralla and Valentine, 1991).

Other genes of oxidative-stress protection are known to be regulated by metal-responsive transcription factors and sometimes more than one system may be operative (Moradas-Ferreira et al., 1996). CTT1, can be activated by both H₂O₂ and heat shock through the general stress-response element (Schuller et al., 1994).

Concerning GSH, their recycling is dependent on the maintenance of an intracellular pool of NADPH mainly via the pentose phosphate pathway, in which the reaction catalyzed by the G6-PDH is the rate-limiting step (Jamieson, 1998). Mutations in ZWF1, the gene which encodes G6-PDH, make cells hypersensitive to oxidants such as H₂O₂ (Juhnke et al., 1996). In contrast, overexpression of ZWF1 gene in G6-PDH deficient cells restored the ability to induce adaptation to H₂O₂ stress (Izawa et al., 1998).

The genes GSH1 and GSH2, encoding enzymes of GSH biosynthesis, were identified in S. cerevisiae (Ohtake and Yabuuchi, 1991; Inoue et al., 1998). In this species, the expression of GSH1 is induced by Cd (Stephen and Jamieson, 1997) and is controlled by the Yap1 transcription factor (Wheeler et al., 2003). Both gsh1 and yap1
mutants show hypersensitivity to Cd (Wu et al., 1993). The gsh1 mutants are also sensitive to oxidative stress imposed by H₂O₂ and t-butyl hydroperoxide (Izawa et al., 1995). Deletion of GSH2 does not affect resistance to H₂O₂ and t-butyl hydroperoxide, when appropriate concentrations of the dipeptide γ-glutamylcysteine are present, protecting cells against oxidative injury (Grant et al., 1997). However, expression of GSH1 and GSH2 in the wild-type strain was induced by H₂O₂ and t-butyl hydroperoxide, and was under control of Yap1p (Sugiyama et al., 2000).

There are a number of mechanisms by which the PCs biosynthetic pathway may be regulated; the first is likely the regulation of GSH biosynthesis. Zhu and collaborators (1999) demonstrated that when expression of the enzymes of the GSH biosynthetic pathway increased, the PCs biosynthesis and Cd tolerance also increased. According to these results, regulation of GSH biosynthesis is a plausible endogenous mechanism by which PC expression may be modulated. Exposure of Arabidopsis to Cd and Cu led to an increase in transcript levels of GSH1 and GSH2 (Xiang and Oliver, 1998). The PC synthase catalyzes the transpeptidation reaction of the γ-Glu-Cys moiety of a GSH molecule onto another GSH molecule, forming (γ-Glu-Cys)₂-Gly or onto another (γ-Glu-Cys)ₙ-Gly molecule, forming the n+1 oligomer (Grill et al., 1989).

Metal detoxification also occurs via-metal mediated transcriptional activation of MT genes by increasing synthesis of MTs (Zhang et al., 2001). Transcriptional induction of MT genes is mediated by the metal-responsive transcription factor 1 (MTF-1), an essential Zn finger protein that binds to specific DNA motifs termed metal-response elements. Transcriptional induction of MTs genes by Zn can be achieved by elevated Zn concentration alone, but induction by Cd or Cu requires the presence of a Zn-satured metallothioneins (Zhang et al., 2003). This is explained by the preferential binding of Cd or Cu to MTs, with the concomitant release of Zn, which in turn, leads to the activation of the transcription factor MTF-1. The release of Zn from cellular components, including MTs, and the sequestration of Zn by newly produced apometallothioneins may be a basic mechanism to regulate MTF-1 activity upon cellular stress. Metallothioneins are not only passive targets of MTF-1, but may rather contribute to the regulation of its activity (Zhang et al., 2003).

In response to Cu toxicity, CUP1 gene is transcriptionally activated in S. cerevisiae and MT are formed (Thiele, 1992). Studies with SOD1 mutants in presence of Cu also suggest that CUP1-encoded MT can function as an antioxidant (Liu and
Thiele, 1997). A second *S. cerevisiae* MT is encoded by *CRS5* gene and is also induced by Cu, even though to a less extent than *CUP1* (Culotta *et al*., 1994). The gene *CRS5* was transcriptionally repressed by oxygen (Culotta *et al*., 1994). Both *CUP1* and *CRS5*, are transcriptionally activated by Ace1-transcription factor responsible for Cu induction of *SOD1* expression (Gralla *et al*., 1991). Since Cu catalyses reactions that generate free radicals from O$_2^{-}$, MTs and Cu/Zn-SOD remove these components. The presence of Cu bound to the Mac1p and Ace1p suggests that these transcription factors may be redox active and, therefore, may play a role in the ability of cells to sense oxidants (Jungmann *et al*., 1993).

### 1.3.4.3. DNA damage induced by ROS and programmed cell death

Several types of enzymatic repair processes developed during evolution are essential to maintain the fidelity and integrity of genetic information. DNA is the only molecule with capacity of self-repair, replacing damaged segments; therefore, if a mutagen induces a DNA lesion and the lesion is repaired before “fixation”, there may be no effect on DNA. This is especially true after low-level of mutagen exposure, where excision repair enzymes are not saturated by a significant number of DNA damaged sites. Exposure of an organism to genotoxic chemicals may include a cascade of DNA-damaging events; initially, structural alterations are formed, then DNA damage is processed and subsequently expressed in mutant gene products (Shugart, 1995).

DNA plays an important role in life and reproduction of each organism; in light of this fact it is of extreme importance to study the effect of oxidative stress and metal induced-oxidative stress on DNA damage. Reactive oxygen species produced *in vivo*, at levels that cannot be dealt conveniently by endogenous antioxidant systems, can lead to damage of lipids, proteins, carbohydrates and nucleic acids (Bai *et al*., 2003). These reactive species may affect all cellular functions, since they are non-specific in their action, although DNA oxidative damage is the most critical target. The non-radical singlet oxygen $^{1}$O$_2$ and the radical $^{·}$OH are the major damaging oxidative species. These species can be generated inside cells during normal aerobic metabolism and fulfill essential prerequisites to be genotoxic agents. Moreover H$_2$O$_2$, and O$_2^{-}$ also induce a spectrum of DNA lesions, such as single-strand breaks, double-strand breaks, crosslinking of DNA and damages to bases (Jornot *et al*., 1998).
Furtheremore, ROS can be assumed as signalling molecules which activate crucial components of programmed cell death (PCD) or in alternative can act indirectly by modifying the cellular redox potential, which regulates key regulatory proteins involved in PCD (Madeo et al., 1999). Low external doses of H$_2$O$_2$ or depletion of glutathione triggers *S. cerevisiae* into PCD, whereas depletion of ROS prevents PCD (Madeo et al., 1999). A recent study reported that ROS accumulation is apparent in almost every apoptotic scenario (Ludovico et al., 2005). In filamentous fungi, such as *Aspergillus nidulans* and *A. fumigatus*, the involvement of ROS in PCD has also been reported (Semighini et al., 2006; Mousavi and Robson, 2003). Furtheremore, the ability of some antioxidant enzymes, such as catalase, to block PCD argues for the central role for oxidative stress in PCD (Buttke and Sandstrom, 1994).

Metal effects in fungi associated with PCD processes are poorly documented. However, exposure to Cd, Cu, Zn and Pb in plants (Gichner et al., 2006), to Cd, Zn, Cu in mammalian cells (Wätjen et al., 2002; Wolfe et al., 1994) or to Zn in HEP-2 cells (Rudolf et al., 2005) may induce phenotypical alterations characteristics of PCD processes.

Programmed cell death is characterized by phenotypical alterations, such as chromatin condensation (Clifford et al., 1996), DNA fragmentation, formation of membrane-enclosed cell fragments (apoptotic bodies) and caspase activation (Tsujimoto, 1997). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff et al., 1994).

Caspases have been considered important mediators of apoptosis, playing a critical role in the downstream execution of the PCD pathway in higher eukaryotes (Earnshaw et al., 1999). Caspase activity is responsible directly or indirectly for cleavage of several intracellular proteins, including proteins of the nucleous, endoplasmic reticulum and cytosol, which are characteristically proteolysed during PCD (Rosse et al., 1998). The genome of *S. cerevisiae* encodes a single metacaspase, Yca1p (Madeo et al., 2002). The yeast apoptotic responses often dependent on Yca1p (Silva et al., 2005; Madeo et al., 2002), though not always (Wissing et al., 2004), indicating a non-exclusive role for metacaspase in PCD in response to toxics. Disruption of YCA1 in *S. cerevisiae* rescue yeasts from PCD, confirming its functional role as an executor of PCD (Vanovska and Hardwick, 2005). Two metacaspases have been found in *A. fumigatus* (Mousavi and Robson, 2003), and two caspases-like
(caspase 3 and caspase 8) activities have been identified in *A. nidulans* during sporulation (Thrane *et al*., 2004).

Programmed cell death allows the rapid removal of unwanted or damaged cells that could otherwise inflame the surrounding cells with their cytoplasmic contents (Madeo, 1997). This process is considered an altruistic mechanism, since spares energy sources for the undamaged cells, and may constitute an evolutionary advantage.

Several investigations revealed that oxidation of thiols other than GSH can mediate induction of PCD, suggesting that the intracellular thiol redox status would be the key factor of the cell death signalling pathways (Sato *et al*., 1995). In fact, PCD can be induced by growing a *gsh1* deletion mutant in the absence of GSH (Madeo *et al*., 1999) or by the oxidation of cellular sulphydryl groups (Sato *et al*., 1995). Thiols have been proposed to play a protective role in oxidative DNA damage by quenching radical species in solution and repairing deoxyribose and nucleo-base radicals. In fact, in the presence of Zn or Cu, MTs can act as effective antioxidants preventing apoptotic mechanisms (Santon *et al*., 2004). Moreover, an active pentose phosphate pathway is required for double-strand-break rejoining in mammalian cells exposed to a mild thiol oxidant (Ayene *et al*., 2000).

1.4. Aim and outline of the thesis

In this study, we assessed the effects of heavy metals in aquatic hyphomycetes by examining several cellular targets and putative defense mechanisms against metal stress to better understand the ability of these group of fungi to survive in metal-polluted environments.

Chapter 1 provides information on the role of aquatic hyphomycetes in freshwater ecosystems and focuses on the negative effects of metal pollution to biota. Particular attention is given to the cellular mechanisms involved in metal detoxification and tolerance. In Chapter 2, the effects of metals, such as Cu, Zn, Cd and Ni, on growth and sporulation of several aquatic hyphomycete species are evaluated. This allowed the selection of fungal species with different sensitivities to metals, to further investigate the interactions between aquatic hyphomycetes and metals. Chapter 3 focuses on the role of antioxidant defenses against Cu and/or Zn stress in *Varicosporium elodeae* and *Heliscus submersus*. Firstly, we assessed the ability of metals to induce reactive oxygen
species (ROS) and plasma membrane disruption. Subsequently, we evaluated the role of catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase to deal with acute- and chronic-metal stress. In Chapter 4, we examined the ability of three aquatic hyphomycete species (*Varicosporium elodeae, Heliscus submersus* and *Flagellospora curta*) to adsorb and accumulate Cu or Zn. Then, we assessed the effects of these metals on H^+\,-ATPase activity and on the levels of thiol-containing compounds. In Chapter 5, we tested whether Cu and Zn stress is able to induce programmed cell death in aquatic hyphomycetes through the evaluation of typical apoptotic markers, namely ROS production, caspase activation, alterations in nuclear morphology and the occurrence of DNA strand-breaks. Finally, in Chapter 6, the main conclusions are presented to provide a global perspective of this work.

**References**


General introduction


General introduction


adsorption and morphological alterations of cells of copper-stressed *Mucor rouxii*. 
Environmental Toxicology and Chemistry 16: 435-441.

Gange E, Mavrogianiis LA, Fragiadakis GS, Alexandraki D. 1997. The yeast Fre1p/Fre2p
cupric reductases facilitate copper uptake and are regulated by the copper-modulated

decussatus*? Ecotoxicology and Environmental Safety 57: 399-409.

Ghislain M, Goffeau A. 1991. The pma1 and pma2 H(+) ATPases from *Schizosaccharomyces
pombe* are functionally interchangeable. The Journal of Biological Chemistry 266:
18276-18279.

Gichner T, Patková Z, Száková J, Demnerova K. 2006. Toxicity and DNA damage in tobacco
and potato plants growing on soil polluted with heavy metals. Ecotoxicology and
Environmental Safety 65: 420-426.

Graça MAS, Pozo J, Canhoto C, Elosegi A. 2002. Effects of *Eucalyptus* plantations on detritus,

Gralla EB, Thiele DJ, Silar P, Valentine JS. 1991. ACE1, a copper-dependent transcription
factor, activates expression of the yeast copper, zinc superoxide dismutase gene.
Proceedings of the National Academy of Sciences of the United States of America 88:
8558-8562.

Gralla EB, Valentine JS. 1991. Null mutants of *Saccharomyces cerevisiae* Cu, Zn superoxide
dismutase: characterization and spontaneous mutation rates. Journal of Bacteriology
173: 5918-5920.

Gralla EB, Kosman DJ. 1992. Molecular genetics of superoxide dismutases in yeasts and related

Grant CM, MacIver FH, Dawes IW. 1997. Glutathione synthetase is dispensable for growth
under both normal and oxidative stress conditions in the yeast *Saccharomyces
cerevisiae* due to an accumulation of the dipeptide γ-glutamylcysteine. Molecular
Biology of the Cell 8: 1699-1707.

Gray CH, Borges-Walmsley MI, Evans GJ, Walmsley AR. 2003. The *pfr I* gene from the
human pathogenic fungus *Paracoccidioides brasiliensis* encodes a half-ABC transporter
that is transcribed in response to treatment with fluconazole. Yeast 20: 865-880.

peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine


Hirata D, Yano K, Miyakawa T. 1994a. Stress-induced transcriptional activation mediated by YAP1 and YAP2 genes that encode the Jun family of transcriptional activators in *Saccharomyces cerevisiae*. Molecular and General Genetics 242: 250-256.


Pereira MD, Eleutherio ECA, Panek AD. 2001. Acquisition of tolerance against oxidative damage in *Saccharomyces cerevisiae*. BMC Microbiology 1:11.
General introduction


General introduction


Chapter 2

Effects of metals on growth and sporulation of aquatic hyphomycetes
Abstract

In this work, we investigated the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species. Effects of metals on growth were assessed in solid and liquid media with different composition (1% malt extract- ME and a mineral medium supplemented with vitamins and 2% glucose- MK), and fungal sensitivity to metals was compared. The exposure to Zn or Cd inhibited sporulation of Heliscus submersus and Tricladium chaetocladium, being the effects stronger in the latter species. In solid medium, mycelial growth was linear and, in most cases, metals negatively affected fungal growth. The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% (EC$_{50}$), showed that Ypsilina graminea and Varicosporium elodeae were the most resistant species to Zn, while Alatospora acuminata, H. submersus and Flagellospora curta appeared to be the most sensitive species to this metal. On the contrary, H. submersus was the most resistant fungus to Cu. Generally, lower toxicity of Zn or Cu than Ni or Cd was found. Moreover, the patterns of species resistance to metals in either liquid or solid medium with similar composition were identical. However, EC$_{50}$ values were about 20-times higher in solid medium than in liquid medium. Changes in nutrient supplies to fungi affected metal toxicity, as shown by higher EC$_{50}$ values in MK than in ME. In addition, fungal tolerance to metals varied with fungal species and metal type, and the tolerance to one metal did not confer tolerance to all metals, suggesting that different mechanisms and/or cellular targets might be implicated in fungal tolerance to different metals.
2.1. Introduction

Heavy metals can be released to the environment from natural processes, but mainly from human activities, such as agriculture, mining and industry. Metal pollution constitutes a serious environmental danger (Ayres, 1992), because metals are not biodegraded, and can be accumulated in living organisms, passing through food chains. Essential metals, such as Zn, Cu and Ni, and non-essential, as Cd, can exert toxicity when present above certain threshold concentrations. Freshwaters are frequently the destination of metals released into the environment. The functioning of these ecosystems depends on recycling of nutrients and energy from allochthonous plant detritus. In this process, a group of fungi known as aquatic hyphomycetes play a critical role. These fungi produce extracellular enzymes able to degrade plant detritus and transform them into a more suitable food source for invertebrate detritivores (Suberkropp, 1998).

Several studies demonstrated that metals can negatively affect growth and reproduction of aquatic hyphomycetes. A decrease in fungal radial growth after exposure to Cd (≥ 50 µM), Cu (≥ 50 µM), Zn (≥ 150 µM) (Miersch et al., 1997) or Ni (>200 µM) (Rodrigues, 2002) has been observed. In addition, Cd (> 0.9 µM; Abel and Bärlocher 1984) and Zn (25 µM Rodrigues, 2002; 150 µM; Duarte et al., 2004) inhibit conidial production of aquatic hyphomycete species. Moreover, data from literature pointed to a higher toxicity of metals to conidial production than to micelial growth (Abel and Bärlocher, 1984; Bermingham et al., 1996; Rodrigues, 2002). However, metal toxicity depends on the fungal species, metal type and several environmental factors, including pH and nutrient availability, which are expected to affect fungal activity and metal bioavailability (Gadd, 1993).

In this work, we investigated the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species. Effects of metals on growth were assessed in solid and liquid media and fungal sensitivity were compared by determining metal inhibition parameters, namely the concentration inhibiting growth in 50% (EC_{50}), the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC).
2.2. Materials and methods

2.2.1. Fungal species and culture maintenance

The aquatic hyphomycetes *Flagellospora curta* J. Webster (UMB-39.01), *Heliscus submersus* H. J. Huds (UMB-135.01), *Tricladium chaetocladium* Ingold (UMB-86-01), *Varicosporium elodeae* W. Kegel (UMB-142.01), *Ypsilina graminea* (Ingold, P. J. Mc Dougall and Dann) Descals, J. Webster and Marvanová (UMB-111-01) and *Alatospora acuminata* Ingold (UMB-173-01) were used in this study. *F. curta*, *H. submersus*, *T. chaetocladium* and *Y. graminea* were isolated from the Este River at the industrial park of the town of Braga, where metal concentrations in the stream water attained 80 µM for Zn, 150 µM for Cu, 52 µM for Ni and 0.53 µM for Cd (Gonçalves, 2001). *A. acuminata* was isolated from a clean site located at the source of the same river and *V. elodeae* was isolated from a clean stream in the Peneda-Gerês National Park. *H. submersus*, *F. curta* and *T. chaetocladium* were isolated from leaves, while *Y. graminea*, *V. elodeae* and *A. acuminata* were isolated from foams. All species were isolated from single spores. Details on fungal species and characterization of water chemistry of their origin sites are in Pascoal et al. (2005).

In the laboratory, fungi were maintained on solid medium containing 2% (w/v) (ME) and 1.5 % (w/v) agar, at 18ºC under permanent artificial light.

2.2.2. Growth experiments

2.2.2.1. Effects of metals on fungal growth in solid medium

The effects of Zn, Cu, Ni and Cd on fungal growth were evaluated in 5 species of aquatic hyphomycetes: *H. submersus, T. chaetocladium, V. elodeae, Y. graminea* and *A. acuminata*. Fungi were grown on 1% ME, 1.5% agar at pH 5.0, supplemented or not with different concentrations of metals. Solid media were inoculated with a 7 mm agar plug (2 week-old cultures) placed centrally on Petri dishes and incubated at 18ºC. Every 3 days, mycelium radial expansion was measured in four replicates.

2.2.2.2. Effects of metals on fungal growth in liquid medium

Effects of metals on fungal growth were evaluated in two different liquid media, namely 1% ME, and mineral medium with vitamins and 2% glucose (MK).
Fifty ml Erlenmeyer flasks containing 20 ml of culture medium were inoculated with conidial suspensions (final concentration, 6 conidia ml\(^{-1}\)) of \textit{V. elodeae}, \textit{Y. graminea}, \textit{H. submersus} and \textit{F curta}. The effect of Zn, Cd and Cu on fungal growth was determined in 1% ME supplemented or not with metals at different concentrations for \textit{V. elodeae}, \textit{Y. graminea} and \textit{H. submersus}. The effect of Zn and Cu in mineral medium was evaluated for \textit{V. elodeae}, \textit{H. submersus}, \textit{F. curta} and \textit{Y. graminea}. In 1% ME fungal growth of \textit{V. elodeae} and \textit{Y. graminea} in presence of Cd and Zn was followed during 8 days (18°C and 160 rpm), while for the other species in this medium and in medium with glucose the biomass was quantified at a fixed day (8\(^{th}\) day). After grown, mycelia were harvested by filtration, washed twice with deionised water, dried at 85°C to constant mass and weighed.

MK was composed of a base medium (0.5% (w/v) (NH\(_4\)) SO\(_4\); 0.5% (w/v) KH\(_2\)PO\(_4\); 0.05% (w/v) MgSO\(_4\) 7H\(_2\)O; 0.013 (w/v) CaCl\(_2\).2H\(_2\)O and deionised water, q.b), supplemented with 2% (w/v) glucose and 0.05% (v/v) of each of the three solutions: oligoelement solution A (1% (v/v) H\(_3\)BO\(_3\); 0.2% (v/v) KI; 0.4% (v/v) NaMoO\(_4\).2H\(_2\)O and deionised water q.b); oligoelement solution B (0.08% (w/v) Cu SO\(_4\).5H\(_2\)O; 0.4% (w/v) FeCl\(_3\).6H\(_2\)O; 0.8% (w/v) MnSO\(_4\).4H\(_2\)O; 0.8% (w/v) ZnSO\(_4\).7H\(_2\)O; 0.8% (v/v) HCl 10\(^{-3}\) N and deionised water q.b); and vitamin solution (0.001% (w/v) biotin; 0.08% (w/v) calcium pantothenate; 4% (w/v) myoinositol; 0.16% (w/v) niacin; 0.16% (w/v) piridoxin hydrochloride; 0.16% (w/v) thiamine hydrochloride, deionised water q.b).

The malt extract and the base media were autoclaved (1 atm, 20 min) while oligoelement solutions, vitamins, and metal solutions were sterilized by filtration.

### 2.2.3. Effects of metals on fungal sporulation

To test the effects of metals on fungal sporulation, fungi were grown in solid medium with 2% ME at pH 5.0 and 18°C during 20 days. Sporulation experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of 10 mM KH\(_2\)PO\(_4\) (pH 5.0), supplemented or not with different concentrations of Zn and Cd, as indicated in the results. Four agar plugs (Ø 5 mm) of each fungal culture were inoculated in each flask, which was kept aseptically under continuously aeration, with aquarium pumps, for 10 days. Every 2 days, all inocula were transferred to fresh solutions with the same concentration of Zn or Cd. To estimate fungal sporulation, conidial suspensions were
mixed with Tween 80 (0.8%) to release the conidia adhered to the glass, and adequate volumes of solutions were filtered (pore size, 5 µm; Millipore). Conidia on filters were stained with 0.1% cotton blue in lactic acid and counted (144 mm²) under a microscope (magnification 100 or 400 X).

Phosphate solutions were autoclaved and metal solutions were sterilized by filtration (0.22 µm pore size membrane). Three independent experiments were performed.

2.2.4. Data analysis

The rate of fungal radial growth (kr) was estimated by linear regression of mycelial radial growth along time. Metal concentrations inhibiting fungal growth in 50% (EC₅₀) were estimated by the Probit Method. The values of EC₅₀ values were compared by t-tests, at 2 levels of comparisons, or by one-way ANOVA when more than 2 levels were considered (Zar, 1996). After one-way ANOVA, a Tukey test was used to identify where the significant effects occur. To determine values of NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) at a fixed time, values of biomass production were compared by one-way ANOVA, followed by a Dunnett’s post-test to identify significant effects ($p<0.05$; Zar, 1996). Data were Ln-transformed to achieve normal distribution and homocedasticity.

To determine metal effects on fungal sporulation, conidial production was converted in percentage of the control (100%), divided by 1000 and normalized by arcsine square root transformation. Data were analyzed by two-way ANOVA, with exposure time and metal concentration as factors. Bonferroni post-test were used to discriminate significant differences ($p < 0.05$; Zar, 1996).

Statistic analysis was done using Prism4 for Windows (GraphPad Software Inc., San Diego).
2.3. Results

2.3.1. Effects of metals on fungal growth

2.3.1.1. Fungal growth in solid medium

In the absence of metals, rates of radial growth (kr) of the aquatic hyphomycetes on 1% ME varied from 1.23 to 0.44 mm d\(^{-1}\) (Table 2.1), being the highest value observed for *V. elodeae* and the lowest one for *A. acuminata*.

Table 2.1. Rates of radial growth (kr) of aquatic hyphomycetes on 1% ME

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>kr ± SE (mm d(^{-1}))</th>
<th>(r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Varicosporium elodeae</em></td>
<td>1.23 ± 0.024</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Ypsilina graminea</em></td>
<td>0.95 ± 0.017</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Tricladium chaetocladium</em></td>
<td>0.80 ± 0.017</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Heliscus submersus</em></td>
<td>0.60 ± 0.040</td>
<td>0.87</td>
</tr>
<tr>
<td><em>Alatospora acuminata</em></td>
<td>0.44 ± 0.011</td>
<td>0.98</td>
</tr>
</tbody>
</table>

*\(r^2\), coefficient of determination; values are mean ± SE, n=80.*

In general, the presence of Zn in the culture medium led to a reduction in the kr for all fungal species (Figure 2.1). *A. acuminata* was the most sensitive species, showing a 95% reduction in the kr at Zn concentration of 1500 µM, while a similar inhibition on kr of the other species was only attained with metal concentrations of about 5-times higher. Moreover, Zn till concentrations of 1500 µM increased the kr of *T. chaetocladium* (Figure 2.1).

Metal concentration inhibiting mycelial growth in 50% (EC\(_{50}\)) for Zn varied from 4258 to 670 µM, with the highest value observed in *Y. graminea* and the lowest one in *H. submersus* (Table 2.2). The analysis of no observed effect concentration (NOEC) and low observed effect concentration (LOEC) values for Zn corroborated the highest sensitivity of *H. submersus* to this metal (Table 2.3).
Figure 2.1. Effect of Zn on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (▼), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18°C. Results are mean ± SE of four replicates.

Table 2.2. Metal concentrations (µM) inhibiting mycelial growth in 50% (EC₅₀) for Zn, Cu, Ni and Cd in *V. elodeae*, *Y. graminea*, *H. submersus*, *T. chaetocladium* and *A. acuminata* after 10 days of metal exposure.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Zn EC₅₀</th>
<th>Cu EC₅₀</th>
<th>Ni EC₅₀</th>
<th>Cd EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. elodeae</em></td>
<td>4134±132</td>
<td>1189±49</td>
<td>101±9</td>
<td>120±3</td>
</tr>
<tr>
<td><em>Y. graminea</em></td>
<td>4258±245</td>
<td>1049±35</td>
<td>106±5</td>
<td>77±5</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>670±78</td>
<td>2236±126</td>
<td>844±29</td>
<td>360±16</td>
</tr>
<tr>
<td><em>T. chaetocladium</em></td>
<td>1963±446</td>
<td>1406±13</td>
<td>24±3</td>
<td>222±4</td>
</tr>
<tr>
<td><em>A. acuminata</em></td>
<td>1192±466</td>
<td>495±5</td>
<td>215±71</td>
<td>66±3</td>
</tr>
</tbody>
</table>

Values are means ± SE.
Effects of metals on growth and sporulation of aquatic hyphomycetes

Table 2.3. Values of NOEC and LOEC obtained in solid medium in presence of Zn, Cu, Ni and Cd in V. elodeae, Y. graminea, H. submersus, T. chaetocladium and A. acuminata after 10 days of metal exposure (µM).

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Zn NOEC (µM)</th>
<th>Zn LOEC (µM)</th>
<th>Cu NOEC (µM)</th>
<th>Cu LOEC (µM)</th>
<th>Ni NOEC (µM)</th>
<th>Ni LOEC (µM)</th>
<th>Cd NOEC (µM)</th>
<th>Cd LOEC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. elodeae</td>
<td>1000</td>
<td>1500</td>
<td>300</td>
<td>700</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>81</td>
</tr>
<tr>
<td>Y. graminea</td>
<td>-</td>
<td>500</td>
<td>200</td>
<td>250</td>
<td>25</td>
<td>50</td>
<td>-</td>
<td>81</td>
</tr>
<tr>
<td>H. submersus</td>
<td>150</td>
<td>300</td>
<td>900</td>
<td>1500</td>
<td>300</td>
<td>600</td>
<td>364</td>
<td>405</td>
</tr>
<tr>
<td>T. chaetocladium</td>
<td>1500</td>
<td>3000</td>
<td>700</td>
<td>900</td>
<td>-</td>
<td>100</td>
<td>138</td>
<td>162</td>
</tr>
<tr>
<td>A. acuminata</td>
<td>500</td>
<td>600</td>
<td>-</td>
<td>40</td>
<td>100</td>
<td>150</td>
<td>-</td>
<td>41</td>
</tr>
</tbody>
</table>

The effects of Cu on the kr of these aquatic hyphomycetes indicated that H. submersus and V. elodeae were the most resistant species to Cu and A. acuminata the most sensitive one (Figure 2.2). Copper at concentrations until 500 µM and 200 µM stimulated the kr of H. submersus and T. chaetocladium, respectively.

Values of EC$_{50}$ for Cu ranged from 2236 to 495 µM, with the highest value for H. submersus and the lowest for A. acuminata (Table 2.2). Analysis of LOEC also supported that A. acuminata was the most sensitive species and H. submersus the most resistant one (Table 2.3).

Figure 2.2. Effect of Cu on the rates of radial growth (kr) of V. elodeae (□), Y. graminea (▲), H. submersus (∇), T. chaetocladium (◊) and A. acuminata (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.
Analysis of Figure 2.3 shows that the highest kr inhibition for Ni was found in *Y. graminea* and *T. chaetocladium*. In the presence of 200 µM of Ni, an inhibition in kr of at least 90% was found for these species. The lowest kr inhibition by Ni was found in *H. submersus* which was the only species whose kr was stimulated by Ni (Figure 2.3). Values of EC$_{50}$ ranged from 844 to 24 µM with the highest values for *H. submersus* and the lowest for *T. chaetocladium* (Table 2.2). Analysis of NOEC and LOEC values also pointed to *H. submersus* as the most resistant aquatic hyphomycete species to Ni (Table 2.3).

Effects of Cd on kr indicated that *H. submersus* was the most resistant species while *A. acuminata* was the most sensitive species to this metal (Figure 2.4). The concentration needed to inhibit the kr in 50% was 4 times higher in *H. submersus* than for *A. acuminata*. Analysis of Figure 2.4 also shows that the tested Cd concentrations significantly decreased the kr of all species, excluding concentrations till 138 µM for *T. chaetocladium*. EC$_{50}$ values for Cd varied from 360 to 66 µM, with the highest value for *H. submersus* and the lowest for *A. acuminata* (Table 2.2). Analysis of LOEC confirmed the highest resistance of *H. submersus* to Cd and the highest sensitivity of *A. acuminata* (Table 2.3).

![Figure 2.3](image_url)

Figure 2.3. Effect of Ni on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18°C. Results are mean ± SE of four replicates.
Figure 2.4. Effect of Cd on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.

When analyzing the degree of toxicity of Zn, Cu, Ni and Cd for each fungal species in terms of EC$_{50}$ values, the following toxicity patterns can be established: *V. elodeae* Ni $\geq$ Cd $>$ Cu $>$ Zn; *Y. graminea* Cd $\geq$ Ni $>$ Cu $>$ Zn; *H. submersus* Cd $\geq$ Zn $\geq$ Ni $>$ Cu; *A. acuminata* Cd $\geq$ Ni $\geq$ Cu $>$ Zn; *T. chaetocladium* Ni $\geq$ Cd $>$ Cu $\geq$ Zn. This indicates higher sensitivity of aquatic hyphomycetes to Cd and Ni than to other metals, except for *H. submersus* (Table 2.2).

### 2.3.1.2. Fungal growth in liquid medium

Two liquid media were used to test the effects of metals on the growth of aquatic hyphomycete species, namely 1% malt extract (ME) and mineral medium with vitamins and 2% glucose (MK).

When *V. elodeae* and *Y. graminea* were grown in 1% ME in the absence of metal, visible growth occurred after 2 days in the former species (Figure 2.5A) and after 4 days in the latter species (Figure 2.5B). After 8 days of growth *V. elodeae* produced more biomass (0.76 mg dry mass ml$^{-1}$) than *Y. graminea* (0.35 mg dry mass ml$^{-1}$) (Figure 2.5). Cadmium exposure led to a decrease in biomass production in *V. elodeae* and *Y. graminea*, and the effects were more pronounced with increasing Cd concentrations (Figure 2.5). After 8 days, the highest Cd concentration (28 µM) led to an inhibition of biomass production in 55 and 86% in *V. elodeae* and *Y. graminea*, respectively (Figure
Furthermore, an increase in the growth lag phase with increasing Cd concentrations was observed for *Y. graminea* (Figure 2.5 B).

Figure 2.5. Effects of Cd on biomass production by *V. elodeae* (A) and *Y. graminea* (B) in 1% ME. Symbols are: ■, control cultures; ▲, 8 µM; △, 16 µM; ◊, 24 µM; ●, 28 µM in graph A and ■, control cultures; ▲, 4 µM; •, 20 µM; ●, 28 µM in graph B. Results are mean ± SEM of three replicates.

Figure 2.6 show the effects of Zn on the growth of *V. elodeae* and *Y. graminea* in 1% ME. Exposure to concentrations of Zn higher than 50 µM led to a decrease in biomass production in *V. elodeae*. For *Y. graminea* all the tested Zn concentrations inhibited biomass production (Figure 2.6 B). An increase in the growth lag phase for the highest Zn concentrations was observed for *Y. graminea*.

Figure 2.6. Effects of Zn on biomass production by *V. elodeae* (A) and *Y. graminea* (B) in 1% ME. Symbols are: ■, control cultures; △, 50 µM; ◊, 100 µM; ●, 150 µM; □, 175 µM in graph A and ■, control cultures; △, 50 µM; ◊, 250 µM; ●, 300 µM in graph B. Results are mean ± SEM of three replicates.
Growth inhibition parameters, namely EC$_{50}$, LOEC and NOEC, determined after 8 days exposure to metals, showed that V. elodeae, Y. graminea and F. curta were more sensitive to Cd than Zn. Moreover Y. graminea was the more resistant species to Zn and F. curta the most sensitive one (Table 2.4). Concerning Cu effect H. submersus was the more resistant species and V. elodeae the more sensitive one (Table 2.4). Values of LOEC and NOEC confirmed that Y. graminea, H. submersus and F. curta were the most resistant species respectively from Zn, Cu and Cd (Table 2.4).

The effects of Zn and Cu on biomass production by V. elodeae, H. submersus, F. curta and Y. graminea were also assessed in MK and results are shown in table 2.5. Analysis of EC$_{50}$ values showed that V. elodeae was the most resistant species to Zn, with an EC$_{50}$ value 16-times higher than that of H. submersus, which was the most sensitive species. However, H. submersus was the most resistant species to Cu, with an EC$_{50}$ 8-times higher than that of F. curta, the most sensitive species to this metal (Table 2.5). Consistently, the highest NOEC and LOEC values were obtained for Zn and Cu in V. elodeae and in H. submersus. Moreover, the most sensitive species to Zn (H. submersus) and to Cu (F. curta) showed the lowest LOEC values (Table 2.5).

Furthermore, results showed that higher concentrations of Zn than Cu were necessary to promote 50% of biomass inhibition for all species, excluding H. submersus (Table 2.5).

Table 2.4. Concentrations (µM) inhibiting biomass production in 50% (EC$_{50}$), concentrations that had no effect on biomass production (NOEC) and the lowest concentrations that induced effect on biomass production (LOEC) for Zn, Cu and Cd in V. elodeae, Y. graminea, H. submersus and F. curta grown 8 days in 1% ME.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Zn EC$_{50}$</th>
<th>Zn NOEC</th>
<th>Zn LOEC</th>
<th>Cu EC$_{50}$</th>
<th>Cu NOEC</th>
<th>Cu LOEC</th>
<th>Cd EC$_{50}$</th>
<th>Cd NOEC</th>
<th>Cd LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. elodeae</td>
<td>152±4.9</td>
<td>100</td>
<td>150</td>
<td>54±4.7</td>
<td>20</td>
<td>50</td>
<td>12.3±1.3</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Y. graminea</td>
<td>194±5.5</td>
<td>150</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.5±0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H. submersus</td>
<td>103±1.6</td>
<td>-</td>
<td>50</td>
<td>102±1.7</td>
<td>30</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F. curta*</td>
<td>78.2</td>
<td>-</td>
<td>-</td>
<td>90.4</td>
<td>15</td>
<td>30</td>
<td>19.3</td>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

*Values are mean ± SE (-) not determined, * values from Guimarães-Soares (2005).
Table 2.5. Concentration (µM) inhibiting biomass production in 50% (EC$_{50}$), concentration that had no effect on biomass production (NOEC) and the lowest concentration that induced effect on biomass production (LOEC) for Zn and Cu in V. elodeae, Y. graminea, H. submersus and F. curta grown 8 days in MK.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Zn</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$</td>
<td>NOEC</td>
</tr>
<tr>
<td>V. elodeae</td>
<td>7315±305.7</td>
<td>7000</td>
</tr>
<tr>
<td>Y. graminea</td>
<td>3740±120.9</td>
<td>1200</td>
</tr>
<tr>
<td>H. submersus</td>
<td>465±83.7</td>
<td>-</td>
</tr>
<tr>
<td>F. curta</td>
<td>1304±54.9</td>
<td>1000</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

2.3.2. Effects of Zn and Cd on fungal sporulation

Figure 2.7 show conidial production along time by H. submersus and T. chaetocladium. In the absence of Zn, a peak of sporulation was observed at the 4$^{th}$ day of the experiment, corresponding to 21643 conidia ml$^{-1}$ for H. submersus (Figure 2.7A) and 3528 conidia ml$^{-1}$ for T. chaetocladium (Figure 2.7B). The sporulation capacity of H. submersus was 6-times higher than that of T. chaetocladium.

Metal effects on sporulation of H. submersus were evaluated in concentrations between 0.25 and 25 µM of Zn. A significant decrease in conidial production was found for all treatments and times ($p<0.001$; Figure 2.7A). In T. chaetocladium a significant decrease in conidial production ($p<0.001$) was observed at all tested Zn concentrations Figure 2.7B. Exposure time significantly affected sporulation of T. chaetocladium ($p<0.001$; Figure 2.7B). Exposure to concentrations of 0.025 µM Zn led to an earlier peak of sporulation (2$^{nd}$ day) while lower Zn concentrations, delayed the peak of sporulation (6$^{th}$ day) (Figure 2.7B).
Effects of metals on growth and sporulation of aquatic hyphomycetes

Cumulative conidial production by *H. submersus* exposed to Zn for 10 days significantly decreased, particularly at higher Zn concentrations (Figure 2.8). In *T. chaetocladium*, Zn at all concentrations drastically inhibited cumulative conidial production, however no significant differences were found between the treatments (Figure 2.8).

Figure 2.7. Effects of Zn on sporulation by *H. submersus* (A) and *T. chaetocladium* (B). Symbols are: ■, control; ♦, 0.25 µM; ▼, 2.5 µM; ▲, 25 µM in graph A; ■, control; ▼, 0.00025 µM; ▲, 0.0025 µM; ♦, 0.025 µM in graph B. Results represent the mean ± SEM of three replicates.

Figure 2.8. Cumulative production of conidia by *H. submersus* (■) and *T. chaetocladium* (▲) in the presence of Zn, after 10 days exposure.
Effects of Cd for *H. submersus* were evaluated in concentrations between 1 and 100 µM along 10 days. The exposure to low Cd concentration did not change the magnitude and position of sporulation peak (*p>*0.05; Figure 2.9A), although significantly lower conidial production was found (*p*<0.001; Figure 2.9A). For 10 µM of Cd, conidial production was very low, with 2 and 1 conidia ml⁻¹ produced after 4 and 6 days, respectively (Figure 2.9A). Concentrations higher than 10 µM completely inhibited sporulation.

In *T. chaetocladium*, the effects of Cd were tested in concentrations between 0.005 µM and 50 µM. Conidial production by this fungal species was only found after 10 days of exposure to the lowest Cd concentration (0.005 µM Cd), with 6 conidia produced per ml of sporulation medium (Figure 2.9B).

### 2.4. Discussion

In natural environments, fungi rarely encounter conditions that allow optimal growth, since they are conditioned by abiotic factors, nutrient availability and pollutants (Gadd *et al.*, 2001). Metals are common in urban aquatic ecosystems since they are not biodegradable and can persist in the environment. Pollution by metals is reported to
Effects of metals on growth and sporulation of aquatic hyphomycetes

decrease fungal diversity in streams (Sridhar et al., 2000; Niyogi et al., 2002; Pascoal et al., 2005). Also, fungal activity, as reproduction and biomass buildup, can be negatively affected by high levels of metals in the stream water (Bermingham et al., 1996; Miersch et al., 1997; Duarte et al., 2004, 2008). Nevertheless, fungi are ubiquitous and can occur in metal-polluted habitats (Sridhar et al., 2000). In the present work, we assessed the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species to understand their ability to survive in metal-polluted streams.

In the absence of metals, conidial production of *H. submersus* was 6-times higher than that of *T. chaetocladium*, which may be due to the marked differences in their conidial size. Indeed, conidia of the former species are, at least, three-times smaller than that of *T. chaetocladium*. This observation is in agreement with previous studies reporting different life strategies of aquatic fungi; generally, fungi with small-size conidia appear to invest in reproduction more than in growth (Chauvet and Suberkropp, 1998; Trenton et al., 2004; Duarte et al., 2006). The exposure to Zn or Cd inhibited sporulation of both fungal species. Conidial production of *T. chaetocladium* was more inhibited by metals than that of *H. submersus*. In addition, Cd was more toxic than Zn to fungal reproduction, which is consistent with the general higher toxicity of Cd than Zn found by other authors in aquatic hyphomycetes (Rodrigues, 2002).

In solid medium, mycelial growth was linear and, in most cases, metals negatively affected fungal growth. Nevertheless, our results showed that low concentrations of Zn, Cu and Ni were able to stimulate the rate of radial growth of some aquatic hyphomycete species, such as *T. chaetocladium, H. submersus* and *A. acuminata*, which is consistent with the role of these metals as micronutrients (Gadd, 1993). Indeed, Zn and Cu are essential components of enzymes, such as cytochrome oxidase and Cu/Zn superoxide dismutase (Walker et al., 1996) and Ni is an essential micronutrient for many microorganisms serving as enzyme cofactor that catalyzes a diverse array of reactions (Hausinger and Zamble, 2007). The general lower toxicity of Zn and Cu than other metals found in this work is corroborated by previous reports (Gadd, 1993; Miersch et al., 1997; Blaudez et al., 2000; Colpaert et al., 2000; Rodrigues, 2002; Guimarães-Soares, 2005). The sensitivity of aquatic hyphomycetes to metals, assessed as EC$_{50}$ values, showed that *Y. graminea* and *V. elodeae* were the most resistant species to Zn, while *A. acuminata, H. submersus* and *F. curta* appeared to be the most sensitive species to this metal. On the contrary, *H. submersus* was the most resistant species to
Cu. Moreover, the patterns of species resistance to metals found either in liquid or solid medium with similar composition were identical. However, EC$_{50}$ values were about 20-times higher in solid medium than in liquid medium, probably because agar might decrease metal bioavailability to fungi (Gadd, 1993).

Changes in nutrient supplies to fungi are expected to change metal toxicity. Indeed, maximum EC$_{50}$ values in MK were at least of one order of magnitude higher than in ME. Gadd et al. (2001) showed that metal toxicity decreased if the amount of carbon source (glucose) increased in the culture medium; MK has higher glucose content than ME, which probably favoured the aquatic hyphomycete nutritional status, allowing them to tolerate higher metal stress.

The high sensitivity of $A$. acuminata to all metals may be related to the fact that this species was isolated from decomposing leaves collected in a clean stream. Consistently, $H$. submersus and $F$. curta isolated from a metal-polluted stream showed high tolerance to the most toxic metals (Cu, Ni and Cd). These findings suggest that fungi adapted to metal-polluted environments tolerate higher metal concentrations. However, this was not always the case. For example, $Y$. graminea isolated from the same metal-polluted stream was tolerant to Zn but not to Cd. Also, $V$. elodeae a species isolated from a clean site was able to tolerate high levels of Zn but not of Cu. This may indicate that fungal tolerance to metals can vary with fungal species and metal type.

Blaudez et al. (2000) using 39 isolates (21 of which from contaminated sites) of 5 different species of ectomycorrhizal fungi showed that EC$_{50}$ values for isolates of polluted sites did not differ from those of non-contaminated sites. Further studies using isolates adapted to different metal-stress conditions may help to clarify whether metal tolerance of aquatic hyphomycetes is more dependent on fungal species or strain.

Our results also indicate that fungal tolerance to one metal does not confer tolerance to all metals, suggesting that different mechanisms and/or cellular targets might be implicated in fungal tolerance to different metals. These aspects will be investigated in the next chapters of this thesis, aiming to better understand the interactions between aquatic hyphomycetes and toxic metals.
References


Chapter 3

Biochemical responses to Cu and Zn stress in aquatic fungi: the major role of antioxidant defenses

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 hours) 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.
3.1. 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 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 cellular and organellar membranes, resulting in a loss of membrane integrity and impairment of membrane function (Ohsumi et al., 1998). 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 (Penninckx
The role of antioxidant defenses against metal stress

and Elskens, 1993). 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 heterogeneous 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 Bärlocher, 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.

3.2. Materials and methods

3.2.1. Fungal species, growth conditions and metal exposure

The aquatic hyphomycetes were isolated from single spores collected in streams in the Northwest of Portugal. *Varicosporium elodeae* W. Kegel (UMB-142.01) was isolated from foam sampled in a clean stream at the Peneda-Gerês National Park, while *Heliscus 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 80 µM and 150 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 (CuCl₂) and zinc (ZnCl₂) 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. 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 hours. 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% (EC₅₀) or 80% (EC₈₀), 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.

3.2.2. 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 hours) and long-term (8 days) exposure to metals at EC₅₀ and EC₈₀, 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 hours. Subsequently, mycelia were dehydrated in ethanol (v/v) as follows: 30%, 5 hours; 60%, 2 hours; and 100%, 1 hour. 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).

3.2.3. 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 (1x 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 EC₅₀ 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).

### 3.2.4. Reactive oxygen species production

ROS production was monitored with the MitoTracker Red CM-H₂XRos (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-H₂XRos (final concentration, 3.3 µg ml⁻¹) for 15 min at room temperature. Mycelia were then exposed to EC₅₀ and EC₈₀ concentrations of Cu or Zn for 30 and 90 min and scanned under an epifluorescence microscope.

### 3.2.5. 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; 18000 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 H₂O₂ 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$_2$O$_2$; 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$_2$O desionized; 50 µl 1 M Tris-HCl pH 8.0; 10 µl NADP$^+$ (disodium); 10 µl MgCl$_2$.6H$_2$O; 50 µl 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.

3.2.6. 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 homocedasticity (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 concentration corresponding to EC$_{50}$ and EC$_{80}$ of biomass inhibition were determined by non-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).

3.3. Results

3.3.1. 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 (Figure 3.1). 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 3.1). *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* (Figure 3.1). 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 3.1). Higher concentrations of Zn than Cu were necessary to promote identical toxicity effects in both fungi.

Figure 3.1. Biomass production by the aquatic hyphomycetes *H. submersus* and *V. elodeae* exposed for 8 days to Cu and Zn. Mean ± SEM, $n = 3$. 

The role of antioxidant defenses against metal stress

Table 3.1. 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*

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (µM)</td>
<td>EC_{80} (µM)</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>54 ± 4.7</td>
<td>85 ± 2.6</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>102 ± 1.7</td>
<td>174 ± 3.6</td>
</tr>
</tbody>
</table>

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

3.3.2. 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 hours 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 Figure 3.2, 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 cellular permeabilization to propidium iodide (PI) of mycelia exposed for short-term (30 - 150 min) to Cu or Zn. Exposure of *V. elodeae* or *H. submersus* to Cu resulted in severe disruption of plasma membrane integrity, particularly in the former species, while Zn elicited a much less pronounced response (see Figure 3.3-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).
The role of antioxidant defenses against metal stress

Figure 3.2. 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, X5,000.

Figure 3.3. 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, X400. II, ROS production assessed by CM-H$_2$XRos in mycelia unexposed (A) or exposed for 30 min to EC$_{50}$ of Cu (B) or Zn (C); magnification, X1000.
3.3.3. 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 MitoTracker Red CM-H$_2$XRos staining (see Figure 3.3-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 culture medium. The presence of this antioxidant agent resulted in an increase in biomass production in both species (Figure 3.4), 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).

![Figure 3.4. 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.](image-url)
3.3.4. 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 3.2). Short-term exposure (14 hours) of *H. submersus* to Cu led to a general increase in the SOD and CAT activities (Figure 3.5A, 3.5C). 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 (Figure 3.5A, 3.5C). In the case of *V. elodeae*, SOD activity remained unaltered (Figure 3.5B), while an increase in CAT activity was observed under short-term exposure to Cu (Figure 3.5D). In addition, we found that the activity of G6PDH was stimulated after long-term exposure of *V. elodeae* to the lowest Cu concentration (Figure 3.5F).

Long-term exposure to Zn enhanced the activity of G6PDH (Figure 3.6E, 3.6F) and CAT (Figure 3.6C, 3.6D) 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 (Figure 3.6C). Conversely, SOD did not appear to be involved in Zn stress (Figure 3.6A, 6B).

Table 3.2. 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.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>CAT (U mg protein⁻¹)</th>
<th>SOD (U mg protein⁻¹)</th>
<th>G6PDH (U mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. elodeae</em></td>
<td>21 x 10⁻⁶ ± 4.9 x 10⁻⁶</td>
<td>0.10 ± 0.036</td>
<td>20 x 10⁻⁵ ± 4.3 x 10⁻⁵</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>11 x 10⁻⁶ ± 4.7 x 10⁻⁶</td>
<td>0.08 ± 0.009</td>
<td>15 x 10⁻⁵ ± 2.4 x 10⁻⁵</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 3.
The role of antioxidant defenses against metal stress

Figure 3.5. Activity of the enzymes SOD (A, B), CAT (C, D), and G6PDH (E, F) in *Heliscus submersus* and *Varicosporium elodeae* unexposed (black) or exposed for short- (14 hours) 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.
3.3.5. 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 hours) to equitoxic mixtures of metals (EC₅₀ or EC₈₀) was similar to that of single Cu exposure (Figure 3.5-3.7), except for CAT whose activity was severely inhibited in the former species (Figure 3.7C). In addition, metal effects on enzymatic activities were generally more pronounced after exposure to mixtures than to Cu alone.

Figure 3.6. 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 hours) and long-term (8 days) to Zn at concentrations of EC₅₀ (white) or EC₈₀ (grey). Mean + SEM, n = 3. Significant differences: *, p < 0.05 or **, p < 0.01.
The role of antioxidant defenses against metal stress

Figure 3.7. 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: *, $p < 0.05$.

3.4. 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 19076 µM Zn and 93.8 µM Cu, Sridhar *et al.*, ...
The role of antioxidant defenses against metal stress

Krauss et al., 2001; industrial park of Braga, Northwest Portugal: 80 µM Zn and 150 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. Even so, no noticeable adsorption of these metals to cell walls was found, minimizing the protective role of this 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 (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.

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 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-Fereira 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 Bebiano, 2004). The first two enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Penninckx and Elskens, 1993; Bai et al., 2003); G6PDH is essential for the replenishment of NADPH intracellular pool to maintain cellular redox balance (Penninckx and Elskens, 1993). In our study, control cultures of *V. elodeae* had higher
activities of CAT, SOD and G6PDH than those of *H. submersus* (Table 3.2). 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 and Bärlocher, 2005) and it may have antioxidant defenses against environmental stressors, including metals. Consistently, metal exposure did not 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 and Zn diminished the activity of glutathione reductase (GR) in *Scenedesmus* sp. (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 or 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.
The role of antioxidant defenses against metal stress

References


The role of antioxidant defenses against metal stress


Chapter 4

Copper and Zn affect the activity of plasma membrane

$H^+$-ATPase and thiol content in aquatic fungi
Aquatic hyphomycetes are the major microbial decomposers of plant litter in streams. In this work, we selected three aquatic hyphomycete species with different ability to tolerate, adsorb and accumulate Cu and Zn, and we investigated the effects of these metals on the H⁺-ATPase activity and on the levels of thiol-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 thiol 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., H. submersus exposed to Cu and V. elodeae exposed to Zn. Short-term (10 min) exposure to Cu completely inhibited the activity of H⁺-ATPase of H. submersus and V. elodeae, while Zn only led to a similar effect on that of 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.
4.1. Introduction

In current days, it is hard to find pristine habitats due to the increased anthropogenic pressure on the environment. For instance, human activities from urbanization, agriculture and industry have greatly contributed to the increase of metals in aquatic ecosystems, leading to negative effects on the biota and ecological processes they govern.

Fungi play a critical role in organic matter turnover in streams. Among fungi, aquatic hyphomycetes appear to have the greatest ecological role as decomposers of plant detritus in streams (Baldy et al., 2002; Pascoal and Cássio, 2004). Even though metal pollution lowers biodiversity and activity of aquatic hyphomycetes, the occurrence of these fungi have been consistently reported in metal-polluted streams (Sridhar et al., 2005; Pascoal et al., 2005a). This probably explains the increased number of studies focusing on the responses of aquatic fungi to metal stress over the last few years.

All metals are toxic above certain concentrations but some, like Zn and Cu, are required as micronutrients for the metabolism and growth of cells (Gadd, 1993). Therefore, living organisms, including fungi, have to tightly regulate intracellular metal concentration in such a way that a safe uptake of metal ions needed in their 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 compartmentation, whose relative contributions for metal detoxification can vary with metal type and fungal species (Gadd, 1993). Therefore, we are still far from fully understanding the mechanisms underlying metal tolerance/resistance in fungi, despite the large amount of information on metal effects in living organisms.

Plasma membrane is the foremost barrier between cytoplasm and the environment and it constitutes the first functional site of contact between metal ions and cells. In fungi, the proton-pump ATPase (H⁺-ATPase) is the major protein component of the plasma membrane, attaining 15 to 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 because it controls essential cellular functions, such as nutrient uptake and intracellular pH regulation (Serrano, 1988; Portillo, 2000). It has been reported that H⁺-
ATPase activity influences fungal tolerance to several environmental stressors, including heat (Piper, 1993), ethanol (Rosa and Sá-Correia, 1992), weak acids (Holyoak et al., 1996; Viegas et al., 1998) and metals (Karamushka and Gadd, 1994; Fernandes et al., 1998).

Metal toxicity to 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 and Bagchi 1995; Azevedo et al., 2007). Transition metals, as Cu and Fe, greatly enlarge ROS production through the Fenton reaction (Bai et al., 2003). However, even non-redox active metals, such as Zn, can lead to ROS production by depleting free radical scavengers, like thiol (SH) compounds (Dietz et al., 1999). Among cellular macromolecules, the polyunsaturated fatty acids of biological membranes are preferential targets for ROS attack (Howlett and Avery, 1997). In fungi, lipid peroxidation induced by Cu leads to a decline in plasma membrane lipid order (Howlett and Avery, 1997) and a subsequent increase in the non-specific permeability of this membrane (Ohsumi et al., 1988). Therefore, Cu readily permeates the plasma membrane acting as a potent depolarizer of cell electrical potential (Kennedy and Gonsalves, 1987). In Saccharomyces cerevisiae, mild Cu stress stimulates H⁺-ATPase, probably for the re-establishment of the cellular electrochemical gradient, but the activity of this pump declines at maximal Cu concentration that allows yeast growth (Fernandes et al., 1998).

Stadler et al. (2003) showed that specific-Cys residues of the H⁺-ATPase are targets for Fe- and Cu-Fenton reagents leading to the enzyme inactivation. Consistently, reduced-thiol groups revealed to be essential for maintaining H⁺-ATPase activity under Fe stress in wheat-root plasma membranes (Yang et al., 2003). In aquatic hyphomycetes, metal tolerance has been associated with increased levels of SH compounds in fungal cells (Miersch et al., 2001; Braha et al., 2007; Guimarães-Soares et al., 2007) and recent evidences point to glutathione, phytochelatins and metallothioneins as putative metal sequesters or ROS scavengers (Jaeckel et al., 2005; Guimarães-Soares et al., 2006).

In the present work, we selected three aquatic hyphomycete species with different sensitivities to Cu and Zn and we examined their ability to adsorb and accumulate these metals. Subsequently, we assessed effects of Cu and Zn on H⁺-ATPase activity and on the levels of SH-containing compounds. We expect that more tolerant species to metals
have higher ability to adsorb metal ions, minimizing their uptake, and/or higher intracellular levels of SH-containing compounds to deal with metal accumulation within cells. In addition, an increased activity of the plasma membrane proton pump is expected to occur to counteract metal-induced dissipation of the electrochemical gradient, which is essential for fungal survival.

4.2. Materials and Methods

4.2.1. Fungi and culture maintenance

The aquatic hypnomycetes *Heliscus 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 the Northwest of Portugal. The two former species were isolated from leaves collected in the Este River, downstream the industrial park of Braga, at a site with high nutrient loading (Pascoal et al., 2005b) and heavy metals in the stream water (Gonçalves, 2001). The latter species was isolated from foams collect in a clean stream in 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 ºC under artificial light.

4.2.2. Growth conditions and metal exposure

Conidial suspensions (6 conidia ml⁻¹, final concentration) of each aquatic hyphomycete species were placed into Erlenmeyer flakks containing mineral medium with vitamins and 2% (w/v) glucose (van Uden, 1967) at pH 5.0, with or without addition of Cu (CuCl₂) or Zn (ZnCl₂). Metal concentrations ranged from 50 to 2000 µM for Cu and from 250 to 10000 µM for Zn. The flasks were kept for 8 days at 18ºC, 160 rpm (Certomat BS 3, B. Braun Biotech International) under artificial light. At the harvest time, fungal cultures were at the end of exponential growth phase (not shown).

To assess long-term effects of metals on the H⁺-ATPase activity and thiol-compound production, fungal mycelia were exposed for 8 days to Cu or Zn concentrations inhibiting biomass production in 50% (EC₅₀). To assess short-term effects, mycelia grown 8 days without metal addition were transferred to fresh medium.
Effects of Cu and Zn on $H^+$-ATPase activity and on the levels of thiol compounds

and exposed to EC$_{50}$ of Cu or Zn for 10 min in the case of H$^+$-ATPase assay or for 14 and 62 h to estimate concentration of thiol compounds in fungal mycelia.

To quantify fungal biomass, mycelia were harvested by filtration, washed twice with deionised water and dried at 85$^\circ$C to constant mass, before weighed (0.001 g).

4.2.3. Assessment of $H^+$-ATPase activity

The H$^+$-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 by homogenizing mycelia in deionised 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 e Zonen 024). The pH electrode was immersed in a water-jacketed chamber magnetically stirred. One millilitre of the 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 (1M to 10 mM) or HCl (1M 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 of $H^+$ s$^{-1}$ mg$^{-1}$ dry weight by comparing the acidification curve after glucose addition to cell suspensions with that of the addition of known amounts of 10 mM HCl.

4.2.4. Preparation of cell free-extracts and quantification of thiol compounds

Fungal mycelia were harvested by filtration, washed twice with deionised water and pressed between two layers of filtering-paper to remove water surplus. Then, 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 20 mM Tris, 1 mM EDTA, pH 7.5, and cell-free extracts were obtained by 2 centrifugation steps (6200 g for 10 min; 18000 g for 50 min) at 4$^\circ$C.

The concentration of total thiols (T-SH) and nonprotein thiols (NP-SH) was determined according to Sedlak and Lindsay (1968), with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). To determine T-SH compounds, 50 µl of the cell-free extracts were mixed with 150 µl of 0.2 M Tris, pH 8.2, and 10 µl of 0.01 M DTNB in a final volume of 1.0 ml of absolute methanol. After 15 min, the mixtures were centrifuged (3000 g, 15 min) and the absorbance measured at 412 nm (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, 500 µl of the cell-free extracts were mixed with 400 µl of milli-Q water and 100 µl of 50% trichloroacetic acid. The mixtures were shaken during 12 min before centrifuged (3000 g, 15 min). Then, 200 µl of the supernatant was mixed with 400 µl of 0.4 M Tris, pH 8.9, and 10 µl of 0.01 M DTNB, and absorbance was measured within 3 min. The concentration of protein-bound thiols (PB-SH) was calculated by subtracting the NP-SH to T-SH concentrations. All buffers and solutions were previously gassed 1-2 min with a nitrogen stream.

4.2.5. Metal adsorption and accumulation

Fungal mycelia were harvested by filtration, washed with distilled water (100 ml), washed 3 times with 100 ml of 20 mM NiCl₂ and again with distilled water (100 ml) to remove metals adsorbed to fungi. Fifty milligrams of the mycelium were digested with 4 ml of 65% (v/v) HNO₃ and 2 ml of 30% (v/v) H₂O₂ in a water-bath at 100 ºC during 40 min.

Concentrations of Cu or Zn in the NiCl₂ washings (for adsorption) and in the digested mycelium (for accumulation) were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; PU 7000 ICP- Philips).

4.2.6. Data analysis

Data on the effects of Cu and Zn on the H⁺-ATPase activity and production of thiol compounds were expressed in 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, H⁺-ATPase activity and production of thiol compounds were compared by one-way ANOVA, followed by Dunnett’s test to identify treatments that differ significantly from control (Zar, 1996).

Values of EC₅₀ for Cu and Zn were estimated by the Probit method (Wardlaw, 1985) and compared by one-way ANOVA, followed by Tukey’s test to identify significant differences (Zar, 1996).

Statistic analysis was done using Prism4 for Windows (GraphPad Software Inc., San Diego).
4.3. Results

4.3.1. Metal toxicity, adsorption and accumulation

The sensitivity of the aquatic hyphomycetes *H. submersus*, *V. elodeae* and *F. curta* to Cu and Zn was assessed by comparing metal concentrations inhibiting biomass production in 50% (EC$_{50}$) after 8 days of growth (Table 4.1). *H. submersus*, with an EC$_{50}$ of 1510 µM for Cu, was the most resistant species to this metal, followed by *V. elodeae* and *F. curta*. The most resistant species to Zn was *V. elodeae* (EC$_{50}$ = 7315 µM), while *H. submersus* (EC$_{50}$ = 465 µM) was the most sensitive one.

The mycelium of *H. submersus* showed higher ability to adsorb Cu (70.5 µmol g$^{-1}$) accumulating 2.6-times more Cu (13.5 µmol g$^{-1}$ dry mass) than the less accumulative species (*V. elodeae*) (Table 4.2). Differences in metal adsorption and accumulation between fungal species were more pronounced for Zn than for Cu (Table 4.2). *V. elodeae* had the highest adsorption ability for Zn, being 8.5- and 32-times higher than that of *F. curta* and *H. submersus*, respectively. *F. curta* was able to accumulate 5- and 159-times more Zn in the mycelium (175.1 µmol g$^{-1}$ dry mass) than *V. elodeae* and *H. submersus*, respectively (Table 4.2).

Table 4.1. Concentrations of Cu and Zn inhibiting biomass production by aquatic hyphomycetes in 25% (EC$_{25}$) and 50% (EC$_{50}$). Fungi were grown in mineral medium supplemented with vitamins and 2% glucose during 8 days with or without metal addition.

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

Values are means ± SEM. In each column, different letters indicate significant differences (Tukey’s test, p < 0.05)
Table 4.2. Metal adsorption and accumulation in fungal mycelia. Fungi were grown in mineral medium supplemented with vitamins and 2% glucose without metal during 8 days and exposed 14 h to EC₅₀ of Cu and Zn.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Cu Adsorption (µmol g⁻¹)</th>
<th>Cu Accumulation (µmol g⁻¹)</th>
<th>Zn Adsorption (µmol g⁻¹)</th>
<th>Zn Accumulation (µmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. submersus</em></td>
<td>70.5</td>
<td>13.5</td>
<td>11.2</td>
<td>1.1</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>34.1</td>
<td>5.2</td>
<td>354</td>
<td>36.2</td>
</tr>
<tr>
<td><em>F. curta</em></td>
<td>8.3</td>
<td>9.0</td>
<td>41.7</td>
<td>175.1</td>
</tr>
</tbody>
</table>

Values are the mean of two independent experiments.

4.3.2. Effects of Cu and Zn on H⁺-ATPase activity

The H⁺-ATPase activity was evaluated by measuring the rate of proton efflux after addition of glucose to suspensions of fungal mycelium. The addition of glucose to fungal suspensions led to an efflux of protons, as exemplified in Figure 4.1 for *F. curta*. Proton efflux varied from 0.023 ± 0.006 to 0.061 ± 0.012 nmol H⁺ s⁻¹ mg⁻¹ dry weight, with the lowest value found in *V. elodeae*, the intermediate in *H. submersus* and the highest in *F. curta* (Table 4.3).

Figure 4.1. Typical acidification curve obtained after the addition of 0.2% glucose to mycelial suspensions of *F. curta* grown 8 days with EC₅₀ concentration of Cu.
Effects of Cu and Zn on H⁺-ATPase activity and on the levels of thiol compounds

Long-term exposure (8 days) to EC₅₀ of Cu increased significantly the H⁺ efflux associated with the addition of glucose in *H. submersus* (2.3-times augment) and in *F. curta* (1.7-times augment), but did not affect the activity of this proton pump in *V. elodeae* (Table 4.3). Long-term exposure to EC₅₀ of Zn led to a 2.7-times increase in the H⁺-ATPase activity in *V. elodeae* (Table 4.3). On the contrary, Zn had no significant effect on the H⁺ efflux in *H. submersus* and *F. curta* (Table 4.3).

Table 4.3. Activity of the H⁺-ATPase in aquatic hyphomycetes exposed or not for 8 days to EC₅₀ of Cu or Zn. The proton efflux was measured upon addition of 0.2% glucose, at pH 5.0 and 20 °C.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>H⁺-ATPase (nmol H⁺ s⁻¹ mg⁻¹ dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>0.030 ± 0.006</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>0.023 ± 0.006</td>
</tr>
<tr>
<td><em>F. curta</em></td>
<td>0.061 ± 0.012</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM, n=3, *Dunnett’s test, p < 0.05

Short-term exposure (10 min) to EC₂₅ or EC₅₀ of Cu led to a total inhibition of the H⁺ efflux in *H. submersus* and *V. elodeae* (Table 4.4). In *F. curta*, the exposure to EC₂₅ of Cu had no significant effect on the H⁺-ATPase activity, but a 1.5-times stimulation was found by exposure to EC₅₀ of this metal (Table 4.4). The H⁺-ATPase activity was not affected by short-term exposure to EC₂₅ of Zn in any fungal species. The exposure to EC₅₀ of Zn totally inhibited the proton pump of *H. submersus*, had no effect on that of *V. elodeae* and stimulated that of *F. curta* (Table 4.4).

Table 4.4. Effects of short-term exposure to Cu or Zn on the H⁺-ATPase activity in aquatic hyphomycetes. Assays were carried out at pH 5.0 and 20 °C. Actual values of the H⁺-ATPase activity in the absence of metals are in Table 4.3.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>H⁺-ATPase (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu</td>
</tr>
<tr>
<td></td>
<td>EC₂₅</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>-</td>
</tr>
<tr>
<td><em>F. curta</em></td>
<td>83.5±1.9</td>
</tr>
</tbody>
</table>

Mean ± SEM, n=3, - total inhibition, *Dunnett’s test, p < 0.05
4.3.3. Effects of Cu and Zn on the production of thiol compounds

The levels of thiol compounds were evaluated in 8 days-old mycelia of the aquatic hyphomycetes *H. submersus*, *F. curta* and *V. elodeae* exposed or not to EC_{50} concentrations of Cu or Zn. *H. submersus* grown without added metals had the highest level of total thiol compounds (T-SH), whereas *V. elodeae* showed the lowest level (Table 4.5). The contribution of non-protein (NP-SH) and protein-bound (PB-SH) thiols to the T-SH compounds varied among the species, with NP-SH thiols being 80%, 65% and 50% of the T-SH in *H. submersus*, *F. curta* and *V. elodeae*, respectively.

Table 4.5. Concentration of total (T-SH), nonprotein (NP-SH) and protein-bound (PB-SH) thiols in the mycelia of aquatic hyphomycetes grown 8 days with no addition of metals and transferred to fresh medium for different incubation periods.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Incubation period (h)</th>
<th>Thiol-compounds (µmol g(^{-1}) dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-SH</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>0</td>
<td>6.32±1.22</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4.93±0.60</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>4.36±0.62</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>0</td>
<td>2.59±0.32</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.55±0.12</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>5.35±0.34</td>
</tr>
<tr>
<td><em>F. curta</em></td>
<td>0</td>
<td>5.41±1.11</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5.19±2.07</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>7.54±1.67</td>
</tr>
</tbody>
</table>

Mean ±SEM of at least 3 independent experiments

Exposure to EC_{50} of Cu induced a significant decrease in the T-SH and NP-SH compounds in *H. submersus* at all times (Figure 4.2A). Short-term exposure (14 or 62 h) of *V. elodeae* to Cu led to a significant increase in the levels of all types of thiol compounds, while long-term exposure (8 days) to Cu only led to a significant increase in PB-SH (Figure 4.2B). Moreover, in all fungal species the levels of NP-SH significantly decreased after 8 days of exposure to Cu (Figure 4.2).
Short-term exposure (14 h) to Zn increased the levels of T-SH, by increasing both NP-SH and PB-SH (Figure 4.2B) in *V. elodeae*, and of NP-SH in *F. curta* (Figure 4.2C). Long-term exposure (8 days) to Zn, significantly increased PB-SH level in *H. submersus* (Figure 4.3A) and no other significant effects were found.

Figure 4.2. Concentration of total (diagonal lines), nonprotein thiols (checkers) and protein-bound thiols (horizontal lines) after short-term (14 and 62 h) and long-term (8 d) exposure to EC_{50} of Cu in *H. submersus* (A), *V. elodeae* (B) and *F. curta* (C). Values are % of control. Mean ± SEM, n=3. Dunnet’s test, p < 0.05. Actual values of controls are in Table 4.5.
Effects of Cu and Zn on H^+-ATPase activity and on the levels of thiol compounds

Figure 4.3. Concentration of total (diagonal lines), nonprotein thiols (checkers) and protein-bound thiols (horizontal lines) after short-term (14 and 62 h) and long-term (8 d) exposure to EC_{50} of Zn in *H. submersus* (A), *V. elodeae* (B) and *F. curta* (C). Values are % of control. Mean ± SEM, n=3. * Dunnet´s test, *p < 0.05. Actual values of controls are in Table 4.5.

4.4. Discussion

In previous studies, we demonstrated that the generation of reactive oxygen species (ROS) contribute noticeably to Cu and Zn toxicity in aquatic hyphomycetes (Guimarães-Soares, 2005; Azevedo *et al.*, 2007). The interaction of ROS with biological membranes results in a variety of functional alterations due to either direct interaction with the molecular cell machinery and/or oxidative modification of biological macromolecules (Stark, 2005). Moreover, metals can directly interact with
Effects of Cu and Zn on $\text{H}^+$-ATPase activity and on the levels of thiol compounds

biomolecules, such as enzymes and transport proteins of essential nutrients and ions, compromising their biological functions (Gadd, 1993). A severe disruption of plasma membrane integrity in several species of aquatic hyphomycetes is reported to occur after short-term (30 min) exposure to Cu and Zn, particularly to the former metal (Azevedo et al., 2007); this could compromise the activity of plasma membrane ATPase. In this work, we found that short-term exposure to Cu completely inhibited the activity of the $\text{H}^+$-ATPase of $H.\ submersus$ and $V.\ elodeae$, while Zn only led to a similar effect on that of $H.\ submersus$. In $S.\ cerevisiae$, damage in plasma membrane caused by Cu affected the functioning of the $\text{H}^+$-ATPase (Fernandes et al., 1998). The reduced ATPase activity under Cu stress may be attributed either to the drastic decrease in plasma membrane organization due to Cu-induced lipid peroxidation (Howlett and Avery 1997) and/or formation of Cu-ATP complexes (Tallineau et al., 1984). However, since a recovery of plasma membrane integrity was observed after 150 min of exposure to Cu in $H.\ submersus$ (Azevedo et al., 2007), a functional restoration of the $\text{H}^+$-ATPase is expected to occur at longer times. In the present work, 8 days of metal exposure led to strong stimulations of the proton pump in the most tolerant species, i.e. when $H.\ submersus$ was exposed to Cu and $V.\ elodeae$ was exposed to Zn. The activation of $\text{H}^+$-ATPase by metal exposure may be related to its ability to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane (Serrano, 1988; Fernandes et al., 1998), suggesting that $\text{H}^+$-ATPase may be involved in aquatic hyphomycete acclimation to metals. Moreover, the acidification of the extracellular medium by the 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 of pH increase metal bioavailability (Douglas and Wiener, 1991). Accordingly, metal uptake in fungi is often reduced by the decrease of extracellular pH (Cu, Gadd and White, 1985; Zn, Ross, 1994).

In this work, the highest amounts of Cu and Zn adsorbed to fungal mycelia were found in $H.\ submersus$ and in $V.\ elodeae$, respectively. Because these fungi were the most tolerant species to each metal, biosorption appears to be a relevant mechanism to avoid unrestrained uptake of metals minimizing their deleterious effects. Nevertheless, these fungal species accumulated metals in their mycelia, although at much lower amounts comparing to that adsorbed. The ability of aquatic hyphomycetes to take up
Effects of Cu and Zn on H^+·ATPase activity and on the levels of thiol compounds

and store large quantities of metals makes them potential candidates for bioremediation. In this context, *V. elodeae* and *F. curta* had remarkable ability to adsorb and accumulate Zn (a total of 390 and 217 µmol per gram of dry mycelium during only 14 h of metal exposure) comparing with values reported for aquatic fungi (Guimarães-Soares, 2005; Jaeckel et al., 2005) Also, *H. submersus* was able to retain ca. 7-times more Cu than metal tolerant strains of *H. lugdunensis* (Braha et al., 2006). However, adsorption of metals by filamentous fungi depends on several factors, 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).

In aquatic fungi, metal tolerance has been also associated with the synthesis of SH-enriched compounds, which are able to bind metals within cells (Miersch et al., 1997; Miersch et al., 2001; Guimarães-Soares et al., 2006, 2007). 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 comparing to *V. elodeae*, the species isolated from a clean stream. Short-term exposure to Cu led to a decrease in both types of thiol compounds in the most resistant species (*H. submersus*), but not in the most sensitive one (*F. curta*). The decrease in reduced thiol compounds might be due to their oxidation during metal sequestration (Cobbett and Goldsbrough, 2002; Guimarães-Soares et al., 2006) or ROS scavenge (Bai et al., 2003), suggesting that the high constitutive levels of thiols might have helped *H. submersus* to deal with Cu stress. In addition, the decrease in the NP-SH compounds in all fungal species after long-term exposure to Cu is consistent with the ability of peptides with very low molecular weight, most likely glutathione and phytochelatins, to bind Cu in aquatic hyphomycetes (Guimarães-Soares et al., 2006). *V. elodeae*, that 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 reporting that high constitutive levels of thiols or the rapid increase of their production may help aquatic hyphomycetes to deal with metal stress (Guimarães-Soares et al., 2006, 2007).
Effects of Cu and Zn on $H^+$-ATPase activity and on the levels of thiol compounds

References

Dietz KJ, Bair M, Krämer U. 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In Prasad MNV, Hagemeyer J. (Eds.), Heavy Metal Stress in Plants: from Molecules to Ecosystems. Springer-Verlag, Berlin, pp. 73-97.


Effects of Cu and Zn on H⁺-ATPase activity and on the levels of thiol compounds


Chapter 5

Metal stress induces programmed cell death in aquatic fungi
Metal stress induces programmed cell death in aquatic fungi

Abstract

Aquatic hyphomycetes are a phylogenetically heterogeneous group of fungi that occur in clean and metal-polluted streams. We examined the ability of Cu and Zn to induce programmed cell death (PCD) in three aquatic hyphomycete species through the evaluation of typical apoptotic markers, namely production of reactive oxygen species (ROS), caspase activation, nuclear morphological alterations and the occurrence of DNA strand-breaks, evaluated by TUNEL assay. In Heliscus submersus and Flagellospora curta, Cu exposure resulted in a high number of cells with ROS production and caspase activation, but a low number of fungal cells showed nuclear morphological alterations and DNA strand-breaks. Conversely, under Zn stress, aquatic hyphomycetes showed high number of cells with nuclear morphological alterations or DNA strand-breaks. In Varicosporium elodeae, Cu induced caspase activation, nuclear morphological alterations and DNA strand-breaks, but no ROS production. These results may indicate that V. elodeae developed a PCD process independent of ROS production. In addition, under Zn stress, F. curta appeared to develop a PCD process independent of caspase activity.
5.1. Introduction

Human activities contribute to a high release of metals to the environment at rates and concentrations sufficient to make them pollutants (Brown et al., 1999). Certain metals, such as Cu and Zn, are needed for the growth and metabolism of microorganisms (Gadd, 1993); however, above critical levels, they are known to inhibit a variety of metabolic activities affecting cellular processes (Cobbet and Goldsborough, 2002).

The toxicity of metals can result from the generation of reactive oxygen species (ROS) that may cause damage to proteins, nucleic acids and lipids, eventually leading to cell death (Stohs and Bagchi, 1995). Madeo et al. (1999) showed that programmed cell death (PCD) could be induced in yeasts by oxidative stress triggered by exposure to H$_2$O$_2$. ROS can also act indirectly by modifying the cellular redox potential, which modulates key regulatory proteins involved in PCD (Mignotte et al., 1998). In fact, PCD can be induced by growing a gsh1 yeast mutant in the absence of GSH (Madeo et al., 1999) or by the oxidation of cellular sulfhydryl (SH) compounds (Sato et al., 1995).

Moreover, the ability of some antioxidant enzymes, such as catalase, to block apoptotic-PCD argues for the central role of oxidative stress in cell death processes (Buttke and Sandstrom, 1994). In spite of noteworthy evidences pointing to ROS as crucial PCD mediators, it was recently reported an active cell death process independent of ROS in yeasts (Almeida et al., 2007).

Programmed cell death, in which cells actively participate in their own death, is characterized by phenotypical alterations, such as chromatin condensation (Clifford et al., 1996), DNA fragmentation, formation of membrane-enclosed cell fragments (apoptotic bodies) (Kerr et al., 1972) and caspase activation (Earnshaw et al., 1999). Metals are reported to induce PCD processes in various cell systems. For instance, DNA damage was caused by exposure to complexes of 1,10-phenanthroline and metals in yeast and mammalian cells (Barry et al., 2004), by exposure to Cd, Cu, Zn and Pb in tobacco and potato plants (Gichner et al., 2006) or to Cu in rat thymocytes (Wolfe et al., 1994). Also, caspase activation was observed after exposure of HEP-2 cancer cells to Zn (Rudolf et al., 2005).

In contrast to the increasing number of studies that are beginning to unravel the PCD pathways in yeast and mammalian cells, relatively little work has been done in
Metal stress induces programmed cell death in aquatic fungi (Robson, 2006). Genomes of filamentous fungi contain the complement of genes involved in PCD in *Saccharomyces cerevisiae* (Fedorova *et al*., 2005; Glass and Dementhon, 2006), although Aspergilli have many putative components of the mammalian apoptotic machinery, including several proteins not found in *S. cerevisiae* (Fedorova *et al*., 2005).

Processes of PCD involving DNA degradation were associated with heterokaryon incompatibility in *Neurospora crassa* (Marek *et al*., 2003) and caspase-like activities were described during cell death at the stationary-phase of *Aspergillus fumigatus* (Mousavi and Robson, 2003) and during asexual sporulation of *A. nidulans* (Thrane *et al*., 2004). Consistently, searches in *A. nidulans* genome revealed two genes encoding metacaspase proteins (Thrane *et al*., 2004). Despite the evidences that in *A. fumigatus* the loss of cell viability and death caused by toxic levels of H$_2$O$_2$ are associated with phenotypic characteristics of apoptosis, no significant activity against caspase substrates was found (Mousavi and Robson, 2004).

Studies examining whether exposure to environmental stressors triggers the development of apoptotic phenotypes are scarce in filamentous fungi (Ramsdale, 2006; Robson, 2006) and virtually unknown in freshwater fungi. Among these, aquatic hyphomycetes are an ecologically relevant group of fungi that play an important role as intermediaries between plant detritus and invertebrates in either clean or metal-polluted streams (Sridhar *et al*., 2001; Bärlocher, 2005; Pascoal *et al*., 2005b). Previous reports showed that the exposure of aquatic hyphomycetes to metals led to an intracellular ROS accumulation (Guimarães-Soares, 2005; Azevedo *et al*., 2007) and to shifts in the levels of GSH or protein-bound SH compounds (Miersch *et al*., 2001; Jaeckel *et al*., 2005; Guimarães-Soares, 2006, 2007; Braha *et al*., 2007). To test whether Cu and Zn stress is able to induce PCD in aquatic hyphomycetes, we characterized cell death processes in three fungal species through the evaluation of typical apoptotic markers, namely ROS production/accumulation, caspase activation, alterations in nuclei morphology, and the occurrence of DNA strand-breaks. The identification of PCD in aquatic hyphomycetes under metal stress will improve our understanding on the mechanisms of cell death helping to explain the survival of fungi in metal-polluted streams.
5.2. Materials and Methods

5.2.1. Fungal species and conditions of maintenance

The aquatic hyphomycetes *Heliscus 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 from streams in the Northwest of Portugal. The first two species were isolated from leaves collected in the Este River, at a site with high nutrient loading (4.968 mg L\(^{-1}\) N-NO\(_3^-\); 0.249 mg L\(^{-1}\) N-NH\(_4^+\); and 0.176 mg L\(^{-1}\) P-PO\(_4^{3-}\)); Pascoal *et al*., 2005a) and heavy metals in the stream water (5.87 mg L\(^{-1}\) Cu; 2.02 mg L\(^{-1}\) Zn, Gonçalves, 2001) due to urbanization, intensive agriculture and industrial activities. *V. elodeae* was isolated from foams collected in a clean stream (0.099 mg L\(^{-1}\) N-NO\(_3^-\); <0.008 mg L\(^{-1}\) N-NH\(_4^+\); and 0.010 mg L\(^{-1}\) P-PO\(_4^{3-}\); Pascoal *et al*., 2005a) at the Peneda-Gerês National Park.

Fungi were maintained on solid medium containing 2% (w/v) malt extract and 1.5% (w/v) agar, at 18º C under artificial light.

5.2.2. Growth conditions and preparation of fungal mycelium suspensions

Fungal spores (final concentration of 6 conidia ml\(^{-1}\)) were inoculated 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 Cu or Zn. The flasks were incubated on a shaker (160 rpm; Certomat BS 3, B. Braun Biotech International) at 18ºC under permanent artificial light, during 8 days. At this time fungal cultures were at the end of exponential growth phase (not shown).

Stock solutions of copper (CuCl\(_2\)) and zinc (ZnCl\(_2\)) were added to the growth medium at concentrations that inhibited biomass production in 50% (EC\(_{50}\)). Metal concentrations were: 1510 µM Cu and 465 µM Zn for *H. submersus*; 183 µM Cu and 1304 µM Zn for *F. curta*; 457 µM Cu and 7315 µM Zn for *V. elodeae*.

Fungal mycelia were harvested by filtration and homogenized in PBS buffer (0.12% (w/v) Na\(_2\)HPO\(_4\) anhydrous, 0.02% (w/v) KH\(_2\)PO\(_4\) anhydrous, 0.8% (w/v) NaCl and 0.02% (w/v) KCl). Mycelium suspensions were washed twice with cold PBS buffer before the assays.
5.2.3. Production of reactive oxygen species

Reactive oxygen species (ROS) production was monitored with MitoTracker Red CM-H$_2$XRos (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 incubated with 0.25 µg µl$^{-1}$ MitoTracker Red CM-H$_2$XRos for 15 min at room temperature and then scanned under an epifluorescence microscopy (BX 61 Olympus, magnification 1000 X).

5.2.4. Activity of caspases

The fluorochrome-labeled inhibitor of caspases (FITC-VAD-FMK) was used to detect active caspases in situ. Because this compound has affinity to the active centre of caspases, its binding to apoptotic cells can indicate caspase activation (Pozawowski et al., 2003). Since in yeast cells unspecific binding of FITC-VAD-FMK to propidium iodide-positive cells has been reported (Wysocki and Kron, 2004), caspase activity was monitored only in propidium iodide negative cells.

Mycelium suspensions prepared as above were resuspended in 200 µl of staining solution (50 µM FITC-VAD-FMK and 5 µg ml$^{-1}$ propidium iodide) and incubated 40 min at 25ºC in the dark. After this, mycelia were washed twice by centrifugation (6200 g, 10 min) ressuspended in 20 µl of PBS and scanned by epifluorescence microscopy.

5.2.5. Nuclear morphological alterations

The morphology of cell nuclei was assessed with 4′,6-diamidino-2-phenylindole (DAPI). This compound is known to form fluorescent complexes with double-stranded DNA and thus localizes nuclei. Nuclei are considered to have the normal phenotype when is bright and homogenous. Apoptotic nuclei can be identified by the condensed chromatin at the periphery of nuclear membranes or by the appearance of nuclear bodies.

Suspensions of fungal mycelium were fixed in ethanol 70% (v/v) during 30 min at 4ºC. Then, mycelium was centrifuged during 4 min at 11500 g (Bifuge-Pico-Heraeus) and the ethanol was discarded. After that, mycelium was incubated 20 min with 0.1 mg ml$^{-1}$ of DAPI (Sigma) under dark at room temperature. Subsequently, mycelia were
washed twice, resuspended in 20 µl of PBS buffer and scanned by epifluorescence microscopy.

5.2.6. TUNEL and propidium iodide staining

DNA strand breaks were visualized by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) and propidium iodide staining with the In situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). This technique labels free 3’-OH termini with FITC-labelled deoxyuridine triphosphate (dUTP), which was detected by epifluorescence microscopy.

Fungal mycelium was fixed with 3.7% (v/v) formaldehyde and cell walls digested with zymolase during 2 h at 37°C and 150 rpm (Med Line SI-600R). Then, mycelium suspensions were prepared as described above and centrifuged (3000 g for 3 minutes). Subsequently, cytospins of cell suspensions were done using a Shandon Cytospin 2 cytocentrifuge at 1000 rpm for 5 min. Slides were incubated in a permeabilization solution (0.1% (v/v) Triton X-100 in 0.1% sodium citrate (w/v)) during 10 min, rinsed twice in PBS buffer and incubated with the TUNEL reaction mixture. Slides were incubated in a humidified atmosphere in the dark (1 h; 37°C). Ten microlitres of a mixture containing 100 µl of the antifading agent Vectashield, 2 µl of propidium iodide (PI; 50 µg ml⁻¹), to co-localize DNA, and 2 µl RNase (0.5 µg ml⁻¹) was added to each slide. Positive controls were prepared by incubating slides with 30 µl DNase (30 min and 37°C), before incubation with the TUNEL reaction mixture. Mycelia were scanned by epifluorescence microscopy.

5.3. Results

5.3.1. Cu and Zn induce reactive oxygen species production

Exposure to metals led to an intracellular ROS accumulation in mycelia of *H. submersus*, *F. curta* and *V. elodeae*, as shown by red fluorescence after staining with MitoTracker Red CM-H₂XRos (Figure 5.1 and Table 5.1). Zinc exposure induced intracellular ROS production in a low number of fungal cells in all species (Figure 5.1 and Table 5.1). The response to Cu was more heterogeneous; *H. submersus* showed higher number of cells with ROS accumulation, *F. curta* showed low levels of
intracellular ROS, and *V. elodeae* had no noticeable ROS production (Figure 5.1 and Table 5.1).

Figure 5.1. ROS production, assessed by MitoTracker Red CM-H$_2$XRos staining, in mycelia of *H. submersus, V. elodeae* and *F. curta* non-exposed or exposed to EC$_{50}$ of Cu or Zn. ROS-positive cells show red fluorescence.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Cu</th>
<th></th>
<th></th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROS</td>
<td>Caspases</td>
<td>NMA</td>
<td>DNA strand-breaks</td>
</tr>
<tr>
<td><em>H. submersus</em></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>V. elodeae</em></td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>F. curta</em></td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

(- No effect; + Low effect; ++ High effect)
5.3.2. Cu and Zn induce caspase-like activity

Caspase activation was not found in control mycelia of all tested aquatic hyphomycete species, as shown by the absence of green fluorescence after FITC-VAD-FMK staining (Figure 5.2). Metal exposure led to caspase activation, except in *F. curta* mycelia exposed to Zn (Figure 5.2 and Table 5.1). Zinc exposure promoted higher number of caspase-positive cells in *V. elodeae* than in *H. submersus*. On the contrary, Cu induced higher proportion of caspase-positive cells in mycelia of *H. submersus* and *F. curta* than in *V. elodeae* (Figure 5.2 and Table 5.1). In general, Cu induced higher levels of caspase activity than Zn except for *V. elodeae* (Figure 5.2 and Table 5.1).

![Figure 5.2. Caspase activity, assessed by FITC-VAD-FMK, in mycelia of *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to EC50 of Cu or Zn. Caspase-positive cells show green fluorescence.](image)

5.3.3. Cu and Zn induce nuclear morphological alterations revealed by DAPI staining

Nuclei of control mycelia of the aquatic hyphomycetes appeared as single round spots when stained by DAPI (Figure 5.3). The exposure to Cu and Zn induced nuclear alterations in all aquatic hyphomycete species, as arrangements in half-rings or nuclear fragments randomly distributed (Figure 5.3 and Table 5.1). These alterations were
found in lower percentage in mycelia of *H. submersus* and *F. curta* than in *V. elodeae* (Figure 5.3 and Table 5.1).

**Figure 5.3.** Morphology of nuclei revealed by DAPI staining in *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to EC$_{50}$ of Cu or Zn. Arrows indicate nuclei with altered morphology. Inserts show detailed nuclear morphology, with nucleous alterations as half-ring arrangements or nuclear fragments.

### 5.3.4. Cu and Zn induce DNA strand-breaks revealed by TUNEL assay

No detectable TUNEL-positive phenotype was observed in control mycelia of the three aquatic hyphomycete species (Figure 5.4). Metal exposure led to DNA cleavage, as shown by the yellow nuclear fluorescence as the result of superimposition of green and red fluorescence due to simultaneous staining with TUNEL and PI. Zinc induced a greater number of cells displaying DNA strand-breaks in *H. submersus* and *F. curta* than in *V. elodeae*, as shown by higher number of cells with TUNEL-positive phenotype in the two former species (Figure 5.4 and Table 5.1). Exposure to Cu resulted in a small number of cells with a TUNEL-positive phenotype in *H. submersus* and *V. elodeae*, while no DNA cleavage was detected in *F. curta* (Figure 5.4 and Table 5.1).
Metal stress induces programmed cell death in aquatic fungi

Figure 5.4. DNA strand-breaks visualized by TUNEL assay in *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to EC_{50} of Cu or Zn. TUNEL-positive cells are shown by yellow nuclear fluorescence, as the result of superimposition of green (FITC-labelled nucleotides) and red fluorescence due to simultaneous staining with TUNEL and PI.

5.4. Discussion

The maintenance of cellular homeostasis is dependent on the ability of cells to respond to diverse environmental stressors. Metals can cause, directly or indirectly, an increase in ROS production in cells (Stohs and Bagchi, 1995). ROS production by damaging lipids, proteins and nucleic acids (Bai et al., 2003) can affect cellular functions and subsequently may induce PCD (Madeo et al., 1999). Moreover, it has been demonstrated that ROS production during PCD can occur upstream of other apoptotic events, such as activation of caspases (Buttke and Sandstrom, 1994) and nuclear fragmentation (Masato et al., 1998). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff et al., 1994), being the most characteristic traits the fragmentation of nucleus with condensed chromatin, extensive membrane blebbing and DNA strand-breaks (Mignotte et al., 1998).
We previously demonstrated that metal-induced ROS production contributes noticeably to Cu and Zn toxicity in aquatic hyphomycetes (Azevedo et al., 2007). However, to our knowledge, this is the first study linking metal-induced oxidative stress to PCD processes in aquatic fungi. In our study, *H. submersus* and *F. curta* displayed high ROS production/accumulation and caspase activation under Cu stress, but a low number of cells had nuclear morphological alterations and DNA strand-breaks. The exposure of *V. elodeae* to Cu did not induce ROS production/accumulation. However, nuclear morphological alterations, caspase activation and DNA strand-breaks were evident in this fungal species. This is in agreement with data linking PCD to an increase in the proportion of TUNEL-positive nuclei in filamentous fungi under stress (early stationary-phase, Mousavi and Robson, 2003; exposure to sphingoid long-chain bases, Cheng et al., 2003). In addition, the absence of detectable ROS in *V. elodeae* under Cu stress suggests that pro-oxidative conditions are not a general prerequisite for PCD in aquatic hyphomycetes, similarly to that found in *S. cerevisiae* treated with an antifungal agent (Almeida et al., 2007) or aspirin (Balzan et al., 2004).

The exposure of *H. submersus* and *F. curta* to Zn led to more DNA strand-breaks than ROS production and caspase activation. This is the opposite to that happened under Cu stress. Moreover, although no active caspases were found after exposure of *F. curta* to Zn, we cannot discard that a different PCD pathway might occur in this fungal species. Treatments of *A. fumigatus* with H$_2$O$_2$ or amphotericin B induced a strong apoptotic phenotype independent of caspase activity (Mousavi and Robson, 2004). Also, the two major sphingoid bases of fungi with fungicidal activity against *A. nidulans* induced a PCD not dependent on metacaspase activity (Cheng et al., 2003). This suggests that a caspase-independent pathway may operate in filamentous fungi as described in mammalian systems (Cheng et al., 2003).

It has been proposed that yeast cells can commit altruistic suicide to provide nutrients for others, probably younger and fitter cells (Herker et al., 2004). In filamentous fungi PCD can occur after treatments with various stress agents (Cheng et al., 2003; Mousavi and Robson, 2004) and during developmental processes (Mousavi and Robson, 2003; Thrane et al., 2004). Therefore, the occurrence of a tightly regulated death pathway, such as PCD, in aquatic hyphomycetes under metal stress might constitute an advantageous way of fungal acclimation in metal-polluted streams, because it would allow the sacrifice of certain cells for the benefit of whole mycelium.
Metal stress induces programmed cell death in aquatic fungi

(Richie et al., 2007). For the first time, we provided evidences that Cu and Zn can trigger apoptotic-PCD in aquatic hyphomycetes. The most tolerant species either to Zn (V. elodeae, EC$_{50}$ 7315 µM) or Cu (H. submersus, EC$_{50}$1510 µM) exhibited the higher levels of PCD markers. Moreover, different combinations of apoptotic markers were found suggesting the triggering of different cell death pathways in aquatic hyphomycetes. This may be linked to fungal resistance/tolerance to Cu and Zn and are worthy of further studies.

References


Metal stress induces programmed cell death in aquatic fungi


Metal stress induces programmed cell death in aquatic fungi


Metal stress induces programmed cell death in aquatic fungi


Chapter 6

General discussion
In recent years, freshwater pollution by heavy metals has attracted considerable attention because it is a worldwide problem with serious environmental consequences. Heavy metals released in the environment result from natural processes, but mainly from human activities, such as agriculture, mining and industry (Ayres, 1992). 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 on biological systems. Essential metals, such as Cu, Zn and Ni are needed for the growth and metabolism of organisms. However, both essential and non-essential metals (e.g., Cd) can be toxic when present above certain threshold concentrations.

In freshwater ecosystems, fungi, particularly aquatic hyphomycetes, have been recognized as playing a dominant role in microbial decomposition of leaf litter (Baldy et al., 1995; Pascoal and Cássio, 2004; Pascoal et al., 2005a), by degrading plant cell-wall polymers and increasing leaf palatability for invertebrate consumption (Bärlocher, 2005). Even though aquatic hyphomycetes are primarily associated with clean and well-aerated freshwaters, they also occur in metal-polluted streams (Sridhar et al., 2000; Pascoal et al., 2005b). However, pollution by metals is reported to decrease aquatic hyphomycete diversity (Sridhar et al., 2000; Pascoal et al., 2005b) and activity, as reproduction (Rodrigues, 2002; Duarte et al., 2004, 2008) and growth (Miersch et al., 1997; Rodrigues, 2002).

In this work, the exposure to metals inhibited reproduction, as sporulation rates, of the tested aquatic hyphomycetes (Chapter 2). Additionally, we found that fungal reproduction was more sensitive to metals than growth, which is in accordance with several reports (Abel and Bärlocher, 1984; Bermingham et al., 1996; Duarte et al., 2004, 2008). The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% (EC$_{50}$), showed that *Ypsilina graminea* and *Varicosporium elodeae* were the most resistant species to Zn, while *Heliscus submersus* was the most resistant fungus to Cu (Chapter 2). Generally, Ni or Cd were more toxic to fungi than Zn or Cu, which is corroborated by previous reports (Gadd, 1993; Miersch et al., 1997; Rodrigues, 2002; Guimarães-Soares, 2005). Moreover, the patterns of species resistance to metals found in either liquid or solid medium with similar composition were identical (Chapter 2). However, EC$_{50}$ values were about 20-times higher in solid medium than in liquid medium, probably because agar may decrease metal bioavailability to fungi (Gadd, 1993). Changes in nutrient
supplies to fungi affected metal toxicity, as shown by higher EC\textsubscript{50} values found in mineral medium supplemented with vitamins and glucose than in malt extract (Chapter 2). This is in agreement with the decrease in metal toxicity with increased concentration of the carbon source in the culture medium (Gadd \textit{et al.}, 2001).

Data from literature indicate higher metal tolerance in fungi isolated from metal-contaminated sites (e.g., Miersch \textit{et al.}, 1997; Colpaert \textit{et al.}, 2000), but this is not always the case (Miersch \textit{et al.}, 1997; Blaudez \textit{et al.}, 2000; Colpaert \textit{et al.}, 2000). In the present work, \textit{Alatospora acuminata}, isolated from decomposing leaves collected in a clean stream, was very sensitive to all metals (Chapter 2). Consistently, \textit{H. submersus} and \textit{Flagellospora curta} isolated from a metal-polluted stream showed high tolerance to the most toxic metals (Cu, Ni and Cd). These findings suggest that fungi adapted to metal-polluted environments tolerate higher metal concentrations. However, \textit{Y. graminea} isolated from a metal-polluted site was tolerant to Zn but not to Cd. Also, \textit{V. elodeae} a species isolated from a clean site was able to tolerate high levels of Zn but not of Cu. This indicates that fungal tolerance to metals can vary with fungal species and metal type, suggesting that different mechanisms or cellular targets may be involved in fungal tolerance to different metals. Therefore, we selected three aquatic hyphomycete species, namely \textit{H. submersus}, \textit{V. elodeae} and \textit{F. curta}, with different sensitivities to Cu and Zn to investigate the mechanisms underlying metal tolerance in aquatic hyphomycetes.

Copper and Zn induced alterations in cell-wall morphology of the tested aquatic hyphomycete species, as shown by scanning electron microscopy (Chapter 3). The highest amounts of Cu and Zn adsorbed to fungal mycelia were found in \textit{H. submersus} and \textit{V. elodeae}, respectively (Chapter 4). Because these fungi were the most tolerant species to each metal, biosorption may be a relevant mechanism to avoid unrestrained uptake of metals minimizing their deleterious effects. Nevertheless, these fungal species accumulated metals in their mycelia, although at much lower amounts comparing to that adsorbed. The ability of aquatic hyphomycetes to take up and store large quantities of metals makes them potential candidates for bioremediation. In this context, \textit{V. elodeae} and \textit{F. curta} had remarkable ability to adsorb and accumulate Zn comparing with values reported for aquatic fungi (Guimarães-Soares, 2005; Jaeckel \textit{et al.}, 2005) Also, \textit{H. submersus} was able to retain ca. 7-times more Cu than metal-tolerant strains of \textit{H. lugdunensis} (Braha \textit{et al.}, 2007). However, adsorption of metals by filamentous fungi
depends on several factors, 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 found in *V. elodeae* and *H. submersus* under different environmental conditions (Chapter 3).

The primary mechanism of Cu toxicity in fungi is the disruption of cellular or organellar membranes (Ohsumi et al., 1988). In agreement, our results 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 (Chapter 3). This effect can be 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). In this study, we clearly demonstrated that generation of ROS contributed noticeably to metal toxicity in aquatic hyphomycetes, particularly under Cu stress, as indicated by the increase of biomass production in the presence of an antioxidant agent (Chapter 3). Moreover, metals can directly interact with biomolecules, such as transport proteins of essential nutrients and ions, compromising their biological functions (Gadd, 1993). In this work, we found that short-term exposure (10 min) to Cu completely inhibited the activity of the H^+\text{-ATPase} in *H. submersus* and *V. elodeae*, while Zn only led to a similar effect on that of *H. submersus* (Chapter 4). However, since a recovery of plasma membrane integrity was observed after 150 min of exposure to Cu *H. submersus* (Chapter 3), a functional restoration of the H^+\text{-ATPase} is expected to occur at longer times. Indeed, 8 days of metal exposure led to strong stimulations of the proton pump in the most tolerant species, i.e. when *H. submersus* was exposed to Cu and *V. elodeae* was exposed to Zn (Chapter 4). The activation of H^+\text{-ATPase} by metal exposure may be related to its ability to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane (Serrano, 1988; Fernandes et al., 1998), suggesting that H^+\text{-ATPase} may be involved in aquatic hyphomycete acclimation to metals.

In aquatic fungi, metal tolerance has been also associated with the synthesis of thiol (SH)-enriched compounds, which are able to scavenge ROS or bind metals within cells (Miersch et al., 1997; Miersch et al., 2001; Guimarães-Soares et al., 2006, 2007). In the present work, *H. submersus* and *F. curta*, the two species isolated from a metal-
polluted stream, had higher levels of non-protein (NP-SH) and protein-bound (PB-SH) thiols before metal exposure comparing to *V. elodeae*, the species isolated from a clean stream (Chapter 4). The latter species, that had the lowest constitutive thiol levels, rapidly increased NP-SH and PB-SH levels under exposure to Cu or Zn. These findings reinforce previous observations that high constitutive levels of thiols or the rapid increase of their production may help aquatic hyphomycetes to deal with metal stress (Guimarães-Soares *et al.*, 2006, 2007). In addition, the decrease in the NP-SH containing compounds in all fungal species after long-term exposure (8 days) to Cu is consistent with the ability of peptides with very low molecular weight, most likely glutathione and phytochelatins, to bind Cu in aquatic hyphomycetes (Guimarães-Soares *et al.*, 2006).

All organisms, including fungi, have a set of enzymatic defenses to deal with oxidative stress (Bai *et al.*, 2003). Enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (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 Bebiano, 2004). The first two enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Penninckx and Elskens, 1993; Bai *et al.*, 2003); G6PDH is essential for the replenishment of NADPH intracellular pool to maintain cellular redox balance (Penninckx and Elskens, 1993). Our studies on antioxidant defenses showed that CAT had a greater role alleviating the stress induced by Zn and Cu than SOD (Chapter 3). 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.

In this work, we also tested whether Cu and Zn are able to induce programmed cell death (PCD), a process in which cells actively participate in their own death (Robson, 2006). For that, we examined typical apoptotic markers, namely ROS production, caspase activation, alterations in nuclear morphology and the occurrence of DNA strand-breaks (Kerr *et al.*, 1972; Clifford *et al.*, 1996; Earnshaw *et al.*, 1999; Madeo *et al.*, 1999). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff *et al.*, 1994), being the most characteristic traits the fragmentation of nucleus with condensed chromatin, extensive membrane blebbing and DNA strand-breaks (Mignotte *et al.*, 1998). In our study, *H.*
submersus and *F. curta* displayed high ROS production and caspase activation under Cu stress, but a low number of cells had nuclear morphological alterations and DNA strand-breaks (Chapter 5). In *V. elodeae*, Cu induced caspase activation, nuclear morphological alterations and DNA strand-breaks, but did not increase ROS production (Chapter 5). These results may indicate that *V. elodeae* developed a PCD process independent of ROS production, similarly to that recently described in yeasts (Almeida *et al.*, 2007). The exposure to Zn led to more DNA strand-breaks or nuclear morphological alterations than ROS production and caspase activation in the tested aquatic hyphomycetes (Chapter 5). For the first time, we provided evidences that Cu and Zn can promote PCD in aquatic hyphomycetes. The occurrence of a tightly regulated death pathway, such as PCD, in aquatic hyphomycetes under metal stress may constitute an advantageous way of fungal acclimation in metal-polluted streams, because it would allow the sacrifice of certain cells for the benefit of whole mycelium (Richie *et al.*, 2007). However, further experiments are needed to better understand the role of PCD in aquatic hyphomycete homeostasis under metal stress.

**References**


General discussion


