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Abortiporus biennis tolerance to insoluble metal oxides: oxalate secretion, oxalate oxidase activity, and mycelial morphology

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Abstract The ability of *Abortiporus biennis* to tolerate and solubilize toxic metal oxides (Cu_2O, Al_2O_3, ZnO, $CuFe_2O_4Zn$, CdO, and MnO_2) incorporated into agar media was investigated and the growth rate, oxalic acid secretion, and mycelial morphology were monitored. Among the tested metal oxides, formation of clear zones underneath the mycelium growing on Cu_2O- and ZnO-amended plates was observed. ZnO, CdO and Cu_2O caused the highest rate of fungal growth inhibition. An increased level of oxalic acid concentration was detected as a response of *A. biennis* to the presence of Cu_2O, MnO_2, ZnO and $CuFe_2O_4Zn$ in growth medium. The oxalate oxidase (OXO) was found to be responsible for oxalic acid degradation in *A. biennis* cultivated in metal-amended media. An increased level of OXO was observed in media amended with Cu_2O, ZnO and MnO_2. Confocal microscopy used in this study revealed changes in mycelial morphology which appeared as increased hyphal branching, increased septation and increased spore number.

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Introduction

Abortiporus biennis belongs to white rot *Basidiomycetes*, a group of fungi which possess the unique ability to degrade all wood components (Martinez et al. 2005). Heavy metals present in the fungal environment are essential for fungal metabolism in a trace amounts, but become toxic at concentrations only a few times greater (Baldrian 2003). The effects of heavy metals on the growth and metabolism of wood-rotting fungi are connected with the influence on the activity of lignin degrading enzymes, such as manganese peroxidase and laccase (Hofrichter 2002; Jarosz-Wilkolazka et al. 2002, 2006). Because these enzymes are often used for biotechnological applications, the interactions between heavy metals and fungi are interesting from the viewpoint of biotechnology and physiology.

Organic acids, such as oxalate, have frequently been observed in different wood-rotting fungal strains (Shimada et al. 1997). Oxalic acid can take part in nonenzymatic initiation of lignocellulose depolymerization through participation in radical species production, buffering fungal vicinity, facilitation of oxidative enzyme activities, metal chelating abilities and middle lamellae destruction (Dutton and Evans

1996; Hofrichter 2002; Schlosser and Höfer 2002). On the other hand, oxalic acid secretion in the presence of metals, suggests its role in metal detoxification mechanisms in fungi (Jarosz-Wilkolazka and Graz 2006). Immobilization of metals by the formation of insoluble oxalate-metal complexes, for example copper oxalates, allows fungi to tolerate environments contaminated by high concentrations of toxic metals (Gadd 1993). Due to the numerous roles of oxalate, its concentration in fungal vicinity has to be precisely controlled by the fungus by the action of two main enzymes involved in this process: oxalate decarboxylase or oxalate oxidase (Svedruzic et al. 2005). In this study, *Abortiporus biennis* was selected because of its known sensitivity towards various stress conditions. The aim of this work was to investigate the influence of metal oxides on the fungal mycelium and oxalate metabolism *via* oxalate decarboxylase and oxalate oxidase in *A. biennis* cultures.

Materials and methods

Fungal strain and culture conditions

Abortiporus biennis used in the present study was obtained from the Fungal Collection (FCL) of the Biochemistry Department, Maria Curie-Sklodowska University, Lublin, Poland. Stock cultures were maintained on 2% malt extract agar (MEA) slants at 4°C. The FCL number of this strain is FCL-123.

The inoculation material was precultured on 2% MEA for 1 week at 25°C. The experiments were performed using plastic petri dishes (55 mm in diameter) containing 10 (±1) ml of solid medium according to Lindeberg and Holm (1952) of the following composition (g l^{-1}): 20 agar, 10 glucose, 2.50 L-asparagine, 3.0 $NaNO_3$, 0.50 KCl, 0.45 KH_2PO_4, 0.17 $Na_2HPO_4 \times 17H_2O$, 0.50 $MgSO_4 \times 7H_2O$, 50 μg l^{-1} of thiamine and $CuSO_4$, $MnCl_2$, $ZnSO_4$, $FeSO_4$ as trace elements (5 mg l^{-1}). Six insoluble metal oxides (Cu_2O, Al_2O_3, MnO_2, ZnO, $CuFe_2O_4Zn$, and CdO) were incorporated to the basal medium before sterilization at appropriate concentrations (10, 20, 30 mM). After setting of the agar and prior to inoculation, a sterile dialysis membrane was placed aseptically onto the surface of the agar in each petri dish, which provided a convenient means of removing the mycelium before further analysis (Sayer et al. 1995). Each plate was inoculated with one agar plug (5 mm in diameter) obtained from the leading edge of colony, which had been maintained on MEA for at least 7 days at 25°C. The agar plates were incubated for the next 2 weeks at 25°C and radial growth of the colonies and diameters of any clear zones of solubilization after peeling away the membranes with the mycelium were measured. The pH values underneath the central part of the colonies were measured in duplicate using a combination flat surface pH electrode (Thermo Electron Corporation, USA). Each oxide was tested in three independent experiments.

Metal content of mycelia

Each mycelium was removed from the replicate agar plates by peeling the biomass from the dialysis membrane and washed in distilled water. The mycelia were weighed and then mineralized in HNO_3 at 195°C and 269 psi, for 8 min. After appropriate dilution with distilled water, the solutions were analyzed for metal ion content using inductively coupled plasma-optic emission spectroscopy (ICP-OES).

Extraction and determination of oxalic acid

After removing the fungal colonies from the agar plates by peeling the biomass together with the dialysis membrane, three discs of agar (5 mm in diameter) were incubated in HEPES buffer pH 5 for organic acids extraction. The formation of extracellular oxalic acid was detected using the capillary electrophoresis method on the Thermo Capillary Electrophoresis, Crystal 100 (Thermo Separation Products, San Jose, USA) equipped with a UV-Vis diode array detector as previously described (Jarosz-Wilkolazka and Graz 2006). All samples, buffer solutions, and conditioning solutions were filtered through 0.22 μm syringe filters before use.

Preparation of mycelium crude extract and enzymatic assays

After removing the fungal colonies from the agar plates by peeling the biomass from the dialysis membrane, the mycelium was homogenized in an ice-chilled motor-driven Potter's homogenizer, in

5 mM phosphate buffer pH 7. The homogenates were then centrifuged (10 min, 10,000×g, 4°C) and the supernatant fractions were collected and analyzed for enzymes activities and for the level of proteins. The protein concentration was determined using the Bradford method and bovine serum albumin as a standard (Bradford 1976). Oxalate decarboxylase (OxDC) activity was determined using the stopped assay of Magro et al. (1988), in which formic acid production was linked to the reduction of NAD by formate dehydrogenase. One unit of OxDC activity (U) was defined as the amount of enzyme required to produce 1 μmol of formate per minute and the specific activity was expressed in U mg^{-1} of proteins. Oxalate oxidase (OXO) activity was determined by the formation of H_2O_2 from sodium oxalate and coupled to the oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in a horseradish peroxidase-mediated reaction, according to Requena and Bornemann (1999). One unit of enzyme activity (U) was defined as the amount of enzyme producing 1 μmol of H_2O_2 per minute, and the specific activity was expressed in U mg^{-1} of proteins.

Confocal laser scanning microscopy observations

Dabs from the pieces of the mycelium were taken and placed on clean glass slides. 10 μl of the fluorescent dye Hoechst 33342 (Sigma) was then added. After 5 min incubation in the dark at 37°C in a humidified atmosphere, cover glasses were put on the glass slides. The specimens were then observed under a confocal laser scanning microscope (LSM 5, Carl Zeiss, Jena, Germany). The data were recorded in a fluorescent channel ($\lambda = 488$ nm). Five sections from three example fields were observed for each mycelium cultivated on particular oxides. All results achieved for particular metal oxide showed the same tendency as presented at Fig. 2. As controls, mycelia without metal oxides addition were used and examined.

Results

Growth of *A. biennis* on insoluble metal oxides

Abortiporus biennis was cultivated on Cu_2O-, Al_2O_3-, MnO_2- ZnO-, $CuFe_2O_4$Zn-, and CdO-amended plates in concentrations of 10, 20, and 30 mM. The fungal growth rates are expressed in terms of ratios of the colony growth rate in the presence of metal oxide (R_O) to the growth rate on a control plate without metal addition (R_C). A R_O/R_C value of < 1.0 indicates that the metal compound inhibited the growth of the fungus. The growth extent was dependent on the sort and the concentration of the metal added to the medium (Table 1). There was no inhibition or only a slight decrease in the fungus growth rate in the case of Al_2O_3-, and $CuFe_2O_4$Zn-amended plates at all the tested concentrations ($R_O/R_C \approx 1.0$) and the hyphae became more and more fragile and slender with the increasing concentration of oxides. With regard to the Cu_2O-, MnO_2- ZnO- and CdO-amended plates, the inhibition of *A. biennis* growth extent was higher. The R_O/R_C factor ranged between 0.25 and 0.9 and decreased with the increasing metal concentration. Among the tested compounds, the most toxic was CdO where the R_O/R_C factor was 0.2 for 10 mM oxide and lack of growth was observed in the case of the 20 and 30 mM concentrations of this oxide. The presence of ZnO inhibited the fungal growth to a great extent and R_O/R_C decreased from 0.4 to 0.15 with the increasing oxide concentration. Cu_2O distinctly limited the *A. biennis* growth at the 30 mM concentration and the presence of MnO_2 visibly inhibited fungal growth at the 20 and 30 mM concentrations (Table 1).

Accumulation of metals in the mycelium of *A. biennis*

The accumulation of metals was examined in the fungal mycelium growing in the presence of insoluble metal oxides at two concentrations (10 and 30 mM). The results shown in Table 2 indicate that *A. biennis* exhibited a preferential order of metal accumulation from the insoluble metal oxides which was $Cu_2O > MnO_2 > CdO > ZnO > Al_2O_3$ at the 10 mM concentration of the tested oxides, and $ZnO > Cu_2O > MnO_2 > Al_2O_3$ at the 30 mM concentration. CdO at the 30 mM concentration was not tested because of a total inhibition of fungal growth in these conditions (Table 1).

Table 1 *Abortiporus biennis* growth rate, clear zones formation, oxalic acid concentration and oxalate oxidase (OXO) activity after 14 days of cultivation on metal-amended plates

Oxide	Oxide concentration (mM)	Growth rate[a] (R_O/R_C)	Clear zone[b] (CZ)	Oxalic acid (mM)	OXO activity (U mg^{-1})
Control	(without oxide)		+++	0.8	1.6
Cu_2O	10	0.75	++	1.4	5.9
	20	0.5	++	1.1	5.9
	30	0.25	+++	1.1	6.0
Al_2O_3	10	1	−	0.6	1.3
	20	1	−	0.7	1.8
	30	0.95	−	0.8	1.6
MnO_2	10	0.9	+	2.9	2.9
	20	0.5	+	2.6	4.5
	30	0.5	−	3.2	6.4
ZnO	10	0.4	+++	1.1	2.3
	20	0.18	+++	1.2	2.8
	30	0.15	++	1.4	3.4
$CuFe_2O_4Zn$	10	1	−	1.4	1.6
	20	1	−	1.4	4.2
	30	0.9	−	1.5	5.2
CdO	10	0.2	++	0.9	nd
	20	0	−	nd	nd
	30	0	−	nd	nd

[a] The growth rates are expressed as the ratio of colony growth rate in the presence of metal (R_O) to the growth rate on control plate without metal addition (R_C); R_O/R_C value of <1.0 indicates that the metal compound inhibited the growth of the fungus

[b] CZ— +++, clear zone goes beyond the mycelium; ++, full clear zones under mycelium; +, clear zones only in the mycelium center; −, no clear zone observed; nd, not determined

All values shown are averages from three measurements (with typical relative standard deviation of about 1%)

Solubilization of insoluble metal compounds and oxalic acid secretion

Solubilization of the insoluble metal compounds by *A. biennis* was monitored as the production of a clear zone under the growing colonies on solid, oxides-amended media (Table 1). We observed the appearance of totally or almost totally clear zones underneath the mycelium on the ZnO- and Cu_2O-amended plates. Underneath the mycelium growing on the CdO- and MnO_2-amended plates, clear zones appeared only under the centre of the mycelium. Despite of the whole plate overgrown by the mycelium of *A. biennis* in the case of Al_2O_3 and $CuFe_2O_4Zn$, clear zones of oxide solubilization were not observed at all. On the MnO_2-amended plates, formation of russet precipitations was observed during fungal growth, due to alteration of the manganese oxidation state (Fig. 1).

Due to the involvement of oxalic acid in metal solubilization, the concentration of oxalate was measured in the solid medium underneath the mycelium (Table 1). The highest concentration of oxalate was detected on the MnO_2-amended plates (3.2 mM). On the ZnO- and Cu_2O-amended plates, where solubilization was also observed, the oxalic acid concentration was maintained above 1 mM (range from 1.1 to 1.4 mM) similarly to the $CuFe_2O_4Zn$-amended plates (about 1.4 mM) where no solubilization was observed. On the CdO- and Al_2O_3-amended plates, the oxalic acid concentration was lower than 1 mM and was comparable to control conditions (Table 1).

Assay of oxalic acid degrading enzymes

The oxalic acid degrading enzyme found in the *A. biennis* cultures was oxalate oxidase (OXO), and no oxalate decarboxylase (OxDC) activity was

Table 2 Accumulation of metal oxides by *A. biennis* after 14 days of growth in the presence of oxides in two concentrations (10 and 30 mM). Data represent means ± SD ($n = 3$)

Metal oxides		Metal tested	Metal concentration in fungal mycelium (μg per gram of wet biomass)
Type	Concentration (mM)		
Cu_2O	10	Cu	3,218 ± 1,008.3
	30	Cu	4,778 ± 1,773
MnO_2	10	Mn	313 ± 75
	30	Mn	664 ± 100.4
CdO	10	Cd	307.5 ± 44.5
	30	nd	nd
ZnO	10	Zn	95.5 ± 9.2
	30	Zn	5,927 ± 306.2
Al_2O_3	10	Al	5.2 ± 1.4
	30	Al	6.5 ± 3.5
$CuFe_2O_4Zn$	10	Cu	195 ± 45.3
	10	Zn	21.2 ± 2.8
	10	Fe	13.1 ± 3.1
	30	Cu	428 ± 5.7
	30	Zn	33.5 ± 9.2
	30	Fe	16.5 ± 2.1
Control	0	Cu	3.1 ± 2.8
	0	Fe	6.5 ± 2.5
	0	Zn	4.5 ± 4.9
	0	Al	3.5 ± 0.7
	0	Mn	4.5 ± 4.9

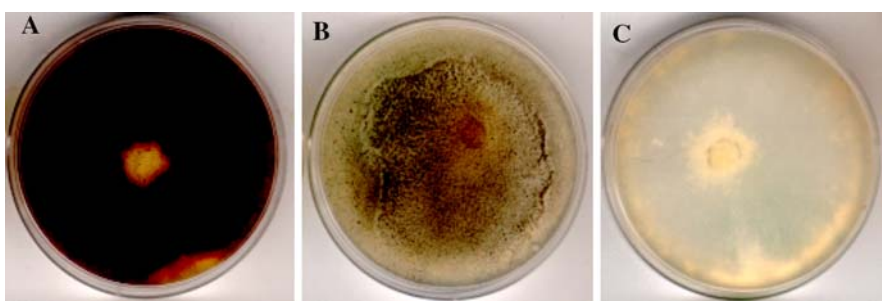

Fig. 1 *Abortiporus biennis* cultures on MnO_2-amended plates with addition of 30 mM MnO_2 (**a**), 10 mM MnO_2 (**b**) in comparison to non-amended control plate (**c**)

detected (Table 1). On the Cu_2O-, MnO_2-, ZnO- and $CuFe_2O_4Zn$-amended plates, increased activities of OXO in comparison to control cultures (without any oxides added) were observed. High activities of OXO were determined in the case of the mycelium growing on the Cu_2O- and MnO_2-amended plates (6.0 and 6.4 U mg^{-1}, respectively). On the MnO_2-, ZnO- and $CuFe_2O_4Zn$-amended plates, OXO activity increased with the increasing metal oxide concentration. In the case of the Al_2O_3-amended cultures, OXO activities were at a similar level as in control cultures. No OXO activity was detected in the case of the CdO-amended cultures (Table 1).

Mycelial morphology after growth on metal-amended media

To compare the influence the addition of the metal oxides on the morphology of *A. biennis* mycelium, confocal microscopy was applied (Fig. 2). The control mycelia were composed of regular, tubular hyphae with many spores (Fig. 2a). Exposure to the

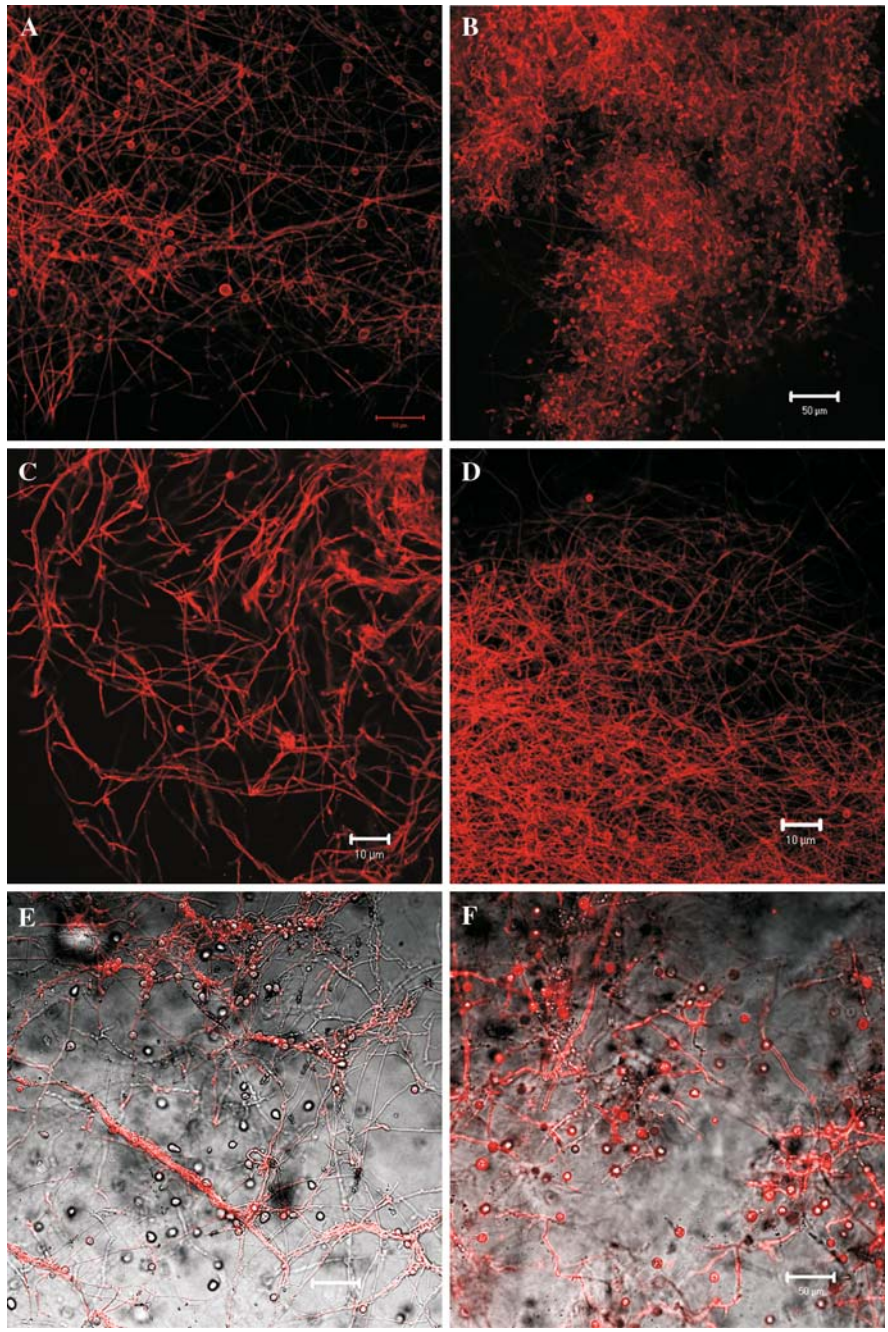

Fig. 2 The morphology of *A. biennis* mycelia under the influence of toxic metal ions**.** A—control mycelium with regular hyphae and spores visible; B—Cu_2O amended cultures with a lot of branches in hyphae and spores with characteristic thick walls; C—MnO_2 treated mycelia showing clear increase in spore number; D—the cultures amended with 30 mM of Al_2O_3 showing coarse and branched cell walls; E—the ZnO treated mycelium with visible huge number of spores and thinner appearance of hyphae; F—CdO-ammended cultures with characteristic increased hyphae branching, irregular septation and hyphal swelling. Scale bares are: A, B, E, F = 50 µm, C, D = 10 µm

tested oxides modified mycelial morphology. An addition of Cu_2O caused increased branching in *A. biennis* hyphae, which became densely packed with spores possessing the thickest cell walls among all the mycelia subjected to the influence of the tested oxides (visible as black points in Fig. 2b). On MnO_2-amended medium the hyphae became shorter in length and branched with a lack of regularity in cell wall thickening. There were also more spores observed in comparison to the control mycelium (without addition of oxides) (Fig. 2c). No distinct changes in mycelial morphology of the fungus growing in the presence of Al_2O_3 were observed, with the exception of the 30 mM concentration. At

this concentration, individual spores appeared and the cell walls were more branched and coarse than those observed in control cultures (Fig. 2d). On the ZnO-amended plates, fungal hyphae were more threadlike, with cell walls revealing irregular thickening in comparison to the control. A correlation between increasing ZnO content in media and the changes in fungal hyphae was also observed. The hyphae treated with ZnO became thinner and had a larger number of spores than the control (Fig. 2e). The CdO-amended cultures were characterized by poorer growth, increased hyphae branching and irregularity of septation. There was also observed hyphal swelling in the presence of Cd ions (Fig. 2f).

Discussion

Metals presented in extend can exert toxic effects in many ways. They can inhibit enzymes and spores germination, cause disruption of membranes, and destructive interact with free radical protection system (Gadd 2007). Fungal capacity to remove soluble and insoluble metal compounds is well known (Jarosz-Wilkołazka and Gadd 2003; Gadd 2004; Fomina et al. 2005b). Fungal mechanisms involved in response to stress induced by toxic metals, comprise reduction of metal uptake or increased metal efflux, metal immobilization *via* biosorption, extracellular precipitation, extracellular sequestration by extracellular metabolites e.g., exopolisaccharides or intracellular seqeuestration by metallothioneins and phytochelatins and vacuolar compartmentation (Gadd 2007). Immobilization of metals to the fungal cell wall elements is one of the better investigated ways that fungi use to withstand stress induced by toxic metals (Baldrian 2003). The range of metal biosorption capacities can differ among different species of fungi. *Abortiporus biennis* was most efficient at accumulating Cu(I) and Zn(II) ions among the tested metal oxides. As regards Cd(II) ions, our earlier investigation had shown that *A. biennis* did not possess any efficient system to keep cadmium ions outside the cell, and revealed a tendency towards bioaccumulation rather than biosorption of Cd(II) ions, which can have a toxic effect on fungal growth and metabolism (Jarosz-Wilkołazka et al. 2006). The main criterion for defining tolerance or sensitivity to toxic metals present in growth medium is fungal growth. The oxides used in this study can be divided into two groups. The first group containing Al_2O_3 and $CuFe_2O_4Zn$, caused no limitation or only a slight limitation of fungal growth, and the second group containing Cu_2O, MnO_2, ZnO, and CdO, inhibited *A. biennis* growth but to different extents. The role of oxalic acid in the metal tolerance mechanism in fungi is well-established (Gadd 1993). An increased concentration of oxalic acid was found on Cu ions (as Cu_2O and $CuFe_2O_4Zn$), Mn ions (as MnO_2) and Zn ions (as ZnO and $CuFe_2O_4Zn$), where except for $CuFe_2O_4Zn$, also solubilization of metal oxides was observed (Table 1). This can implicate involvement of metal oxalates in the solubilization processes of the tested oxides. In our earlier studies, 28 strains of white-rotting fungi were tested, and oxalic acid was selected as a very important metabolite participating in the response of the tested strains to the presence of metals (Jarosz-Wilkołazka and Grąz 2006). Presence of toxic metals may increase oxalic acid secretion by fungi, e.g., copper tolerance of basidiomycetous fungi is linked with oxalate secretion (Green and Clausen 2003). The mechanisms of tolerance to other toxic metals such as cadmium, copper, lead and zinc minerals, can connected be also with their conversion into oxalates, as is the case with the fungus *Beauveria caledonica* (Fomina et al. 2005a). Oxalic acid plays important role in solubilization of radioactive uranium oxides by fungi as demonstrated by Fomina et al. (2007). Apart from oxalic acid the secretion of gluconic, malic, acetic and citric acid was detected, but their contribution to the process of uranium oxide dissolution was not as significant as that of oxalic acid (Fomina et al. 2007). Oxalate degradation in *A. biennis* mycelium under the tested conditions was caused by oxalate oxidase (OXO). Oxalate decarboxylase (OxDC) and oxalate oxidase (OXO) are considered two main fungal oxalate degrading enzymes. A common and well documented enzymatic pathway for oxalic acid decomposition for white rot fungi is the action of oxalate decarboxylase (Dutton and Evans 1996; Dashek and Micales 1997). The activity of this enzyme was characterized in such fungi as *Postia placenta* (Micales 1995, 1997), *Coriolus versicolor* (Dutton et al. 1994), *Flammulina velutipes* (Kesarwani et al. 2000; Chakraborty et al., 2002), *Dichomitus squalens, Phanerochaete sanguinea, Trametes ochracea* (Mäkelä et al. 2002), and *Sclerotinia sclerotiorum* (Magro et al. 1988). Oxalate

oxidase activity has been commonly detected in plant tissues and its role has been connected with oxalic acid degradation during fungal pathogenesis (Hegedus and Rimmer 2005). With regard to fungi, the activity of OXO has so far been well described in the white rot fungus *Ceriporiopsis subvermispora* (Aguilar et al. 1999). The structure of *Ceriporiopsis subvermispora* oxalate oxidase differs from plant enzyme, and belongs, like oxalate decarboxylase to the bicupin family (Escutia et al. 2005). To our knowledge, this is the first report of oxalate oxidase activity in *A. biennis*. The increased activity of OXO observed in this study on manganese-amended media may be connected with the requirement of Mn(II) ions in the active centre of this enzyme (Just et al. 2004; Svedruzic et al. 2005). We also observed a stimulating effect on OXO activity under copper (as Cu_2O) and zinc (as ZnO) amended conditions. Both copper and zinc play a significant role in metabolism as part of the enzymatic active center of selected enzymes such as different oxidases e.g., laccase or superoxide dismutase (Shah and Nerud 2002; Kurmova et al. 2008). The presence of essential heavy metals in the fungal environment is necessary for the development of the ligninolytic activity (Baldrian 2003). Singhal and Rathore (2001) observed increased activities of lignin peroxidase and manganese peroxidase of *Phanerochaete chrysosporium* cultivated on artificial medium with Zn(II) and Cu(II) addition. The positive effect of copper addition on the production of fungal laccase is well known (Palmieri et al. 2000; Galhaup and Haltrich 2001). Manganese as Mn(III) ions is formed in the MnP catalytic cycle as a diffusible agent able to oxidize lignin compounds (Hofrichter 2002). Figure 3 presents the correlation between oxalic acid secreted by fungi and the activity of intracellular OXO. The increased OXO activity on MnO_2-, Cu_2O- and ZnO-amended plates was correlated with a higher oxalic acid level than detected in control media. In the case of plates amended with Mn(II) ions, the highest activity of OXO was observed, which correlated with the highest detected level of oxalic acid in comparison with control conditions. Addition of Cu ions to fungal growth media as Cu_2O and $CuFe_2O_4Zn$ caused an increase in OXO activity comparable with OXO activity on Mn(II)-amended plates (only in the case of Cu_2O) but smaller accumulation of oxalic acid was observed. In Al_2O_3 amended conditions, the level of oxalic acid

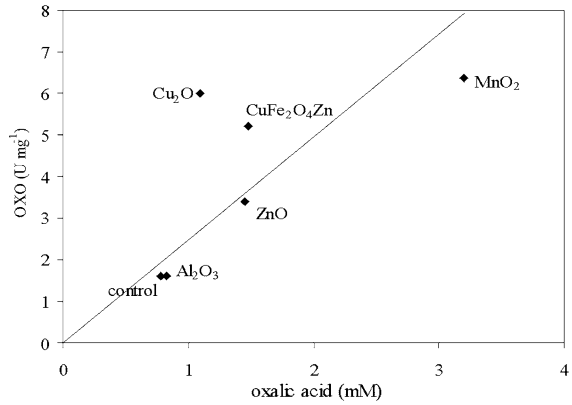

Fig. 3 Correlation between intracellular oxalate oxidase activity (OXO) and oxalic acid secretion by *A. biennis* after 14 days of growth on metal-amended media. The results presented are arithmetic mean from 3 independent measurements (with typical relative standard deviation of about 1%)

and OXO activity was similar to control conditions. In the case of Cd(II) ions, there was no OXO activity and oxalic acid determined because of the total inhibition of fungal growth upon these conditions. The observed correlation between increased OXO activity and an increase in the level of oxalic acid may indicate an inductive character of OXO synthesis. Other studies concerning OxDC activity in fungi have also shown the inductive character of this enzyme activity (Mäkelä et al. 2002).

Inhibition of the fungal growth rate under the influence of metal ions is often accompanied by changes in mycelial morphology (Lafranco et al. 2002; Fomina et al. 2005a). Fungi can employ many morphological strategies in response to presence metals in their environment represented by changes in branching patterns or different degrees to commitment to explorative or exploative growth of mycelium (Gadd 2007). Morphological changes in hyphae, induced by the presence of toxic metals seem to be common among all groups of fungi (Baldrian 2003). In our study, these changes appeared generally as increased hyphal branching, swelling of hyphae, irregularity in septation and an increased number of spores. These alterations were correlated with enhanced metal concentrations. Formation of mycelial cords which exhibited increased excretion of oxalic acid in the fungus *Beauveria caledonica* was observed by Fomina et al. (2005a). Changes in morphology of the ericoid fungus PS IV under Zn-amended growth conditions were reported by

Lafranco et al. (2002). They also observed increased hyphal branching, swelling and septation connected with a higher amount of chitin in zinc-treated hyphae in comparison to control mycelium. The increased chitin content detected in hyphae treated with zinc ions may indicate involvement of cell wall components in fungal response to toxic metal ions.

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