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Biosorption and solubilization of copper oxychloride fungicide by *Aspergillus niger* and the influence of calcium

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Abstract

The biosorption of copper oxychloride fungicide particulates (~1 μm diameter), at concentrations ranging from 25 to 500 ppm active ingredient (ai), by pelleted mycelium of *Aspergillus niger* grown on Czapek Dox medium was evaluated. The concentration of the fungicide adsorbed to the mycelium, remaining suspended or solubilized in the medium, was determined by analysis of its copper content (CuF) using atomic absorption spectrophotometry (AAS). 2-day-old pellets exhibited high biosorption efficiency ranging from 97 ± 1.0 to 88 ± 1.2% of the initially added fungicide concentrations, respectively, within 10 min. However, under the same conditions, amounts of the removed fungicide by 6-day-old mycelial pellets were significantly lower and ranged from 0.5 ± 0.03 to 0.15 ± 0.01%. Scanning electron microscopy studies of 2-day-old pellets supplemented with the fungicide revealed predominant aggregations of clumps and dense particulates on the hyphal tips. The adsorbed CuF of 125 ppm ai fungicide subsequently decreased from 7.5 ± 0.5 to 2.1 ± 0.1 μmol Cu (mg dry wt)$^{-1}$ after 12 h incubation. Simultaneously, the soluble portion of CuF remaining in the medium increased from 0.9 ± 0.6 to 4.9 ± 0.2 μmol Cu ml^{-1}. The presence of 50 mM $CaCl_2$ resulted in a decrease of the adsorbed CuF to 3.5 ± 0.5 μmol Cu (mg dry wt)$^{-1}$ and solubilized copper in the medium increased to 5.9 ± 0.8 μ mol Cu ml^{-1}. Additionally, the cellular copper contents attained after 2 h were 0.08 ± 0.01 and 0.16 ± 0.007 μmol Cu (mg dry wt)$^{-1}$ in absence and presence of calcium, respectively. The addition of calcium to glucose-starved pellets greatly increased the medium [H^+] which was conclusively discussed in relation to Ca^{2+}/H^+ exchange capacity of the fungal cells. These results are of potential environmental, biotechnological and agricultural importance.

Introduction

Bioremediation of polluted effluents and waste waters, based on metal biosorption activity of microbial cells, is of potential significance. Fungi have been extensively studied due to their extensive variety and availability of fungal biomass from industrial processes and fermentation (White & Gadd 1990). The biomasses of different categories of fungi are effective in sorbing metal contaminants such as Sr, Th, Cs, Au, Ag, Cu, Pb, Cd and Zn. Further, the biomass can be used as either living, dead or pelleted whole cells and mobile or immobilized sorbents in batch and continuous culturing (White & Gadd 1990; De Rome & Gadd 1991; Mchale & Mchale 1994; Avery 1995; Morley & Gadd 1995; Volesky & Holan 1995; Gomes & Lindari 1996; Zhou 1999). Mycelium of *Aspergillus niger* has been used in Austria to remove zinc from polluted water, and the results show that the simultaneous presence of other naturally occurring ions does not affect biosorption (Leuf et al. 1991). Transformation and recovery of metallic residues by fungi, which is equally important in bioremediation of polluted habitats, has also been reported (Tsezos & Volesky 1982; Gharieb et al. 1995; Morley et al. 1996). Particulates and insoluble metal compounds, such as zinc dust, $Fe(OH)_3$, magnetite, oxides, and sulphides, can also be adsorbed and physically entrapped in fungal cells, resulting in the formation of bioaggregates and removal of such compounds from solution (Wainwright et al. 1986; Wainwright & Grayston 1989; Singleton et al. 1990; Wainwright 1992).

In some cases, subsequent transformation (solubilization) of such particulates was investigated due to its significance in leaching of metals from industrial wastes and low grade ores (Schinner & Burgstaller 1989; Franz et al. 1991).

Many potentially toxic metals are introduced to the environment in large amounts through fungicidal application. Copper is an essential trace metal for many organisms. However, it is still a potentially toxic metal that can exert an inhibitory effect at higher concentrations, which is the basis for formulation of copper-containing fungicides. Copper oxychloride is probably the most widely employed copper fungicide for a wide range of plant pathogenic fungi. It is a bluish-green insoluble powder consisting of basic copper chloride [$CuCl_2 \cdot 3Cu(OH)_2$], and its fungitoxicity depends on the solubilization and release of ionic copper (Hassall 1990). Mobilization of the potentially toxic metal by fungi should be studied to understand its fate when discharged into the environment. The aim of the present study was first to investigate biosorption and mobilization of copper of this fungicide by the most widespread saprophytic fungus in the terrestrial environment, *Aspergillus niger*. Additionally, the availability and partitioning between mobile and immobile phases of metals is likely to be influenced by the environmental conditions. Therefore, the second aim of this work was to determine the influence of the most agronomically important element, calcium, on such fungal activities.

Materials and methods

Organism, medium and culture conditions

A strain of *Aspergillus niger* was isolated from a cultivated field in Egypt and identified according to Raper & Fennell (1977). It was routinely maintained on Czapek- Dox medium of the following composition (g l^{-1} distilled water): sucrose, 30; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeCl_3 \cdot 6H_2O$, 0.001–15,0 g l^{-1} agar (Lab M No. 2) was added to prepare solid medium. 1 ml aliquot of a spore suspension containing $\sim 3 \times 10^5$ spores was used to inoculate 1 l Erlenmeyer conical flasks, each containing 0.5 l sterile liquid medium and then incubated at 25 °C on an orbital shaker (150 rpm). For determination of dry weight, the mycelial pellets were harvested and washed twice with 20 ml ddH_2O, and dry weight was determined using tared foil cups dried to constant weight at 80 °C.

Biosorption experiments

A stock suspension [50,000 ppm active ingredient (ai)] of insoluble copper fungicide copper oxychloride (particulate size was approximately 1 μm) (Prochemiocos, Ugota, Columbia), was prepared in sterile distilled water. 50 ml of 2-day- or 6-day-old mycelial spherical pellet (approximately 3 mm in diameter) suspensions were transferred in triplicate into 100 ml Erlenmeyer conical flasks. The liquid to solid (v/v) ratio was adjusted to 10 : 1 at which the dry weight was $\sim$2 mg ml^{-1}, and the pH was adjusted to 5.0 by adding small amounts of NaOH and HCl. Aliquot volumes from the stock solution of the fungicide were added to obtain the desired final concentrations (ranging from 25–1000 ppm) and shaken on an orbital shaker (120 rpm). A preliminary experiment showed that disappearance of the bluish colour of the fungicidal suspension due to the fungal pellets took $\sim$10 minutes. Shaking was maintained for 30 min, 3 ml duplicate samples of the suspension being taken from each flask at time intervals, and the fungal pellets were instantly separated using nylon mesh (100 μm aperture size). The fungal pellets were then washed 2$\times$ with 10 ml ddH_2O and then 2$\times$ with 5 ml 0.1N HCl, and subsequently pooled to determine the adsorbed copper onto the mycelia. Total copper contents in the medium were determined by taking 1 ml samples from the supernatant fluid to which aliquote 1 ml of 0.1N HCl was added to solubilize the fungicide particulates that probably remained in the medium. To separate the soluble from insoluble copper in the medium, 1 ml filtrate samples were further filtered and washed with 2 ml ddH_2O through a cellulose nitrate membrane filter (pore size 0.45 μm; Whatman, Maidstone, UK). The membrane filters were then digested in 0.5 ml conc. HNO_3 by heating at 90 °C for 1 h. After cooling, the volumes of all samples were increased to 10 ml with ddH_2O. The copper contents were analyzed using a Pye Unicam SP9 atomic absorption spectrophotometer (AAS) with reference to appropriate standard solutions of $CuCl_2$ in ddH_2O. Long term experiments were carried out using triplicates of 500 ml 2-day-old growing pellet suspension in 1 l Erlenmeyer conical flasks. An appropriate volume from the stock solution of the fungicide was added aseptically to obtain a final concentration of 125 ppm ai fungicide. Other treatments were also prepared in triplicate to which definite volumes of membrane-filtered (pore size, 0.45 μm) 10 M stock solution $CaCl_2$ were added to obtain final concentrations ranging from 0 to 50 mM $CaCl_2$. A

fungicide-calcium combination as well as fungal-free, but fungicide-containing, control treatments were also prepared. After addition of the fungicide and $CaCl_2$, 10 ml duplicate samples were taken from each flask at time intervals. Fungal pellets were separated using nylon mesh and washed with ddH_2O and 0.1 N HCl, whereas the obtained medium filtrate was then further filtered and washed using a cellulose nitrate membrane filter, as described above. Both the dried mycelia (at 80 °C until attaining a constant weight) and the membrane filters were digested with conc. HNO_3 and diluted with an appropriate volume of ddH_2O, and then the copper and calcium contents were determined with AAS.

Electron microscopy

Mycelial pellets from control and 1000 ppm fungicide treatments after accomplishment of biosorption (15 min) were prepared for scanning electron microscopy (SEM) according to Gharieb et al. (1995). These were fixed in 2% (v/v) glutaraldehyde (SEM grade) in 5 mM 1,4-piperazine diethanesulphonic acid (PIPES) buffer, adjusted to pH 6.5 using solid tetramethylammonium hydroxide for 2 h at room temperature. Fixed pellets were washed four times in 5 mmol L^{-1} PIPES (15 min per wash) prior to dehydration in an ethanol/distilled water series (25%, 50%, 75% and 100% (v/v)), samples being incubated overnight in each stage. Following dehydration, samples were taken through an acetone/ethanol series (20%, 40%, 60%, 80% and 100% acetone (v/v): three transfers being made in absolute acetone. The discs (submerged in acetone) were dried using a Samdri- PVT-3B critical point drying apparatus after exposure for one hour in the transitional fluid (liquid CO_2). Dried samples were mounted on aluminium stubs and sputter-coated with palladium-gold alloy using a JFC-1100E coating unit. Samples were finally examined using a Jeol JSM 3500 scanning electron microscope with an accelerating voltage of 25 kv.

Calcium accumulation and proton exchange

The effect of calcium on cellular proton efflux was carried out as described by Karamushka & Gadd (1994). Two-day-old fungal pellets were harvested by filtration through nylon mesh. The spent medium was discarded and the pellets washed twice and finally resuspended in glucose-free 5 mM PIPES buffer adjusted with tetramethylammonium hydroxide pentahydrate to pH 6.5, and the liquid to solid ratio (v/v) was adjusted to 10 : 1. After 1 h incubation at room temperature, appropriate volumes of $CaCl_2$ from a stock solution were constantly added to the buffer-pellet suspension at intervals of 30 sec to give a final concentration increment of 10 mM, and the pH changes were recorded using a pH-meter (Cole–Parmer) with a glass electrode. A blank titration with the buffer alone was also tested, and the average of three titrations for each treatment was reported. All titrations were carried out at 25 °C. The mycelial dry weight and calcium contents were determined after washing 2× with fresh buffer, with drying and digestion as mentioned above.

Results

During the present study, it was noticed that the addition of the fungicide copper oxychloride to the growth medium resulted in the formation of a bluish green coloured suspension. Simultaneously, the presence of 2-day-old pellets gradually removed such a coloured suspension, thereby making the medium completely clear within few minutes. At the same time, the whitish yellow colour characterizing the fungal mycelial pellets changed to blue. The fungicide concentration was expressed by its adsorbed copper content (CuF), remaining suspended or solubilized in the medium. Figure 1a shows that adsorption of 125 ppm ai fungicide by 2-day-old fungal pellets increased, reaching a maximum of 7.5 ± 0.5 μmol Cu (mg dry wt)$^{-1}$ after 10 min. Within this time, the medium content of total CuF consequently decreased from 12.8 to 0.9 ± 0.6 μmol Cu ml^{-1} whereas the insoluble copper was totally removed. However, 6-day-old pellets (Figure 1b) displayed a much lower biosorptive ability with this fungicide. The maximum adsorbed fungicide by such pellets was not greater than 0.5 ± 0.3 μmol Cu (mg dry wt)$^{-1}$, and the medium insoluble copper detected over the incubation time was 10 ± 0.2 μmol Cu ml^{-1} (Figure 1b). The adsorbed fungicide significantly increased by increasing the concentration of the fungicide initially added, and a sigmoidal pattern of CuF adsorption resulted from the addition of fungicide concentrations ranging from 25 to 500 ai ppm (Figure 2). Furthermore, up to 97 ± 1.0% of 50 ppm ai fungicide was biosorbed and removed within 10 min. The ratio of the removed fungicide decreased with increasing concentration of the added fungicide, reaching 88 ± 1.2% at 500 ppm ai. On the other hand, the amount of the fungicide removed by 6d old culture ranged from 0.5 ± 0.03 to

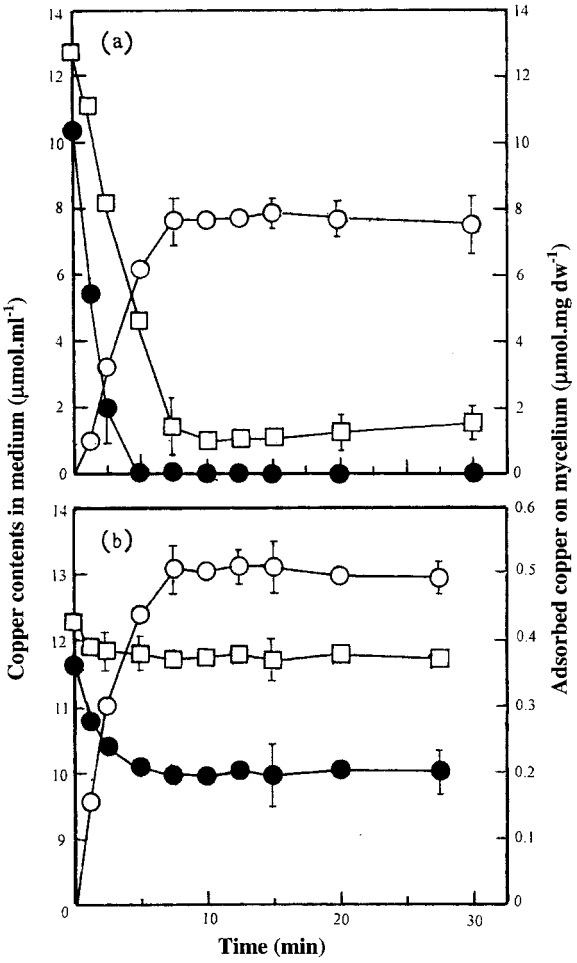

Figure 1. Partition of CuF adsorbed to mycelium (○), insoluble (●), and soluble (□) contents in the medium after the addition of 125 ppm ai fungicide copper oxychloride as an initial concentration to (a) 2-day-old and (b) 6-day-old mycelial pellets of *A. niger* grown on Czapek-Dox liquid medium. Other details as described in methods. Bars indicate standard error of the mean (six replicates) and when not shown were smaller than the symbols.

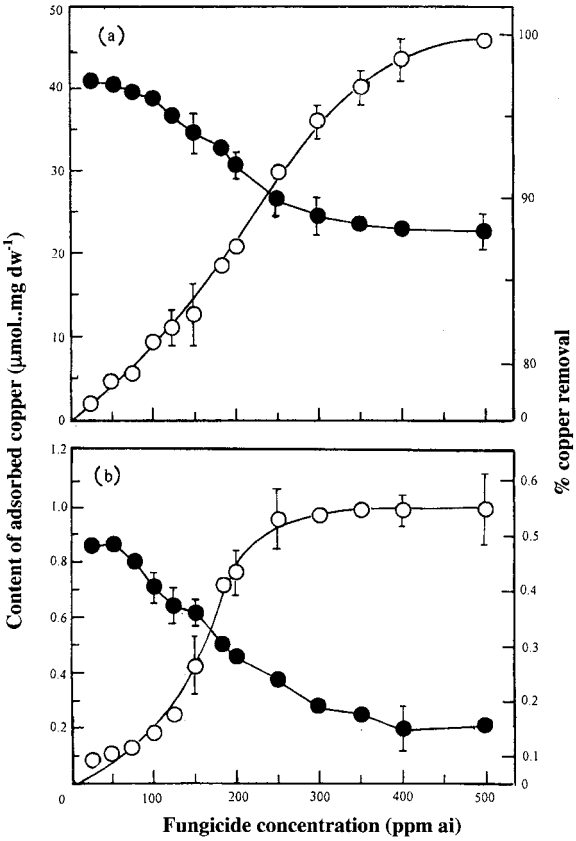

Figure 2. Adsorption (○) and the percentage of removal (●) CuF by (a) 2-day-old and (b) 6-day-old mycelial pellets of *A. niger* grown on Czapek- Dox liquid medium after the addition of different concentrations (ai) of the fungicide copper oxychloride. Other details as in Figure 1.

0.15 ± 0.01% at the same concentrations and conditions used in young pellets (Figure 2b). Scanning electron microscopy examination of 2-day-old fungal pellets challenged with 500 ppm ai fungicide, revealed that particulates were predominantly aggregated onto the fungal mycelia incubated on fungicide-containing medium (Figure 3b,d). Such a particulate aggregation occurred in particular on the hyphal tips rather than on other parts of the mycelium (Figure 3b,d).

The CuF of 125 ppm ai fungicide adsorbed to the cell wall of growing fungal pellets in culture medium displayed a plateau pattern over 24 h incubation time. Figure 4 indicates that beyond the time of maximum biosorption, the copper content on the cell wall gradually decreased from 7.5 ± 0.5 to a minimum of 2.1 ± 0.1 μmol Cu (mg dry wt)$^{-1}$ during the first 12 h incubation. It is worth mentioning that the estimated insoluble CuF portion decreased to zero after 10 minutes incubation as shown in Figure 1. It was also noticed that during the subsequent incubation time the colour of the medium changed from colourless to clear bright blue. Figure 4 indicates that the soluble CuF portion increased from 0.9 ± 0.6 to 4.9 ± 0.2 μmol Cu ml^{-1} at 10 min and 12 h incubation respectively. The presence of 50 mM $CaCl_2$ greatly changed both the cellular adsorbed and medium soluble CuF to 3.5 ± 0.5 μmol Cu (mg dry wt)$^{-1}$ and 5.9 ± 0.8 μmol Cu ml^{-1}, respectively, after 15 minutes incubation (Figure 4). The adsorbed and soluble CuF in the presence of calcium after 12 h incubation were also changed to 1.7 ± 0.1 μmol Cu (mg dry wt)$^{-1}$ and

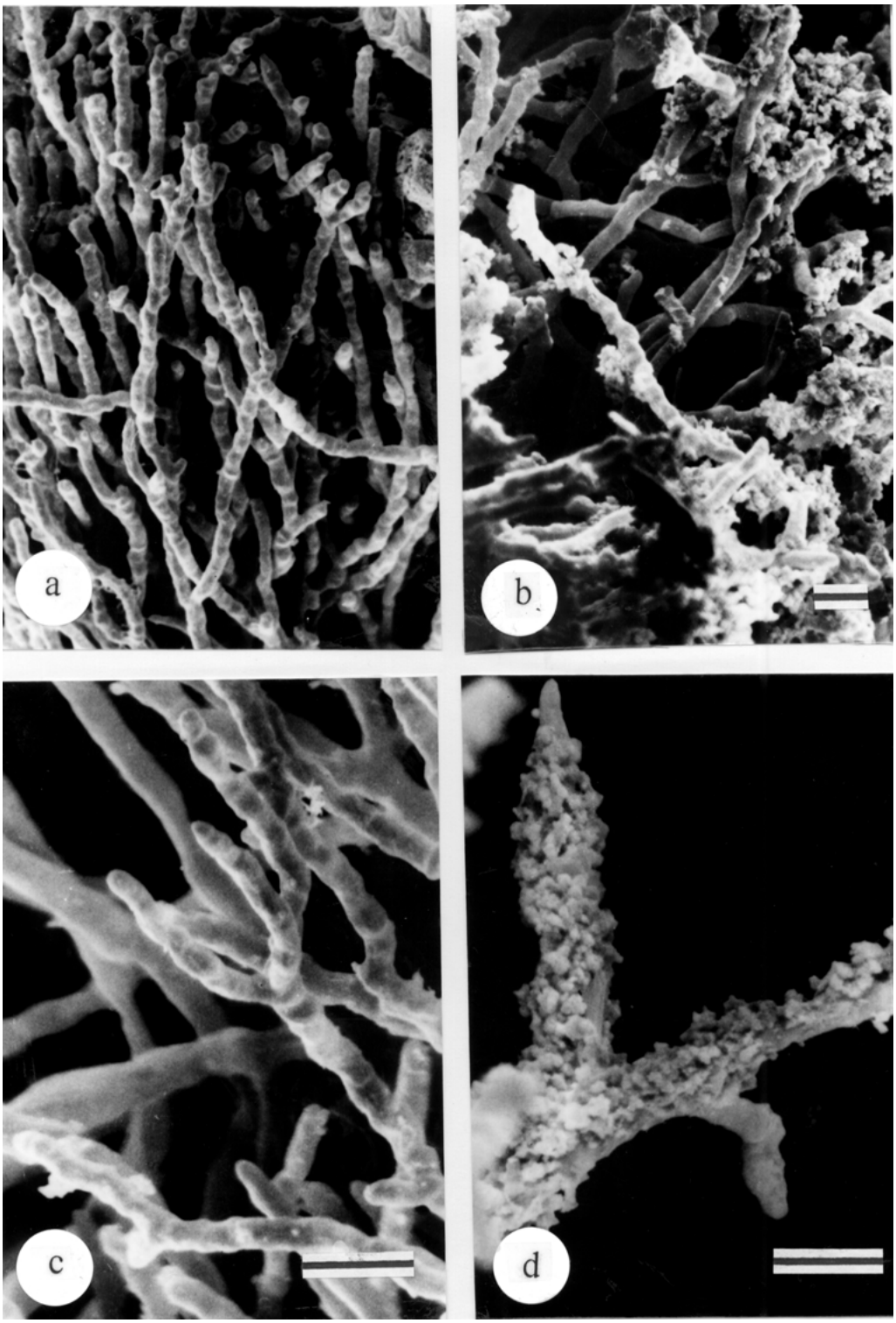

Figure 3. Scanning electron micrographs of 2-day-old mycelial pellets of *A. niger* grown on Czapek-Dox medium and shaken for 15 min in (a,c) control and (b,d) presence of 500 ai ppm fungicide copper oxychloride. It shows accumulation of the fungicide particulates on the fungal hyphal tips (b, d). Micrographs shown are representative of at least 15 replicates. Bars = 10 μm.

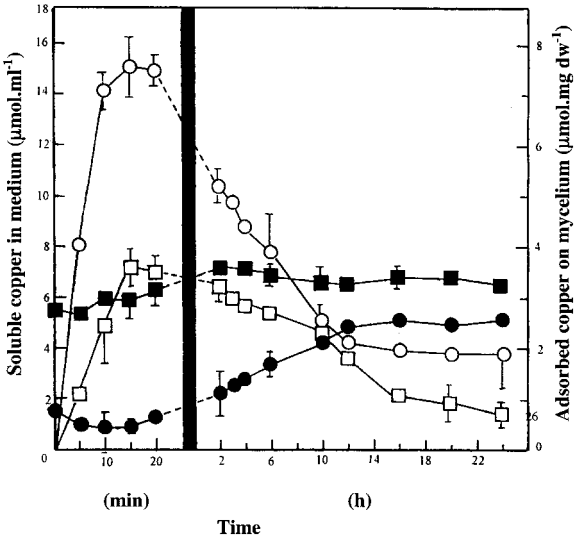

Figure 4. Adsorbed CuF to growing mycelia (○, □), and soluble copper content in the medium (●, ■) after the addition of 125 ppm'ai fungicide copper oxychloride as an initial concentration to 2-day-old mycelial pellets of *A. niger* grown on Czapek-Dox liquid medium and incubated for a further 24 h in absence (○, ●) and presence (□, ■) of 50 mM $CaCl_2$. Other details as Figure 1.

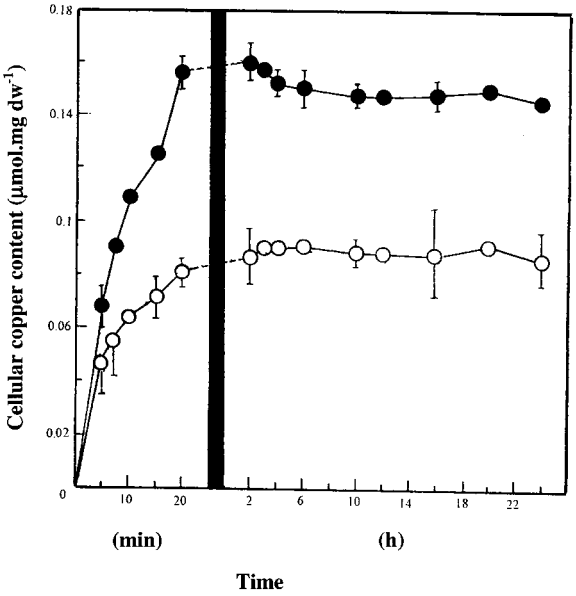

Figure 5. Pattern of cellular copper content after the addition of 125 ppm ai fungicide copper oxychloride as an initial concentration to 2-day-old culture of *A. niger* grown on Czapek-Dox liquid medium and incubated for a further 24 h in absence (○) and presence (●) of 50 mM $CaCl_2$. Other details as described in methods and Figure 1.

6.8 ± 0.2 μmol Cu ml^{-1}, respectively. Initial cellular uptake of copper of the fungicide exhibited two phases (Figure 5), of which the first phase was rapid and lasted 5 minutes after the addition of the fungicide. In the absence of calcium, the second phase was slow attaining a maximum copper content of 0.08 ± 0.01 μmol Cu (mg dry wt)$^{-1}$ after 2 h. However, in the presence of calcium, the copper uptake during the second phase was significantly higher, and the cellular copper content increased to 0.16 ± 0.007 μmol Cu (mg dry wt)$^{-1}$ after 2 h incubation. The dry weight of the fungal mycelial pellets after a further 5 days growth in the presence of elevated concentrations of calcium chloride with 125 ppm fungicide, exhibited a significant increase in the fungicidal toxicity with increasing calcium concentration as shown in Figure 6.

Although the adsorption of 125 ppm ai fungicide caused an initial increase in Czapek-Dox medium, pH 4.4 to 4.8, growth of the fungus in the presence of the fungicide for a further 5 days incubation resulted in decreasing the medium pH to 3.2 (data not shown). However, it was desirable to find out whether the changes in partitioning of copper content observed by calcium addition were a response to change in the medium proton content. Figure 7 reflects the accumulation of calcium by glucose-starved mycelial pellets suspended in 5 mM PIPES buffer (pH 6.5), and the in-

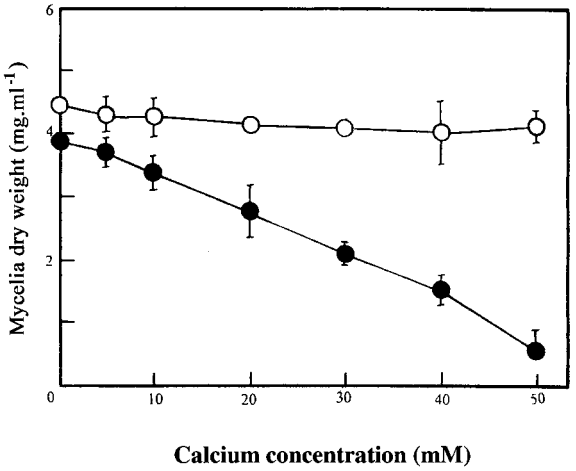

Figure 6. Mycelial dry weight of *A. niger* grown for a further 5 days, after the addition of elevated concentrations of calcium chloride to 2-day-old growing culture on Czapek Dox medium in absence (○) and presence (●) of 125 ai fungicide copper oxychloride. Other details as in Figure 1.

stant response of the suspension pH value. It shows the ability of the cells to accumulate calcium, which was significantly increased by increasing the exogenously added calcium. There was a simultaneous marked increase in $[H^+]$ from 0.8 ± 0.3 to 1.8 ± 0.2 μM caused by increasing the amended calcium from 0.0 to 100 mM $CaCl_2$, respectively. On the other hand, Figure 7 also shows that the addition of elevated concentrations of calcium had no influence on $[H^+]$ of the mycelial pellets-free buffer.

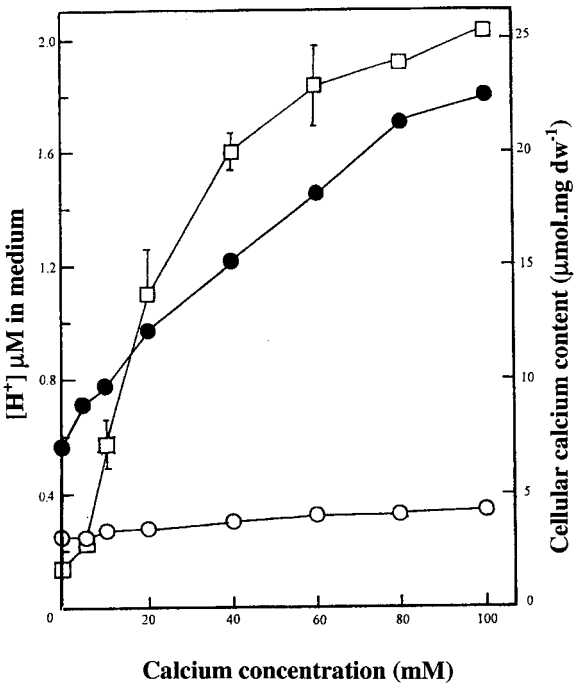

Figure 7. Instant changes in $[H^+]$ after the addition of different concentrations of $CaCl_2$ to 5 mM PIPES buffer (pH 6.5) in absence (○) and presence (●) of 2-day-old mycelial pellets of *A. niger*, and the calcium content accumulated by the fungal cells (□). Other details as in Figure 1.

Discussion

Adsorption of certain insoluble metal compounds by fungi was previously reported. Wainwright & Grayston (1989) reported that mycelial spheres of *A. niger* can adsorb and oxidize the sulphides of copper, cadmium, zinc and lead. Scanning electron microscopy of *Aspergillus funiculosus* and *Fusarium* sp. incubated with 50 mM sodium selenite showed the presence of needle-like crystals of elemental selenium on the surface of hyphae and conidia (Gharieb et al. 1995). Uranium and thorium were deposited as a layer of needle-like fibrils on cell walls of *Rhizopus arrhizus* and *Saccharomyces cerevisiae* (Tsezos & Volesky 1982; Tsezos 1986). Biosorption of soluble heavy metals was generally found to be accomplished by living or dead fungal biomass through metabolism-dependent and metabolism-independent processes, which may be affected by changes in the physical and chemical characteristics of the organism or suspending medium (White & Gadd 1990). On the other hand, adsorption of insoluble particulates was reported to be a property of the fungal cell wall and occurs independently of cellular metabolism (Wainwright 1992). The present study, however, illustrated that the hyphal tips of 2-day-old pellets represent the effective mycelium portion in adsorption and entrapment of the fungicide particulates. Changes in pellet texture, physiological condition of the mycelia or the medium chemical constituent may affect the availability and activity of such a hyphal portion. Therefore, low fungicide biosorption capacity of 6-day-old pellets might be due to alteration in the growth condition, deserving further study in the future. Generally, the process of fungicide biosorption by fungal mycelia could be considered to be a fungal growth-dependence and influenced by the physiological state of the organism. The sigmoidal expression resulted from fungicide adsorption by the fungal cell wall (Figure 2), suggesting that the initial binding of the fungicide has brought about structural changes and an increase in the availability of binding sites, as described by Hughes & Poole (1989), whereas decreasing the percentage of removed fungicide by increasing its concentration indicates, saturation of the receptive sites on the cell wall.

In a preliminary experiment, it was found that the addition of elevated concentrations of HCl in wells in agar plates containing this fungicide resulted in clear extended zones of solubilization. Solubilization of the fungicide copper oxychloride was also previously reported to be mediated by dilute acids (Tomlin 1995). This process is accomplished by protonation and by making the anion less available to the copper ions (Hughes & Poole, 1991). In a biological system, protons can be pumped into the external medium by the proton translocating ATP_{ase} of the plasma membrane (Slayman et al. 1990; Morley et al. 1996) and/or organic acid production such as citric acid, which is a major product of *A. niger* grown in liquid medium (Kubicek & Rohr 1986). On the other hand, Franz et al. (1991) reported that the adsorption of insoluble

metal oxides onto the mycelial pellets of *Penicillium simplicissimum* was required for citric acid production and solubilization. Additionally, oxidation and solubilization of metal sulphides and release of free metals by *A. niger and Trichoderma harzianum* was linked to the ability of the fungal hyphae to adsorb these compounds (Wainwright & Grayston 1989). Therefore, the present results suggest induced-excretion of acidic complexing agents (possibly citric acid) by fungicide adsorption onto the fungal cells and solubilization of the fungicide including the adsorbed particulates. However, in addition to providing protons, organic acid is usually capable of forming a complex with the metal cation, thus affecting its mobility and toxicity (Gadd & White 1989; Sayer & Gadd 1997; Gharieb & Gadd 1999). Moreover, resistance of *A. niger* and other soil fungi to a high concentration of the fungicide copper oxychloride (up to 1000 ppm), by the formation of either organic soluble complexes in liquid medium or insoluble copper oxalate in solid medium, has been evidenced (unpublished data). Therefore, adsorption of the fungicide copper oxychloride onto the fungal mycelia might be an active process necessary for copper tolerance.

The present investigation also showed that the addition of calcium with the fungicide significantly increased fungicide solubilization, the cellular content of copper and the fungicidal toxicity (Figures 4, 5 and 6). In a previous work, Gharieb (1999) found that the presence of exogenous calcium with soluble copper resulted in an immobilization of oxalate inside the fungal cells and exertion of higher copper toxicity. Additionally, Karamushka & Gadd (1994) reported that calcium completely removed the inhibitory effect of copper on glucose-dependent H^+ efflux from *S. cerevisiae*. Therefore, the present results suggest implication of proton efflux rather than organic acids excretion by the fungal cells in response to calcium amendment. Figure 7 illustrated that although the fungal cells were glucose-starved and there were no metabolizable substrates, the addition of calcium increased both the medium $[H^+]$ and the cellular content of calcium. So it could be assumed that increasing the availability of protons by calcium could be *via* Ca^{2+}/H^+ exchange capacity of the fungal cell in addition to the other probable mechanisms including activation of H^+-ATP_{ase}. These responses would lead to increasing solubilization of the fungicide and subsequently releasing free copper ions available for cellular uptake. Alteration of the medium pH by the presence of calcium than that possibly favours the fungicide adsorption and tolerance might also be implicated in increasing the fungicidal toxicity by calcium.

In conclusion, such a finding provides further understanding of the influence of calcium in toxicity of copper-containing insoluble fungicide which may have potential agricultural application. Additionally, this work shows a high ability of fungal mycelia to adsorb particulates of metal-containing fungicides, an aspect which is of environmental and biotechnological significance. However, despite this feasibility, biosorption and removal of metal-containing pesticides can be more suitable using chemicals that do not generate toxic metals. In order for the microbial biosorption process to be applicable, further study of the proper conditions is needed as well as exploration of proper fungal strains which favoured such a process without solubilization and release of hazardous metal ions into solution.

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