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Interactions between a soil fungus, *Trichoderma harzianum*, and IIb metals—adsorption to mycelium and production of complexing metabolites

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Fungi are capable of accumulating metals and, in soil, such accumulation may influence metal speciation and transport. The interactions between a common soil fungus, *Trichoderma harzianum*, and IIb elements were studied in the present investigation. The accumulation of the metals zinc, cadmium and mercury by starved and non-starved mycelium at different pH was determined by a batch technique using radioactive tracers; uptake of the metals was found to be large, with respective distribution coefficients of about $10^{3.5}$, $10^{2.5}$ and $10^{4.0}$ for zinc, cadmium and mercury, respectively. Metal accumulation by a starved system was largely independent of pH in the range 3–9, where in a non-starved system an increased accumulation of zinc (at 10^{-8} M) was observed at low pH (3–5). Potentiometric titrations performed on the two systems revealed significant differences in acid capacities, i.e. values close to zero for the starved system and 500–800 meq kg^{-1} for the non-starved system. The maximum metal uptake was at least 50 mmol kg^{-1} at pH 6.5 (calculated from adsorption isotherms). The present findings suggests that in the non-starved system a metabolite is produced and then released when the pH is within a certain range.

Keywords: adsorption, complexes, *Trichoderma harzianum*, IIb metals

Introduction

Fungi are capable of accumulating significant amounts of metals from their external environment (Gadd & Griffiths 1978, Shumate & Strandberg 1985, Townsley 1985, Gadd 1986a, b, Trevors *et al.* 1986). For example, *Rhizopus arrhizus* mycelium has been found to accumulate a large number of different metal ions in amounts ranging from only a few to as much as 20% of the mycelial dry weight (Tobin *et al.* 1984). This is comparable to the capacity of conventional ion exchangers and much higher than the capacities of anionic abiotic materials (Allard *et al.* 1983). Considering a surface/mass ratio, the metal-accumulating capacity of a fungal mycelium will actually be higher than the capacities of conventional ion exchangers. This knowledge has led to extensive research on the possibility of using fungal mycelia as biosorbents to decontaminate metal-polluted waters and to recover valuable metals from natural or industrial waters (Galun *et al.* 1987, 1988, Kuyucak & Volesky 1988, Huang *et al.* 1990, Siegel *et al.* 1990). However, little attention has been focused on the effects of fungi on the speciation and transport of metals in soil, one of the natural habitats of these organisms. In soil the distribution of metals is determined by their interactions with complexing agents in the water phase as well as with inorganic and organic solid surfaces. Since fungal mycelia can constitute a significant pool of organic surfaces (up to 0.14 $m^2 g^{-1}$ soil, as calculated from Söderström 1979) with a high capacity for metal accumulation, it seems likely that fungi can affect the overall mobility of metals in soil systems.

A variety of microbial processes may lead to the removal of metals from soil solutions. These processes include purely chemical or physical adsorption to

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cell walls and other constituents as well as mechanisms related to metabolism, e.g. transport, internal compartmentalization and extracellular precipitation or solubilization by excreted metabolites. Metal uptake by microorganisms is often divided into two main phases. The first, which is usually rapid and can occur in both living and dead cells, can be described as a metabolism-independent binding (or adsorption) to cell walls and other external surfaces. The second phase involves a slower metabolism-dependent transport across the cell membrane (for a review, see Gadd 1988). In many cases the observed uptake is due solely to surface accumulation processes, i.e. the much slower process of intracellular uptake is of little quantitative significance.

The cell walls of fungi are composed of polysaccharides, proteins and lipids (Farkas 1985) which contain a number of functional groups with potential chemical–metal complexing capacities. The chemical composition of fungal cell walls can, however, vary considerably between species, and it is therefore not surprising that significant differences in uptake of metals have been found between different species and strains and even between cell types within a single organism (Somers 1963, Mowll & Gadd 1983).

In addition to their cell wall constituents fungi also produce extracellular compounds with complexing abilities, and such compounds can affect the speciation and mobility of metals; oxalic and citric acid are examples of these compounds (Gadd 1986a). Organic complex formation can also occur when metals are detoxified, e.g. through the production of metallothioneins and metallothionein-like compounds (Higham *et al.* 1984, Olafson 1986, Harwood-Sears & Gordon 1990).

The aim of the present investigation was to study interactions between IIb elements (zinc, cadmium and mercury) and a common soil fungus, *Trichoderma harzianum*. Cadmium and mercury are both toxic and are the cause of environmental concern; zinc is an analogous element (with a smaller ionic radius) within the same group (IIb). *T. harzianum* is found worldwide in many different types of soil. In most earlier studies on the accumulation of metals by microorganisms, metal concentrations far above natural levels were used, but in the present study, experiments were performed using concentrations representative of natural systems. The adsorption of metals is a pH-dependent process, thus we chose to use a pH range coinciding with the expected natural interval. A batch distribution technique using radioisotopes was selected to determine metal accumulation, since the method allows measurements at very low and controlled total metal concentrations. The distribution studies were performed on fungal mycelium under nutrient stress, i.e. the condition normally prevailing in natural environments. Two stages of fungal activity were considered in the experiments: one where the fungus was starved for 2 days and one using mycelia just taken from the culture medium.

Materials and methods

Growth and preparation of fungal mycelium

Stock cultures of *T. harzianum* (J 65, from the Swedish University of Agricultural Sciences, Department of Microbiology, Uppsala, Sweden) growing on 4% malt extract agar slants (Oxoid, UK) were stored at 4 °C and transferred to fresh agar slants at 3 month intervals. Prior to experiments, spores of fungus grown for 14 days on 4% malt extract agar, were suspended in a freeze medium (in g l^{-1}; KH_2PO_4, 0.18 g; K_2HPO_4, 0.82 g; sodium citrate, 0.59 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; glycerol (87%) 172 ml) in 1 ml portions and frozen at −70 °C; these suspensions were used as a standardized inocula with 4×10^6 spores ml^{-1}. Then 1 ml of the fungal spore suspension was inoculated in 100 ml of a non-chelating glucose-salt medium (Townsley 1985) with the following composition (in g l^{-1}): magnesium glycerophosphate ($C_3H_5(OH)_2PO_4 Mg \cdot 2H_2O$), 0.5; $(NH_4)_2SO_4$, 2.0; KCl, 0.5; $CaCl_2 \cdot 6H_2O$, 0.25; 2(-*N*-Morpholino)-ethanesulphonic acid (MES buffer), 9.76; glucose, 20.0. Trace metals were supplemented by adding 0.1 ml of a solution containing (in g l^{-1}): $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 1.75; $MnSO_4 \cdot H_2O$, 0.076; $CuSO_4 \cdot 5H_2O$, 0.1. The pH was adjusted to 5.5 with KOH or HCl. The inoculated media were incubated at 25 °C with continuous shaking for 72 h.

The mycelia were harvested when in the late logarithmic phase; this was accomplished by centrifugation (15000 × *g* for 10 min at 4 °C) followed by three washings in sterile 0.1 M KCl. Experiments were performed on mycelium immediately after the washing procedure and on mycelium starved for 2 days. Prior to experiments, the latter had been subjected to washing as described and then resuspended in 0.1 M KCl and incubated at 25 °C with continuous shaking for 2 days without the addition of nutrients; this mycelium was harvested by centrifugation and washed two times with 0.1 M KCl. All biomass expressed as dry weight.

Adsorption studies

The uptake of zinc, cadmium and mercury was studied by a radio-tracer batch-distribution technique using ^{65}Zn, ^{109}Cd and ^{203}Hg (from Amersham, UK; all as chlorides), with non-radioactive carriers of the same elements added to achieve the desired total concentrations. The chosen concentrations of the metals were below the solubility products of any possible sparingly soluble metal species at the pH interval in question and also below levels that could

affect the fungal activity. The distribution studies were performed in 50 ml polyallomer centrifuge tubes containing a total sample volume of 20 ml. All equipment was cleaned by soaking in HNO_3:H_2O (1:3; v/v) overnight and then rinsing eight times in Milli-Q water. Solutions of each fungal mycelium (both starved and non-starved, dry weight 1.5–4 mg) were added to an electrolyte solution (0.1 M KCl) to give a final solid/solution ratio of 0.075–0.2 $g l^{-1}$. Portions of acid (0.1 M HCl) or base (0.1 M KOH) were added to obtain different preselected pH values (NB: buffers were not added to achieve rigorous control of pH). An acidic metal stock solution was added to give a final metal concentration in the range of 10^{-6} to 10^{-8} mol l^{-1}. The final volumes were adjusted by adding 0.1 M KCl. After a contact time of 19 h (on a rotary shaker of 120 r.p.m. and room temperature), the mycelia were removed by centrifugation (15 000 × *g* for 10 min) and the pH was measured. Aliquots (1 ml) of the supernatant were sampled and analyzed for their metal content by using a well-type scintillation counter (Compu Gamma, LKB Products, Bromma, Sweden). The pellets (fungal mycelium) were harvested and radioactivity measured repeatedly to assure that metals were accumulated in the fungal fraction and not on the vessel walls. Each set of experiments was repeated at least two times, with three parallel samples in each experiment.

Potentiometric titrations

About 50–100 mg of mycelium (dry weight, estimated after each titration) was suspended in 0.1 M KCl to a final volume of 50 ml. The initial pH was adjusted to about 3.5 with 0.100 M HCl. Portions (0.05 ml) of 0.100 M KOH were added and the pH was measured after each addition.

Total organic carbon (TOC) measurements

Total carbon (TC) and inorganic carbon (IC) were measured in the 0.1 M KCl supernatants after the adsorption experiments (Shimadzu Total Carbon Analyzer; TOC-5000, Japan), and TOC was calculated as the difference between the values.

Chemicals

All reagents used were of analytical reagent grade. Water of Milli-Q quality, obtained from a Millipore reagent-grade water system, was used in all solutions. All stock solutions (containing both radionuclides and carriers) were kept at pH 1 (HCl medium) to eliminate hydrolysis and losses due to adsorption on the vessel walls.

Results

Accumulation of metals by a starved fungal mycelium

Metal uptake, expressed as the distribution coefficient (K_d; defined as the ratio of concentrations; in mol kg^{-1} per mol l^{-1}; see Benes & Majer, 1980) is illustrated in Figure 1(a–c). In the neutral pH range accumulation of zinc, cadmium and mercury corresponded to distribution coefficients ($\log K_d$) of 3.5 ± 0.5, 2.5 ± 0.5 and 4.0 ± 0.5, respectively. In the pH range 3–9, metal uptake was largely unaffected by pH. The distribution coefficients were of the same magnitude within the metal concentration range 10^{-8} to 10^{-6} M.

The metal loading of the mycelia continued to increase with increasing concentrations of metals, which shows that the metal-accumulation maxima of the mycelia were not reached, even at the highest initial concentration (10^{-4} mol l^{-1}) in the aqueous phase. However, an uptake of at least 50 mmol kg^{-1} would generally be expected, and a maximum uptake of about 100 mmol kg^{-1} was observed (see Figure 2).

Accumulation of metals by a non-starved fungal mycelium

The accumulation of zinc in non-starved mycelium as compared to the starved system was enhanced in the pH interval 3–5, giving a log K_d value of almost 7 (see Figure 1a). At pH values above 5, the uptake appeared to be slightly below the level for the starved mycelium, at least for zinc and mercury. This effect was, however, only obtained for the lowest concentration (10^{-8} mol l^{-1}). No significant enhanced adsorption (in the pH range 3–5) was observed for cadmium, but the data for this pH range were scattered and precise pH adjustments were difficult to achieve in the absence of any pH buffer.

Potentiometric titrations

Acid capacity (C, meq kg^{-1}) was calculated using the formula $C = (M_b V_{beq} - M_a V_a)/m$, where M_b is the molarity of the base, M_a is the molarity of the acid, V_{beq} is the volume of base at the point of equivalence, V_a is the volume of acid added and m is the mass of the mycelium. The titrations revealed that the non-starved system had a total capacity of 500–800 meq kg^{-1} fungal mycelium (dry weight; see Figure 3) whereas no such capacity was exhibited by the starved system. At pH about 4, 6 and 8 three equivalence points could be distinguished in the non-starved mycelium (assessed by a calculation program designed for organic polyelectrolyte macromolecules; Ephraim *et al.* 1986).

Surface charge expressed as the excess base (A^-) in the system was calculated according to $A^- = (M_b V_b - M_a V_a + h V_s/\gamma)/m$, where V_b is the volume of base, h is the hydrogen ion concentration,

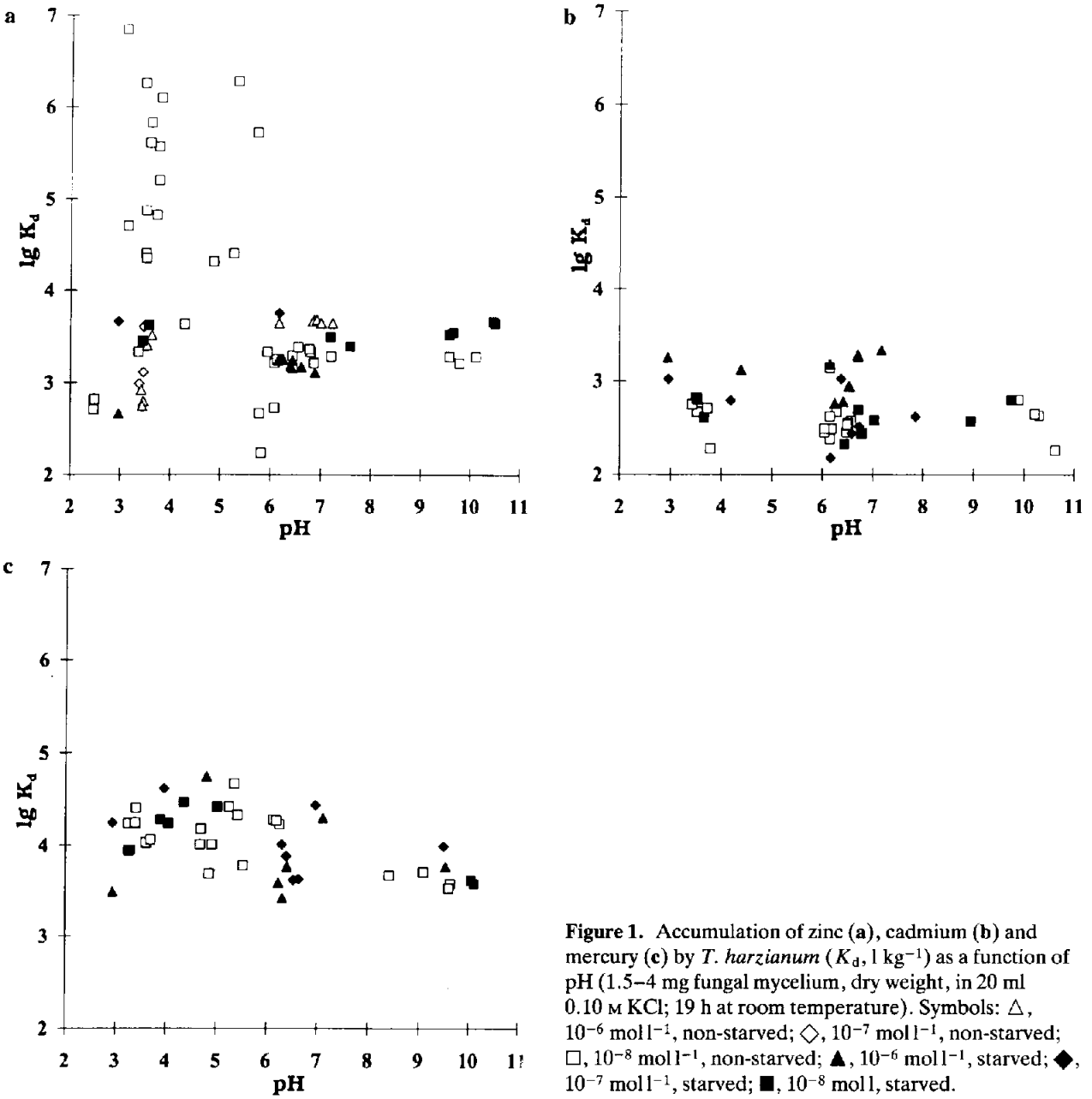

Figure 1. Accumulation of zinc (**a**), cadmium (**b**) and mercury (**c**) by *T. harzianum* (K_d, l kg^{-1}) as a function of pH (1.5–4 mg fungal mycelium, dry weight, in 20 ml 0.10 M KCl; 19 h at room temperature). Symbols: △, 10^{-6} mol l^{-1}, non-starved; ◇, 10^{-7} mol l^{-1}, non-starved; □, 10^{-8} mol l^{-1}, non-starved; ▲, 10^{-6} mol l^{-1}, starved; ◆, 10^{-7} mol l^{-1}, starved; ■, 10^{-8} mol l, starved.

V_s is the total volume of the solution and γ is the activity coefficient (Ephraim *et al.* 1986). This calculation shows (Figure 4) that the surface of the non-starved mycelium was negatively charged in the pH range 3.5–9.5. For the starved system, however, the surface charge changed with increasing pH, from being negative to positive at pH 3.5 and *vice versa* at pH 7.

Discussion

The starved mycelium of the fungus *T. harzianum* exhibited significant metal binding, with a total capacity of at least 50 mmol kg^{-1} and with high metal affinities, indicated by distribution coefficients of about $10^{3.5}$, $10^{2.5}$ and $10^{4.0}$ l kg^{-1} for zinc, cadmium and mercury, respectively. The metal accumulation was largely independent of pH in the range 3–9. This result is surprising since the complexing of metals by organic systems, such as humic acids, decreases at low pH (i.e. close to or below pK_a). In general low pH also decreases any adsorption process in biological systems, although there are exceptions to this. Galun *et al.* (1987) found that the uptake of Cu^{2+} and UO_2^{2+} ions, by a *Penicillium* biomass was virtually insensitive to pH; a neutral

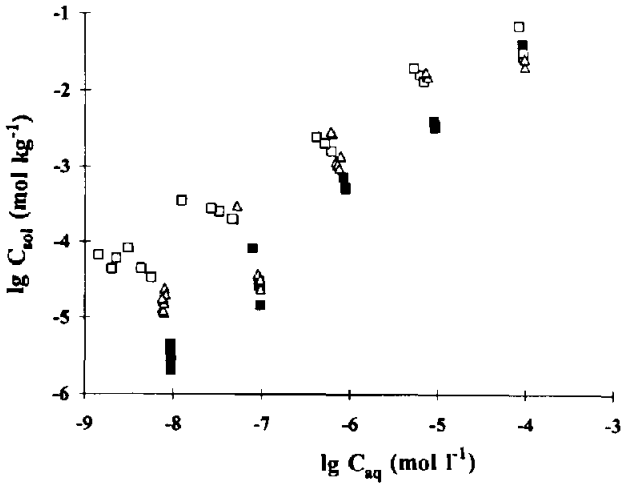

Figure 2. Adsorption isotherms for the accumulation of zinc, cadmium and mercury by *T. harzianum* (1.5–4 mg fungal mycelium, dry weight, in 20 ml 0.10 M KCl; 19 h at room temperature and pH 6.5). △ zinc; ■, cadmium; □, mercury.

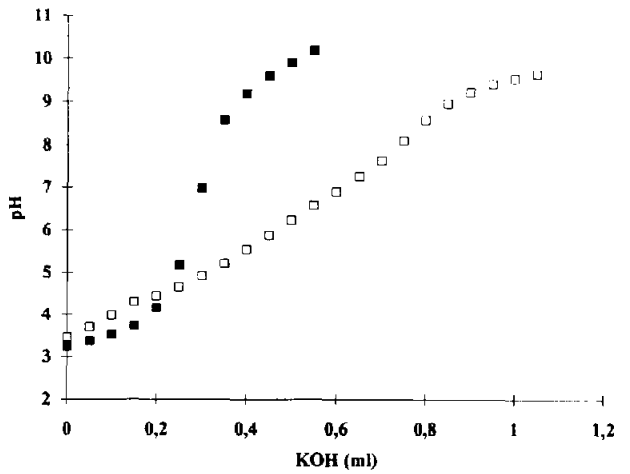

Figure 3. Potentiometric titrations of starved (■) and non-starved (□) *T. harzianum* (50–100 mg fungal mycelium, dry weight, in 50 ml 0.10 M KCl; titrated with 0.100 M KOH and 0.100 M HCl).

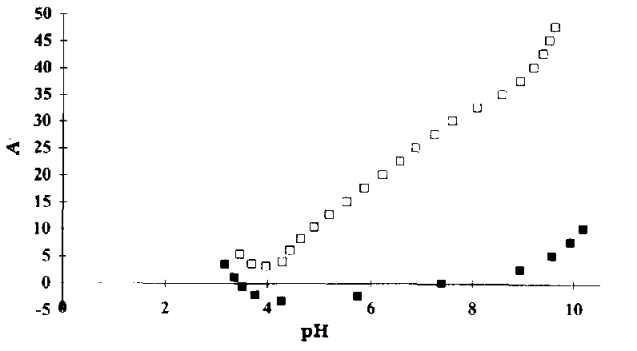

Figure 4. Surface charge of starved (■) and non-starved (□) *T. harzianum*, as a function of pH, calculated according to the formula $(A^- = M_b V_b - M_a V_a + hV_s/\gamma)/m$.

ligand was suggested to be involved in this uptake. Considering europium uptake by a bacterial system (*Klebsiella oxytoca*), it was shown to be considerable at pH 4–7, whereas desorption occured at pHs above 7, which was suggested to be due to the release of a metal-complexing exudate from the bacterial surface at high pH (Wallberg *et al.* 1991).

The metal uptake did not directly reflect the size of the metal ions (radii at coordination number 6 of 0.75, 0.95 and 1.02 Å for zinc, cadmium and mercury, respectively; Jesson *et al.* 1969). Galun *et al.* (1987) have previously reported a decrease in metal-uptake capacity with increasing ionic radius. If that does occur, in the present study the larger ionic radius of cadmium, as compared with zinc, could have been responsible for the lower level of cadmium accumulation, i.e. larger size could have resulted in, for example, a steric hindrance of the formation of a chelate. This could not, however, have been the case for mercury, the largest ion included in our experiments. In contrast other studies of fungal metal accumulation have reported an increase in metal binding with increasing ionic radius (Shumate & Strandberg 1978, Strandberg *et al.* 1981). Obviously there are different mechanisms involved in the accumulation process of the three studied metals.

Complexation of a metal ion, M^{n+}, can be expressed as

$$M^{n+} + R\text{–}H = R\text{–}M^{(n-1)+} + H^+;\ k$$

where R–H denotes an acidic complexing functional group and k the formation constant. It is evident that the present fungal system contains more than one type of complexing sites (not necessarily acidic). The assumed complexation can be described by conditional stability constants (at constant pH) corresponding to the reactions

$$M^{n+} + R^- = R\text{–}M^{(n-1)};\ \beta^*$$

$$R\text{–}H = H^+ + R^-;\ k_a$$

where $k = k_a\beta^*$, and β^* and k_a are the formation constants. Thus, the conditional stability constant β^* is merely a function of the observed K_d value according to

$$\beta^* = K_d/[R^-].$$

When loading is low the number of available sites, expressed as $[R^-]$, would be constant. Thus, the differences between K_d values are equal to the differences between the corresponding β^* values. Conditional formation constants ($\log \beta^*$) of 4.6, 3.6

and 5.4 for zinc, cadmium and mercury, respectively, are obtained at pH 6.5, assuming a capacity of 50 mmol kg^{-1} solid mycelium. These values (as well as the differences between them) are roughly in the same range as the formation constants of complexes formed between the metals (zinc, cadmium and mercury) and well-defined di- and tricarboxylic acids (see, e.g. Perrin 1979).

The use of more sophisticated models for the description of the surface binding, e.g. introduction of corrections for electrostatic interactions of the charged surface with the ions, assumptions of several protonation steps, etc. (see, e.g. Xue *et al.* 1988), could be done. This is probably of no use in the present system, since it is difficult to control the metabolic activity that is inducing the formation and subsequent release of complexing exudates.

In the non-starved mycelium, an enhanced accumulation of zinc, in particular, was observed at pH 3–5. Such an increase at low pH was seen only at the lowest concentration of zinc (and possibly mercury), 10^{-8} mol l^{-1}. This indicates that the mechanism behind the increased accumulation is close to the limit of its metal binding capacity at that concentration. A similarly enhanced accumulation of cadmium at low pH was not observed. However, there are only a few measurements (for all the systems) in the critical pH range of 3–5, which was difficult to maintain in the absence of buffering agents. These results indicate a weak but significant capacity for selective uptake by an agent that is present in the non-starved system. The results of the potentiometric titrations also indicate large differences between a starved and non-starved mycelium.

There are no obvious explanations to the observed increased uptake at low pH. Differences in cell wall composition between starved and non-starved fungi are not likely to account for the described phenomena, since the growth conditions (pre-starvation) were identical. However, in non-starved mycelium, the observed adsorption behavior could have been due to the presence of a compound which was attached to the fungal cell surface in the pH range 3–5. Measurements of TOC and UV spectra of the KCl solution (i.e. the solution to which the fungal mycelium was transferred after separation from the growth medium) show that the fungus produced organic compounds that were released into the surrounding solution. Hence the marked decrease in adsorption at pH values above 5 could have been due to the release of compounds from the cell surface. The fact that the accumulation was below the level observed in the starved mycelium system (at pH values above 5) supports the hypothesis that a metal-complexing compound was actually released from the cell surface at high pH. This agrees with TOC measurements of the supernatant from adsorption experiments with zinc, where a 25% reduction of TOC at low pH values (i.e. an increased zinc accumulation) was observed, as compared to references without zinc, and no reduction in samples at pH values greater than 5. Whether this production and release of organic compounds is related to a mechanism of active transport (with preference for zinc, which is an essential element in contrast to cadmium and mercury) or merely a release of cell wall components, intracellular leakage, etc., needs to be further investigated.

Production of siderophores under a condition of iron defiency has recently been reported for a number of *Trichoderma* strains as well as other fungi (Anke *et al.* 1991, Mor *et al.* 1992). The siderophores are strong complexing agents for iron and possibly also other transition metals. The growth medium for *T. harzianum* in the present study contained enough iron that a siderophore production is not expected, but it can of course not be entirely excluded.

The production of metabolites under nutrient stress has previously been suggested to be important in natural leaching processes (Schinner *et al.* 1989). It is also known that microorganisms survive in the presence of elevated levels of metals, and that they do so by using complexing compounds to detoxify the metals and thereby reduce the amounts of free metals in solution. One such organism is the marine bacterium *Vibrio alginolyctis*, which has been shown to produce a copper-binding protein (Harwood-Sears *et al.* 1990).

Conclusions

The fungus *T. harzianum* is capable of significant accumulation of the studied metals, in the order mercury > zinc > cadmium; the maximum capacity is at least 50 mmol kg^{-1}. This ability is especially remarkable at low pH. The pH dependency of the adsorption observed in the fungus in no way resembles that seen in abiotic systems. This indicates that fungi may in fact play a significant role for the speciation and distribution of metals in soil system, particularly under acidic conditions. The possible generation of complexing compounds that can be released at high pH, and possibly account for the unexpected selective uptake of zinc at low pH, would be of importance, at least for the local micro-environment. Further studies of the exudates from *T. harzianum* are in progress.

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