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Pattern of cadmium accumulation and essential cations during growth of cadmium-tolerant fungi

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Abstract

The present study evaluates the growth response of two strains of filamentous fungi; a *Fusarium* sp. and *Alternaria tenuis*, grown on both solid and liquid Czapek Dox medium amended with different concentrations of $CdCl_2$. Colony extension and the mycelial dry weight of both fungi were significantly inhibited by high concentrations of cadmium. Extended lag phases and low growth rates resulted from cadmium administration. Cadmium drastically affected fungal morphogenesis by the production of stunted sterile thick mycelial filaments of the *Fusarium* sp. and chains of uncharacterized swellings instead of conidia in *A. tenuis*. Experiments showed that cadmium accumulation by the *Fusarium* sp. grown in liquid medium was a concentration dependent, and over the incubation time it displayed a plateau pattern. The cells grown on medium containing 0.25 mmol l^{-1} $CdCl_2$ accumulated up to 89 ± 12 μmol Cd (gm dw)$^{-1}$ after two days, falling to $\sim 29 \pm 10$ μmol Cd (gm dw)$^{-1}$ after five days. At 0.5 mmol l^{-1} $CdCl_2$ treatment the maximum cellular cadmium content was $\sim 132 \pm 14$ μmol (gm dw)$^{-1}$, attained after 3 days, and decreased to $\sim 98 \pm 9$ μmol (gm dw)$^{-1}$ at the end of the incubation time. There was a simultaneous marked drop in cadmium content and pH of the growth medium during the first few days. The presence of cadmium markedly altered the cellular essential cations; K^+ and Mg^{2+} being decreased while Na^+ increased during the growth period. Such findings resulted a reverse pattern of cellular Na^+/K^+ ratio for cells grown on cadmium-containing medium in respect to the control treatment. The results are discussed in relation to a further dimension of cadmium effects that might reflect its toxicity, as well as the implication of cadmium extrusion for tolerance during fungal growth.

Introduction

Cadmium is a potentially toxic metal that has no essential biological functions. The toxicity of heavy metals include the blocking of functional groups of important molecules, e.g., enzymes, polynucleotides, transport systems for essential nutrients and ions and substitution of essential ions from cellular sites (Ochiai 1987). Cadmium induce free radicals toxicity as a result of their reactions with thiols or enzymes which normally protect against these reactive species (Splittgerber & Tappel 1979; Singhal *et al.* 1987). Cadmium toxicity is also believed to be caused by structural lesions in the plasma membrane possibly by binding to organic ligands such as sulphydryl groups (Gadd & Mowll 1983; Rayner & Sadler 1989). However, such a toxic metal still may be tolerated and accumulated by certain living cells including fungi (Gelmi *et al.* 1994; Bender *et al.* 1995; Baldrian *et al.*, 1996; Gabriel *et al.* 1996; Purchase *et al.* 1997). Metallothioneins, phytochelatin and glutathione peptides constitute the most widely used cadmium detoxification molecules in the cytosolic component of the fungal cells (Mehra & Winge 1991; Kneer *et al.* 1992; Wu *et al.* 1995; Inouhe *et al.* 1996; Rama Rao *et al.* 1997). Implication of the cell wall fraction in cadmium binding and detoxification using the yeast *Saccharomyces exiguus* was also reported (Inouhe *et al.* 1996).

Na^+, K^+, Mg^{2+} and Ca^{2+} are present in relatively high concentrations in biological systems and may be classified as bulk metals. These elements are essential for different cellular functions including formation of

charge and concentration gradients across membranes which may be used in transport processes, osmotic responses, maintenance of cytoplasmic pH value, stabilizing the structures of ribosomes and nucleic acids, and activating enzymes concerned with synthesizing DNA, RNA and proteins (Hughes & Poole 1989). Little attention has been paid to changes in the physiologically important cellular cations; K^+, Na^+, Ca^{2+} and Mg^{2+} in response to cadmium administration during the growth period. The purpose of the present work is to elucidate the growth response of filamentous fungi to cadmium, and the relationship between cadmium accumulation, its toxicity, and the cellular content of the essential bulk metal elements during growth.

Materials and methods

Organism, media and growth conditions

Two filamentous fungi were employed in this study; a *Fusarium* sp. and *Alternaria tenuis.* They were isolated from a cultivated soil in Egypt, and identified according to Ellis (1971) and Domash *et al.* (1980). They were 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; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeCl_3$ $.6H_2O$, 0.001. 15.0 g l^{-1} agar (Lab M no.2) was added to prepare solid medium. Stock solutions of the above salts and sucrose were prepared and sterilized separately by autoclaving while a stock solution of 1 M $CdCl_2$ was prepared in distilled water and sterilized by membrane filtration (cellulose nitrate, pore size 0.45 μm; Whatman, Maidstone, U.K.). For mycelial extension experiments, 9-mm disks taken from the margins of a culture growing on solid Czapek-Dox agar (8-day-old) were inoculated into the center of Czapek-Dox agar plates containing 0, 0.25, 0.5 and 1.0 mmol l^{-1} $CdCl_2$. Cadmium chloride ($CdCl_2$) was added to media at 50–55 °C under aseptic conditions. Plates were incubated at 27 °C for 8 days. Radial growth measurements were made daily during the incubation period.

Electron microscopy

Colonies were prepared for scanning electron microscopy (SEM) according to Gharieb *et al.* (1995). Small discs (9 mm diameter) were taken from the margins of 7 day-old colonies grown on cadmium-free and cadmium-containing solid Czapek-Dox medium. These were fixed in 2% (v/v) glutaraldehyde (SEM grade) in 5 mmol l^{-1} 1,4-piperazine diethanesulphonic acid (PIPES) buffer, adjusted to pH 6.5 using solid tetramethylammonium hydroxide, for 2 h at room temperature. Fixed colonies were washed four times in 5 mmol l^{-1} PIPES (15 min per a 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 were made in absolute acetone. The discs (submerged in acetone) were critical point dried using a Polaron E3000 series II critical point drying apparatus after exposure for 1 h in the transitional fluid (liquid CO_2). Dried samples were mounted on aluminum stubs and sputter-coated with palladium-gold alloy using a Polaron E1500 coating unit. Samples were finally examined using a Jeol JSM 35C scanning electron microscope with an accelerating voltage of 25 kv.

Determination of Cd^{2+}, Mg^{2+}, K^+ and Na^+ in the fungal mycelium

Initial inoculum was prepared by inoculating a 2-ml aliquot of a spore suspension (*Fusariumi* sp. $\cong 1.5 \times 10^7$ ml^{-1}; *A. tenuis* $\cong 0.8 \times 10^4$ ml^{-1}) into 250 ml Erlenmeyer conical flask containing 100 ml sterile liquid medium and then incubating for 3 days at 27 °C on a rotary shaker (150 rpm). An appropriate volume from the sterile stock solution of $CdCl_2$ was added aseptically to sterile 0.5 L Czapek-Dox medium in 1 L Erlenmeyer conical flasks to obtain final concentrations of 0.25 and 0.5 mmol l^{-1}. A cadmium-free medium was also prepared as a control treatment. 3-ml aliquot of the initial inoculum was added aseptically to each treatment, and then the flasks were incubated at 27 °C on a rotary shaker (150 rpm). After inoculation, 20-ml samples were taken at time intervals and biomass separated using nylon mesh (100 μm aperture size) and washed 2× with 10 ml dH_2O then dried overnight at 80 °C. The dried mycelia was digested in 0.5 ml 6 M HNO_3 by heating at 90 °C for 1 h. After cooling the digest was diluted with an appropriate volume of dH_2O. Cadmium and magnesium content of diluted extracts was determined using a Pye Unicam SP9 atomic absorption spectrophotometer (AAS; Thermo Unicam, Cambridge, U.K.), whileas K and Na were determined using Jenway PFP7 flame photometer with reference to appropriate standard solutions.

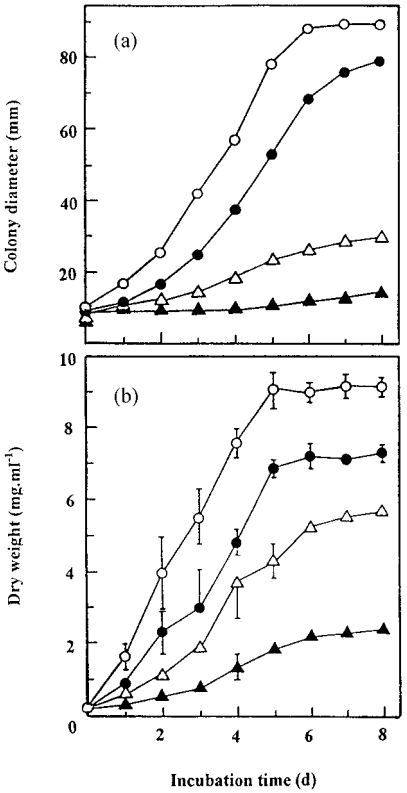

Fig. 1. Radial growth (a) and mycelial dry weight (b) of *Fusarium* sp. grown on solid and liquid Czapek-Dox medium, respectively, containing 0.0 (○); 0.25 (●); 0.5 (△) or 1.0 (▲) mmol l^{-1} cadmium chloride. Disks (9 mm diameter) taken from the margin of 7 days old culture were inoculated on the center of plates containing Czapek-Dox agar (a), whileas 2 ml aliquot from 3 days old culture were used as an inoculum for 0.5 l liquid medium (b). Both media were incubated at 27 °C. Colony diameters and mycelial dry weights were measured daily during the incubation period. Bars indicate standard error of the mean (five measurements) and when not shown were smaller than the symbol dimensions.

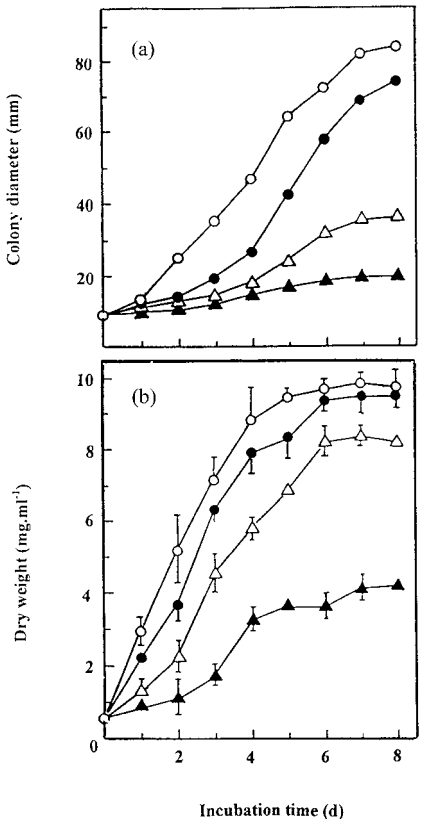

Fig. 2. Radial growth (a) and mycelial dry weight (b) of *Alternaria tenuis* grown on solid and liquid Czapek-Dox agar medium, respectively, containing 0.0 (○); 0.25 (●); 0.5 (△) or 1.0 (▲) mmol l^{-1} cadmium chloride. Other details as in Figure 1.

Results

Figures 1 and 2 display the influence of cadmium on the fungal growth that was assessed in terms of colony diameter and mycelial dry weight over 7 days incubation. Both the fungal strains showed a marked growth reduction in the presence of cadmium compared to the control treatments. The growth inhibition significantly increased with increasing cadmium chloride concentration. Colony extension and the mycelial dry weight of both fungi were moderately affected by 0.25 mmol^{-1} cadmium chloride while the higher concentrations (0.5 and 1.0 mmol l^{-1}) showed a significant growth inhibition. Additionally, there was a higher effect on the colony extension in relation to that on the mycelial dry weight. By the addition of 0.5 mmol l^{-1} $CdCl_2$ the obtained colony diameters after 7 days were 30 and 35% of controls for *Fusarium* sp. and *A. tenuis*, respectively. The presence of 1.0 mmol l^{-1} cadmium chloride resulted in an extended lag period for mycelial extension, to about 5 days for *Fusarium* sp. and about 3 days for *A. tenuis* followed by slight growth rate to manifest high growth inhibition at the end of the incubation time. Generally lag times in term of mycelial dry weight for both fungi were shorter than that of the mycelial extension at the same concentration of cadmium.

Supplementation of the growth medium with Cd^{2+} affected not only the fungal growth parameters but also caused significant changes in fungal morphology and reproductive ability. In cadmium-free medium both fungi were characterized by the appearance of normal aerial mycelia and abundant sporulation (Figures 3A-C). The presence of 1 mmol Cd^{2+} in the growth medium inhibited the production of character-

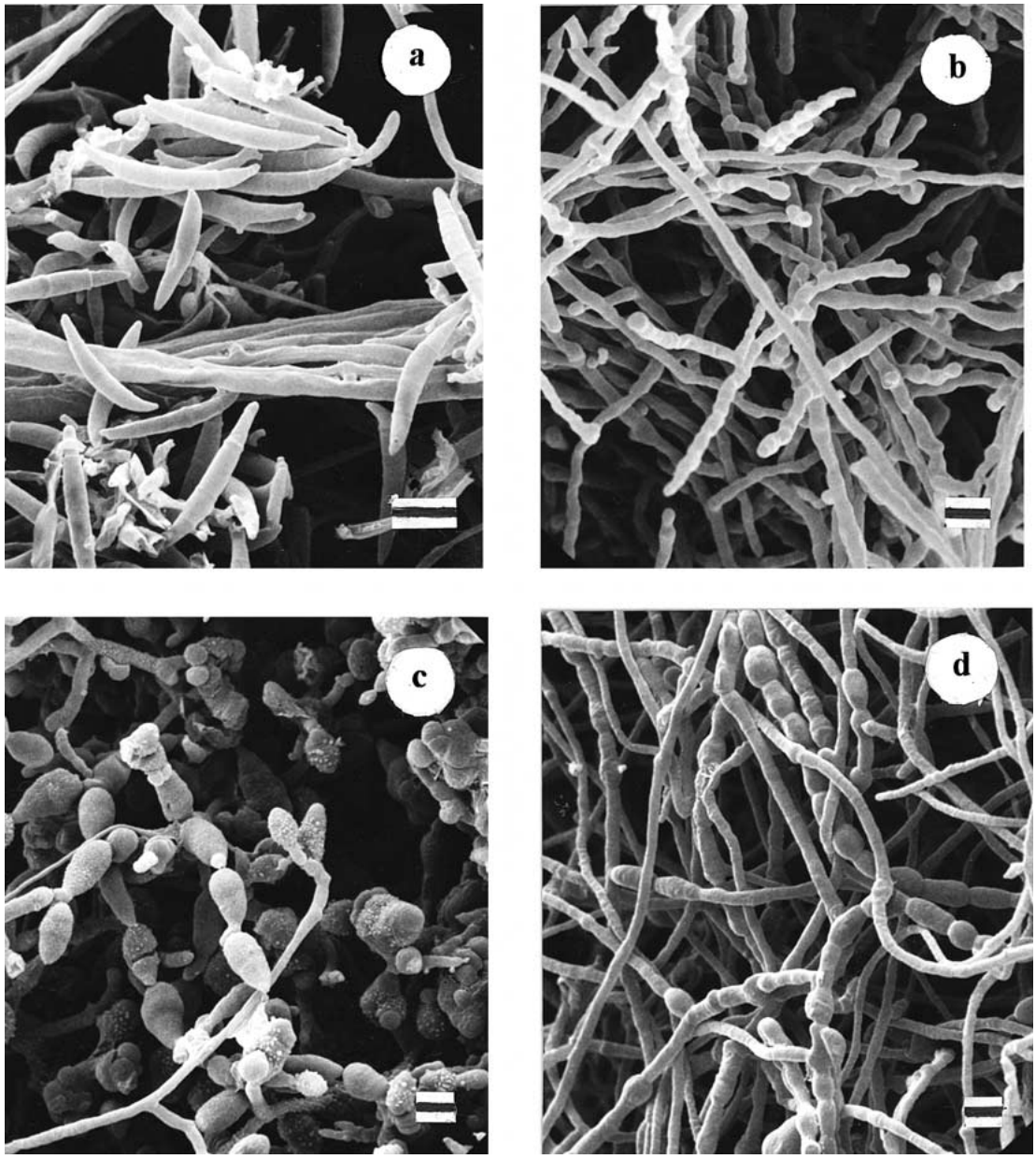

Fig. 3. Scanning electron micrographs (SEM) of *Fusarium* sp. (a, b) and *Alternaria tenuis* (c, d) grown on cadmium-free (a, c) and Czapek Dox agar medium-containing 1.0 mmol l^{-1} cadmium chloride (b, d) for 8 days at 27 °C. Other details as in Figure 1A. Bars = 10 μm.

istic conidia of *Fusarium* sp. as well as the appearance of stunted side branches that are likely to be distorted conidiophores. Moreover, abundant swellings were noticed along such conidiophores-like structures (Figure 3B). The same influence of Cd^{2+} was also detected on the morphology of *A. tenuis*. This fungal species produces characteristic chains of loose ellipsoidal shaped conidia in cadmium-free treatment. However, the addition of cadmium to the growth medium inhibited the formation of such conidia, and instead a chain of uncharacterized swellings were formed (Figure 3D). The growth of *Fusarium* sp. in cadmium-free medium also increased the medium pH to about pH 8.5 after 7 days incubation (Figure 4). However, the addition of cadmium chloride to the growth medium decreased the pH despite displaying the same pattern of pH changes over the incubation time. Figure 4 shows that the medium pH was signif-

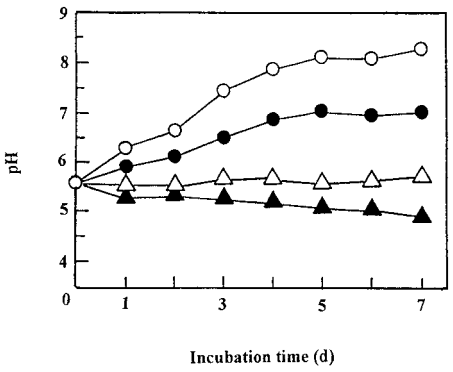

Fig. 4. Change in pH during growth of *Fusarium* sp. over 7 days incubation in 0.0 (○); 0.25 (●); 0.5 (△) or 1.0 (▲) mmol l^{-1} cadmium chloride-containing Czapek-Dox medium. Other details as in Figure 1B.

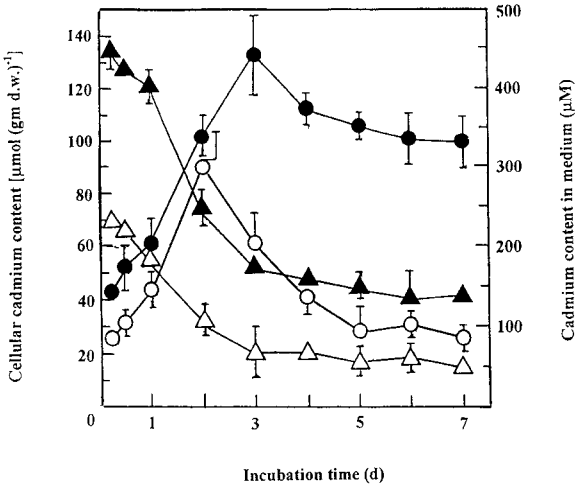

Fig. 5. Cadmium accumulation by mycelia [μmol (gm dw)$^{-1}$] of *Fusarium* sp.during growth for 7 days at 27 °C on Czapek Dox liquid medium containing 0.25 (○) or 0.5 (●) mmol l^{-1} cadmium chloride. Change in cadmium concentration in the growth medium containing initial concentrations of 0.25 (△) and 0.5 (▲) mmol l^{-1} during the fungal growth was also shown. Bars indicate standard error of the mean (three measurements) and when not shown were smaller than the symbol dimensions. Other details are described in the Methods.

icantly decreased down to 4.9 after 7 days incubation in the presence of 0.5 mmol l^{-1} $CdCl_2$.

The cadmium content in mycelia of *Fusarium* sp. grown on Dox medium containing 0.25 and 0.5 mmol l^{-1} $CdCl_2$ over 7 days incubation time is shown in Figure 5. The magnitude of Cd^{2+} accumulated into the fungal cells increased with increasing cadmium concentration in the culture medium, and significantly increased over the first few days of incubation. The accumulated cadmium in cells grown on medium containing 0.25 mmol $CdCl_2$ became a

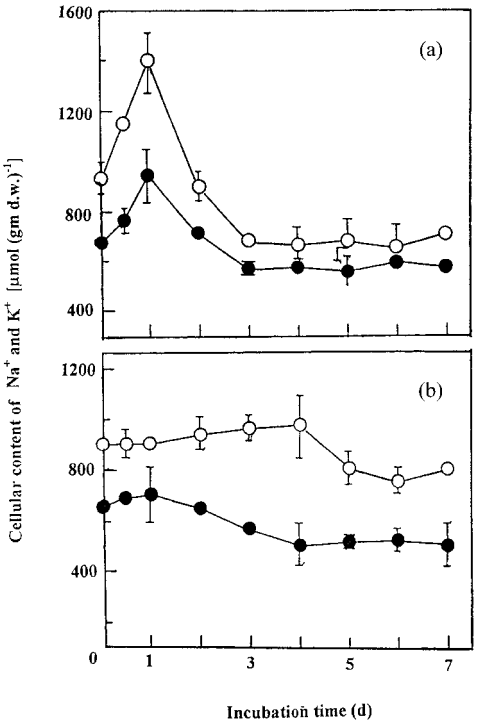

Fig. 6. Change in K^+ (•) and Na^+ (○) contents in mycelia of *Fusarium* sp. during growth for 7 days at 27 °C in liquid Czapek-Dox medium containing (a) 0.0 or (b) 0.5 mmol l^{-1} cadmium chlorid. Other details as in Figure 5.

maximum after 2 days incubation [89 ± 12 μmol Cd(gm dw)$^{-1}$]. Beyond this time the cellular content of cadmium was markedly reduced to 29 ± 10 μmol Cd(gm dw)$^{-1}$ on the fifth day of incubation. At 0.5 mmol l^{-1} $CdCl_2$ treatment the maximum cellular cadmium content was 132 ± 14 μmol Cd(gm dw)$^{-1}$ attained after 3 days. After that there was a slight reduction in the cadmium content of cells to 98 ± 9 μmol Cd(gm dw)$^{-1}$ at the end of incubation time. Simultaneously there was a concomitant and significant decrease in the cadmium content in the growth medium over the first 3 days of incubation to the lowest levels attained after 4 days (50 and 130 μ mol l^{-1} at 0.25 and 0.5 mmol l^{-1} treatments, respectively).

Intracellular contents of Na^+ and K^+ of the growing cells on cadmium free medium during the incubation period revealed a similar pattern. There was a marked increase in both cations after the first 24 h followed by a sharp decrease becoming at their lowest values on the third day (Figure 6A). On the other hand Figure 6B shows that the presence of 0.5 mmol l^{-1} cadmium chloride in the growth medium reduced the mycelial content of these monovalent cations during

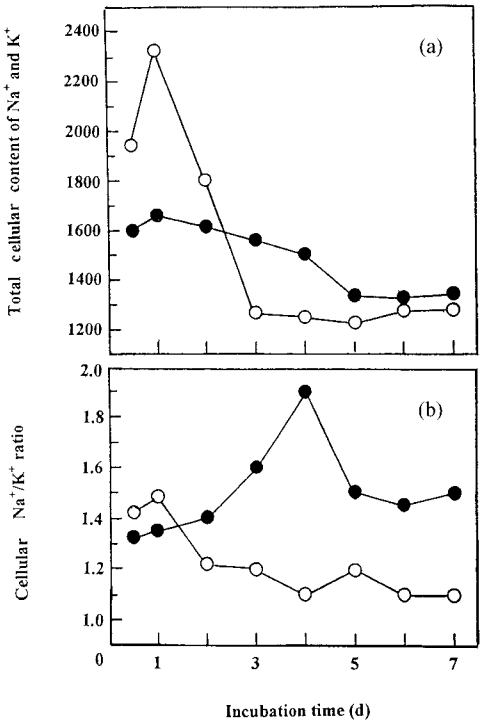

Fig. 7. Change in (a) total content of cellular K^+ and Na^+ and (b) cellular Na^+/K^+ ratio in mycelia of *Fusarium* sp. during growth for 7 days at 27 °C in liquid Czapek Dox medium containing 0.0 (○) or 0.5 (●) mmol l^{-1} cadmium chloride.

the growth period compared to the control treatments. Additionally, the pattern of cellular Na^+ content was more or less in reverse to that of K^+ over the incubation period (Figure 6B). Total cellular Na^+ and K^+ as well as Na/K^+ ratio are represented in Figure 7. While there was a great drop in the total monovalent cations during the first two days of growth on cadmium-free medium such a drop was lower and occurred gradually over 5 days (Figure 7A). Representation of Na^+/K^+ ratios displayed the presence of small peak after the first day of incubation in absence of cadmium (Figure 7B). However, the addition of cadmium to the growth medium caused a significant alteration and a reverse pattern of Na^+/K^+ ratio over the incubation period in relation to that of the control treatment, becoming maximum (1.9) on the fourth day of incubation.

Levels of cellular Mg^{2+} decreased significantly by the addition of cadmium despite displaying a similar pattern to the control treatment over the incubation period (Figure 8). About a 50% reduction in the cellular content of Mg^{2+} was resulted from the administration of 0.5 mmol l^{-1} cadmium chloride to the growth

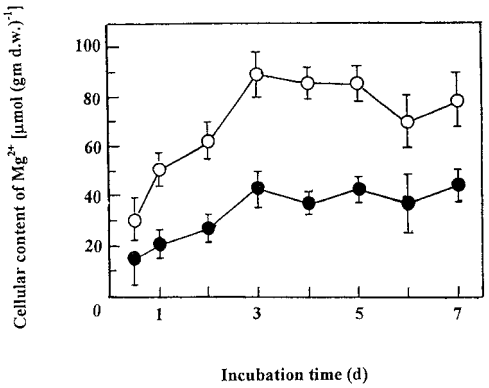

Fig. 8. Change in cellular content of Mg^{2+} in mycelia of *Fusarium* sp. during growth for 7 days at 27 °C in liquid Czapek-Dox medium containing 0.0 (○) or 0.5 (●) mmol l^{-1} cadmium chlorid. Other details are described in the Methods.

medium. During the growth time there was an increase in cellular Mg^{2+}, and the maximum values were obtained after 3 days and they were 88 ± 8 and 43 ± 7 μmol Mg(gm dw)$^{-1}$, for the control and cadmium-containing treatment, respectively.

Discussion

Cadmium is an element with no known biological function and may be toxic even at low concentrations. It has been described as a potent inhibitor of several enzyme systems resulting from affinity towards sulphydryl groups, hydroxyl groups and ligands containing nitrogen (Singhal *et al.* 1987). In recent years, particular attention has been paid to heavy metal tolerance, and numerous strategies have been exhibited by fungi. Preventing metal entrance or reducing metal uptake and sequestration of the toxic metal inside microbial cells are the most important mechanisms of detoxification (Gadd 1994; Gharieb & Gadd 1998). The present results showed higher effect of cadmium on the mycelial extension than the dry weight. It might be due to a synthesis of macromolecules being implicated in detoxification inside the cells grown in cadmium-containing medium. The accumulation of Cd^{2+} into the cells has been linked with different mechanisms of detoxification such as formation of insoluble Cd sulphide around the cell wall/membrane or Cd phosphate inside the cell (Aiking *et al.* 1984; Kosman 1994). Tohoyama *et al.* (1996) reported that a Cd resistant strain of *S. cerevisiae* cells is protected against toxicity by the production of large amounts of Cd-binding metallothionein. Synthesis of glutathione

and short, cysteine-containing γ-glutamyl, peptides known as 'cadystins' or 'phytochelatins' by animal cells and some fungal strains such as *Schizosaccharomyces pombe*, *Candida glabrata, S. cerevisiae* and *Neurospora crassa* to counteract cadmium toxicity has also been shown (Singhal *et al.* 1987; Mutoh *et al.* 1991; Mehra & Winge 1991; Wu *et al.* 1995). Singhal *et al.* (1987) reported that mice intracellular glutathione functions in protection against Cd^{2+} toxicity, and this tripeptide provides a first line of defense against Cd^{2+} before induction of metallothionein synthesis occur. Additionally Ramadan *et al.* (1988) reported that the growth of *Aspergillus carbonarius* and a strain of *Penicillium* in the presence of cadmium resulted in an accumulation of extraordinary high contents of proteins, lipids and carbohydrates whereas the activities of certain enzymes, lipases, amylases and proteases were inhibited.

Cadmium accumulation by the fungal cells has been previously reported (Gelmi *et al.* 1994; Gabriel *et al.* 1996). The present investigation showed that the cellular cadmium content of the *Fusarium* sp. increased progressively during the fungal growth. It was also positively correlated with cadmium concentration in the growth medium, until reaching a plateau or a threshold concentration beyond which it decreased. This result suggested that some cadmium accumulated by the cells was expelled outside. Such a mechanism of cadmium efflux and extrusion outside the cell has been previously shown in *Staphylococcus aureus* (Chopra 1971; Rayner & Sadler 1989; Gelmi *et al.* 1994) and the fungi *Aureobasidium pullulans* and *Candida albicans* (Mowll & Gadd 1984; Gelmi *et al.* 1994). It was also reported that the efflux mechanism is probably not sufficient when cells are subjected to very high (millimolar) levels of cadmium (Rayner & Sadler 1989), that is in agreement with the present findings. Therefore the results suggest that cadmium efflux is an inductive mechanism being activated by the presence of a threshold concentration gradient of cadmium across the cell membrane. Such a mechanism is efficient in avoiding the dangerous accumulation of further cadmium ions inside the fungal cell. Generally, it could be suggested that cadmium entering the fungal cell is firstly detoxified by synthesis of chelating proteins, short peptides, sulphide or phosphate during early stages of growth. Additionally, an active mechanism of Cd^{2+} extrusion outside the cell also appears to be involved in cadmium tolerance.

Concomitant with cadmium accumulation, the cellular K^+ was decreased while Na^+ was increased inside the cells. This might be an integration with cadmium transport since H^+ and K^+ are involved in ionic gradients and the membrane potential (Gadd 1990). In several bacteria, including *Staphylococcus aureus* and *Bacillus subtilis*, active transport of cadmium depends on the cross membrane electrical potential and this uptake system is highly specific for Cd^{2+} (Gadd 1990). Short term uptake experiments, reported by Brady and Duncan (1994), showed that copper and cadmium were accumulated by *S. cerevisiae* with a subsequent increase in the permeability of the plasma membrane to release K^+, Mg^{2+} and Ca^{2+}. Releasing of cellular K^+ as an effect of cadmium uptake was also previously shown in *Aureobasidium pullulans* (Mowll & Gadd 1984) and suggested as a stoichiometric exchange of Cd^{2+} and K^+ ions across the cell membranes. It was also reported that potassium release has been associated with membrane disruption and loss of viability (Gadd & Mowll 1983). This is not applied to the results found in this study, and the decreasing cellular potassium is suggested to maintain the ionic balance across the cell membrane. However, potassium is an essential element for living cells performing important functions including osmoregulation, activator of enzyme synthesis and is also involved in the stabilization of intracellular structures, e.g. ribosomes (Hughes & Poole 1989). On the other hand, increasing the cellular sodium and the reverse results of the cellular Na^+/K^+ ratio suggest a possible non-target effect of cadmium since sodium toxicity and the stability of the living cell highly depends on such a ratio.

Reducing the cellular content of Mg^{2+} by administration of cadmium reflects either the competitive action of cadmium in magnesium uptake or exchange of these divalent ions across the plasma membrane. Competition of the uptake of a toxic metal and an essential element has been documented since divalent cations may enter cells by a common mechanism (Gadd 1994). In *S. cerevisiae* it was evident that calcium competed for the cadmium transport sites and it is therefore likely that cadmium was transported via an existent calcium uptake system (Norris & Kelly 1977). Additionally Cu^{2+} accumulation by the same fungal strain resulted in a slower release of approximately 60% of cellular Mg^{2+} (Brady & Duncan 1994). Reduction in Mg^{2+} content in the root of seedlings of the plant *Betula pendula* by the presence of Cd^{2+} was also shown (Gussarsson 1994). The present work indicates a significant reduction in the cellular Mg^2 by Cd^2 during the growth phases that is in agreement with the previous findings. The essentiality of high amounts

of Mg^{2+} during the growth of microbial cells was established; its function including stabilization of the structure of ribosomes and nucleic acids and activation of many enzymatic processes involve hydrolysis and synthesizing DNA, RNA and proteins (Hughes & Poole 1989).

Abnormal cell morphology, particularly production of giant cells, in response to heavy metal toxicity has been documented. The morphological malformation of fungi grown in cadmium-containing medium was previously reported (Ramadan *et al.* 1988; Gabriel *et al.* 1996). Cadmium toxicity was attributed to the affinity of cadmium to sulphydryl groups which would alter the structural and functional components of the cell. Predominance of -SH groups in the walls of the tips phialides of *Penicillium notatum* was shown by Pitt (1969). He therefore suggested that these groups serve a function in maintaining plasticity in areas that proliferate cells rapidly during sporulation. Inhibition of the fungal sporulation and the appearance of swollen segments might therefore be resulted in part from binding of Cd^{2+} to -SH groups probably localized in the conidiation sites of the fungal mycelia. Generally, formation of enlarged globose cells in the fungal mycelia was previously reported as a response to one of several conditions unfavourable to growth such as low pH, potassium deficiency, oxygen deficiency, high osmotic pressure or the presence of inhibitors (Bent & Morton 1963). Therefore, declining medium pH which would be on the expense of cytosolic pH, and alteration the cellular contents of essential cations, K^+, Na^+ and Mg^{2+} resulting from cadmium uptake would explain a non-target effect of cadmium which possibly interfere with fungal morphogenisis.

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