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Growth inhibition of the filamentous fungus Aspergillus nidulans by cadmium: an antioxidant enzyme approach

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The heavy metal cadmium is very toxic to biological systems. Although its effect on the growth of microorganisms and plants has been investigated, the response of antioxidant enzymes of Aspergillus nidulans to cadmium is not well documented. We have studied the effect of cadmium (supplied as CdCl$_2$) on catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR). 0.005 mM CdCl$_2$ had a very slight stimulatory effect on the growth rate of A. nidulans, but at concentrations above 0.025 mM, growth was totally inhibited. The accumulation of Cd within the mycelium was directly correlated with the increase in the concentration of CdCl$_2$ used in the treatments. Although a cadmium-stimulated increase in SOD activity was observed, there was no change in the relative proportions of the individual Mn–SOD isoenzymes. Higher concentrations of CdCl$_2$ induced a small increase in total CAT activity, but there was a major increase in one isoenzymic form, that could be separated by gel electrophoresis. GR activity increased significantly following treatment with the highest concentration (0.05 mM) of CdCl$_2$. The increases in SOD, CAT, and GR activities suggest that CdCl$_2$ induces the formation of reactive oxygen species inside the mycelia of A. nidulans.

Key Words—Aspergillus nidulans; catalase; glutathione reductase; oxidative stress; superoxide dismutase

Introduction

Cadmium (Cd) is a heavy metal, which is highly toxic to nearly all organisms at low concentrations and may also be carcinogenic for mammals, including humans (Momose and Iwahashi, 2001; Waalkes, 2000). Cd can markedly inhibit the growth of plants, where it accumulates mainly in the roots (Cardoso et al., 2002; Pereira et al., 2002; Rauser, 2000; Vitória et al., 2001).

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and it can rapidly induce the synthesis of a group of cysteine-rich metal binding peptides, termed phytochelatins (Höfgen et al., 2001; Leustek et al., 2000). Cd has been shown to generate reactive oxygen species (ROS) in plants, as an indirect result of lipid peroxidation and chlorophyll degradation (Somashekaraiah et al., 1992). As a response to ROS production in plants, from either environmental stresses or metabolic processes, a group of enzymes, which are localized in different cell compartments, such as catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2) (Azevedo et al., 1998), have been shown to respond differently, depending on the plant species, tissue and developmental stage (Vitória et al.,
Cd can also inhibit the growth of the fungi, *Saccharomyces cerevisiae* (Soares, 2002) and *Aspergillus niger* (Tsekova et al., 1999). Cd inhibited both the growth and citric acid production of *A. niger*, for which the protein and DNA content of the cells was reduced, whilst lipids and polysaccharides were increased (Tsekova et al., 2000). The effect of Cd on the mycelial growth of 64 strains of 25 different species of potential pathogenic soil fungi was tested by Plaza et al. (1998) and the sensitivity was shown to vary greatly. The free radical scavenger N-acetylcysteine has been shown to prevent the toxicity of Cd in *S. cerevisiae*, whilst mutants lacking SOD activity, or the capacity to synthesise glutathione, were hypersensitive to Cd. Taking into account the observation that cells grown in the absence of oxygen were more resistant to Cd, these results suggest that oxidative stress is involved in the toxic action of Cd on *S. cerevisiae* (Brennan and Schiestl, 1996).

Momoese and Iwahashi (2001) studied the changes in gene expression in *S. cerevisiae* by DNA microarray analysis. Glutathione synthesis, common stress response and almost all the genes involved in the synthesis of sulphur amino acids were greatly induced after exposure to Cd. In total 42 other genes were upregulated more than 4-fold by the presence of Cd. In a comparable study, Vido et al. (2001) examined the individual proteins that were affected by the application of Cd to *S. cerevisiae*; 52 proteins were shown to be induced and 43 repressed. Again the enzymes of glutathione and sulphur amino acid biosynthesis were strongly induced. The transcription factor Yap1p was shown to play a key role in the induction of antioxidant defence genes involved in Cd tolerance.

SOD catalyzes the conversion of the superoxide ion to hydrogen peroxide. Three classes of SOD isoenzymes have been shown to exist in most organisms; Mn–SOD, Cu/Zn–SOD, and Fe–SOD, which may be located in one or more cellular compartment. However in the majority of fungi, only a mitochondrial Mn–SOD and a cytoplasmic Cu/Zn–SOD have been isolated, although a recent study in *Candida albicans* has indicated the expression of a third gene encoding an atypical cytosolic Mn–SOD in the stationary phase of growth (Lamarre et al., 2001). Mutants of the yeast *S. cerevisiae* and the filamentous fungus *Neurospora crassa* lacking Cu/Zn–SOD are sensitive to oxygen and superoxide generating agents, suggesting that this enzyme is responsible for the principal superoxide dismutating activity during oxidative stress (Chary et al., 1994; Lee et al., 2001; Park et al., 1998). Cu/Zn–SOD has been purified and characterized from a range of *Aspergillus* species (Holdom et al., 1996), whereas Oberegger et al. (2000) demonstrated that the depletion of iron, but not carbon or nitrogen starvation of *A. nidulans*, induced the synthesis of Cu/Zn–SOD.

Detoxification of H2O2 is a fundamental aspect of the cellular antioxidant responses in which CAT plays a major role producing H2O and O2. Initial studies indicated that *A. nidulans* contained two (Kawasaki et al., 1997) or possibly three genes encoding catalase (Takasuka et al., 1999). However more recently, Kawasaki and Aguirre (2001) showed that a triple catABC mutant of *A. nidulans* was able to grow normally with peroxide generating substrates and provided evidence for a fourth gene, catD. The *A. nidulans* gene catA was shown to be only expressed in spores, whilst catB was induced and translated in growing mycelia. The application of hydrogen peroxide, paraquat, heat shock, or uric acid induced the transcription of catB but not catC. CatD activity was induced during the late stationary phase by glucose starvation, high temperature and to a lesser extent by hydrogen peroxide (Kawasaki and Aguirre, 2001).

GR using NADPH as a substrate is important in maintaining glutathione in the fully reduced state. *S. cerevisiae*, *glr1* mutants lacking GR activity were sensitive to paraquat and hydrogen peroxide, but were still able to grow normally under aerobic conditions (Grant et al., 1998; Jamieson, 1998). Transcription of the *GLR1* gene was induced by oxidative stress including hydrogen peroxide and was shown to be under the control of the yap1–1 protein (Grant et al., 1996; Jamieson, 1998). However, for *Schizosaccharomyces pombe*, the presence of active GR was shown to be essential for growth under normal aerobic growth conditions. Although expression of the *pgr1*+ gene encoding GR was shown to be induced by various oxidants, heat shock, high osmolarity and starvation, hydrogen peroxide itself had no effect (Lee et al., 1997). These data indicate that there is a clear difference between the regulation and role of GR in *S. cerevisiae* and *Sch. pombe*.

Although the responses of a range of yeasts to ROS and to Cd, have been extensively investigated, far less work has been carried out on the *Aspergillus* fungi.
Members of the genus are found typically as saprophytes on decaying organic material in the soil. The genus also contains a range of pathogens that can cause diseases in humans. The bioremediation of heavy metals has received a great deal of attention, especially with the increase in contamination of waste waters. Several microorganisms, including species of Aspergillus, have been examined for their ability to remove metals from solution (Kapoor and Viraraghavan, 1997; Kapoor et al., 1999). In the bioremediation field, it is important to identify the metabolic and molecular effects caused by heavy metals and the consequent cellular responses by distinct microbial strains, thus achieving an understanding of the different mechanisms involved in resistance to heavy metal stress. A. nidulans is relatively non-pathogenic and has been genetically well characterized and represents a model filamentous ascomycete. As far as we are aware no attempt has been made to examine in detail the effect of Cd on antioxidant enzymes in any species of Aspergillus, although mutants resistant to Cd have been isolated (Cooley et al., 1986a, b). In this study, we report new findings concerning the effect of Cd on growth and on the activity of antioxidant enzymes CAT, SOD, GR of A. nidulans.

Materials and Methods

Reagents. All chemicals used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Aspergillus nidulans strain. The Aspergillus nidulans Master Strain E (MSE) was employed for all experiments. The choice of the strain was based on the presence of at least one genetic marker for each chromosome, which will allow future genetic analysis related to this study. This strain was developed at the Genetics Department, University of Glasgow, G12 8QQ, UK.

Culture conditions and cadmium treatment. The complete medium (CM) as described by Pontecorvo et al. (1953) was used for A. nidulans growth. The initial conidial concentration was estimated in a Neubauer's chamber. A suspension containing 10^6 conidia per ml was inoculated into 100 ml of CM, in 250 ml Erlenmeyer flasks. After 12 h of incubation in a shaker (120 rpm) at 37°C, CdCl₂ at different concentrations (0.005, 0.010, 0.025, and 0.05 mM) was added to the medium and the cultures were allowed to grow for a further period of 24 h, or longer in preliminary experiments, under the same conditions. Mycelium samples were collected after 6, 9, 12, and 24 h. The mycelia was separated by filtration on Whatman no. 1 filter paper, washed with copious distilled-deionized water, frozen in liquid nitrogen and stored frozen at -70°C for further enzyme extraction. The dry weight for each time point and CdCl₂ concentration tested was obtained at 55°C, until a constant weight was achieved.

Enzyme extraction. The frozen mycelia were extracted and used for enzyme analysis. The extractions were carried out as described by Chaparro-Giraldo et al. (2000) in a pestle and mortar at 4°C, and the mycelia were homogenized in 100 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA, 3 mM dithiothreitol, and 4% (w/v) insoluble polyvinylpyrrolidone. The homogenates were centrifuged at 10,000 x g for 30 min to remove cell debris. The supernatants were collected and the aliquots were kept frozen at -70°C prior to enzyme analysis.

Protein determination. The protein concentration was determined spectrophotometrically at 595 nm as described by Bradford (1976) using the Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE). For enzyme activity staining of CAT, SOD, and guaiacol peroxidase, non-denaturing native PAGE was carried out in gels containing 8% polyacrylamide with a 4% stacking gel. Electrophoresis was performed at 4°C and a constant current of 15–30 mA applied for 4–12 h depending on the enzyme to be stained.

SDS-PAGE under denaturing conditions was performed in 10% polyacrylamide gels. Electrophoresis buffers and gels were prepared as described by Laemmli (1970). Equal amounts of protein (100 μg) were loaded onto each lane of the gels, for both native and denaturing gels.

Superoxide dismutase activity staining. SOD activity was determined as described by Azevedo et al. (1998). The non-denaturing gels were incubated in the dark in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% (v/v) TEMED, at room temperature for 30 min. At the end of this period, the reaction mixture was poured off, and the gels were rinsed with distilled-deionized water and then illuminated in water until the development of colourless bands of SOD activity in a purple-stained
gel were visible. In order to classify SOD isoenzymes from *A. nidulans*, a sample of the control treatment was subjected to non-denaturing PAGE and the SOD bands classified as described by Azevedo et al. (1998). Prior to SOD staining, the gel was cut into three slices; the first was stained for SOD as above; the second and third slices were incubated in 100 mM potassium phosphate buffer (pH 7.8), containing either 2 mM KCN or 5 mM H₂O₂, for 20 min. At the end of this period, both slices were washed in distilled-deionized water and then stained for SOD activity. The pre-treatment of the gel in H₂O₂ and KCN before SOD staining, allowed the classification of the SOD bands into Cu/Zn–SOD, Fe–SOD or Mn–SOD. Cu/Zn–SOD is inhibited by both inhibitors, Fe–SOD isoenzyme is resistant to KCN and inhibited by H₂O₂, and Mn–SOD is resistant to both inhibitors.

**Catalase activity staining.** CAT activity in non-denaturing PAGE gels was determined as described by Vitória et al. (2001). The gels were incubated in 0.3% H₂O₂ for 10 min and developed in a 1% (w/v) FeCl₃ and 1% (w/v) K₃[Fe(CN)]₆ solution for 10 min.

**Guaiacol peroxidase activity staining.** Peroxidase activity was determined at 25°C as described by Ridge and Osborne (1970). Gels were incubated at room temperature in a reaction mixture of 50 mM sodium phosphate buffer pH 6.0, containing 0.5% (v/v) guaiacol and 0.1 M H₂O₂. With the appearance of the bands, the gels were transferred to distilled-deionized water.

**Glutathione reductase assay.** GR activity was determined as described by Barata et al. (2000). GR activity was assayed spectrophotometrically at 30°C in a mixture consisting of 3 ml 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5'-dithiol-bis (2-nitrobenzoic acid), 1 mM oxidized glutathione and 0.1 mM NADPH. The reaction was started by the addition of 50 µl of mycelium extract. The changes in absorbance due to the rate of reduction of oxidized glutathione were monitored at 412 nm for 2 min.

**Cd accumulation.** The accumulation of Cd in *A. nidulans* was analyzed as described by Vitória et al. (2001) using an inductively coupled plasma mass spectrometer (ICP-MS).

**Statistical analysis.** The experiments described were repeated in two blocks, each with three replicates for spectrophotometric analysis. Data were statistically analyzed by SAS and the results expressed as mean (±SE) of three independent replicates for dry weight and GR measurements.

**Results**

**Growth of A. nidulans in the presence of CdCl₂.**

A preliminary experiment was carried out to establish the range of CdCl₂ concentrations and exposure time that were able to inhibit mycelium growth. It was observed that concentrations of 0.05 and 0.1 mM CdCl₂ strongly inhibited the growth of the *A. nidulans* mycelia (Data not shown). Based on this result, *A. nidulans* strain MSE was grown in CM, which was supplemented with varying concentrations of CdCl₂ up to 0.05 mM, for a 24 h period. The lowest concentration of CdCl₂ tested (0.005 mM), caused a slight stimulation of growth during the whole time of treatment (Fig. 1A), however, at a CdCl₂ concentration of 0.01 mM, a reduction of growth was observed throughout the treatment. At CdCl₂ concentrations above 0.025 mM, the growth of mycelium appeared to be totally inhibited even after only 6 h of treatment (Fig. 1A). By the end of the 24 h period, at the two highest CdCl₂ concentrations, there was evidence of a loss of weight, indicating that autolysis may have been taking place. It was also clearly observed that the number of germinating conidia increased in 0.005 mM CdCl₂, followed by a dramatic re-
duction in germination and growth above 0.025 mM CdCl₂ (Fig. 1B). Statistical analysis for mycelium dry weight showed a high significance in relation to time of exposure to CdCl₂ (prob. > F=0.00002), concentration of CdCl₂ (prob. > F=0.00001) and the interaction between these two factors (prob. > F=0.00084), indicating that concentrations of 0.025 mM CdCl₂ and above, and the time of treatment, are critical for growth (Table 1). Furthermore, statistical analysis (Table 1) also indicated that at 6 h of Cd exposure the concentration of CdCl₂ did not cause any effect on mycelium dry weight; however, for the other exposure times (9, 12, and 24 h) a significant effect of CdCl₂ concentration was observed (decrease in mycelial dry mass weight with the increase in CdCl₂ concentration) (Table 1).

**Cd accumulation in A. nidulans**

The accumulation of Cd in A. nidulans was determined in mycelia grown in the presence of 0, 0.005, 0.01, 0.025, and 0.05 mM CdCl₂ for 24 h (Table 2). Even at the lowest treatment of 0.005 mM CdCl₂, accumulation of Cd was observed with a massive accumulation being determined at 0.05 mM CdCl₂. The accumulation was approximately proportional to the external concentration applied and a 10–100-fold increase in concentration from the external medium was detected.

**Protein content**

When expressed in relation to fresh weight, there was little difference in the protein content of the A. nidulans mycelia in any treatment up to 0.025 mM CdCl₂. However, a reduction of protein content was observed in the 0.05 mM CdCl₂ treated cells after 12 h (Data not shown). Evidence of proteolytic breakdown of the higher molecular mass proteins in the 12 and 24 h, 0.05 mM CdCl₂ treated cells was observed (Data not shown).

**Superoxide dismutase activity**

Protein extracts of the A. nidulans mycelia exposed to CdCl₂ concentrations for a 24 h period were subjected to non-denaturing PAGE and stained for SOD activity. Control SOD activity staining carried out with a large amount of A. nidulans protein, allowed the identification of three SOD bands (Fig. 2). The incubation of identical gels in H₂O₂ and potassium cyanide before incubating the gels in the staining reaction mixture allowed the classification of the all three SOD bands as Mn–SOD isoenzymes, since the activity bands were not be inhibited by either hydrogen peroxide or potassium cyanide (Fig. 2). A soybean leaf SOD extract was
Fig. 2. Classification of individual SOD isoenzymes of A. nidulans. Extracts of A. nidulans mycelium were subjected to non-denaturing PAGE and stained for SOD isoenzymes. (1) Control SOD activity staining, (2) plus 2 mM KCN, and (3) plus 5 mM H_2O_2. Bands have been numbered from the least electronegative band (I). All isoenzymes were classified as Mn–SOD. These numbers are referred to in the text.

Fig. 3. Activity of individual SOD isoenzymes isolated from A. nidulans mycelium following non-denaturing PAGE (activity staining was performed as described in MATERIALS AND METHODS). Lanes 1 to 5 correspond to 0, 0.005, 0.01, 0.025, and 0.05 mM CdCl_2 treatments, respectively.

tested simultaneously with the A. nidulans extract to serve as a control of SOD isoenzyme activities (Data not shown). The analysis of SOD activity of A. nidulans extracts exposed to control conditions and CdCl_2 treatments revealed that bands II and III accounted for almost 100% of the SOD activity (Fig. 3). It can be seen that the total SOD activity was not affected after 6 h, by any of the CdCl_2 treatments. After 9 h, there was an increase in total SOD activity in all the CdCl_2 treated cells, although it was most apparent with 0.025 and 0.05 mM CdCl_2; a similar, slightly greater increase was also seen after 12 h. Although after 24 h of growth, a lower total SOD activity was detected, when compared to the 12 h treatment, a continuous trend of increased

Fig. 4. Activity of CAT isoenzymes isolated from A. nidulans mycelium following non-denaturing PAGE (activity staining was performed as described in MATERIALS AND METHODS). Lanes 1 to 5 correspond to 0, 0.005, 0.01, 0.025, and 0.05 mM CdCl_2, respectively. Bands have been numbered from the least electronegative band (I).

SOD activity with increased CdCl_2 concentrations was still observed. The increase in activity could not be correlated to any particular SOD isoenzyme. Furthermore, no other changes, including the appearance or disappearance of SOD activity bands, were observed in the SOD isoenzyme pattern in response to CdCl_2 treatment.

Catalase activity
Catalase activities of the same A. nidulans mycelial extracts used for SOD activity staining were also determined following non-denaturing PAGE. Activity staining revealed three bands of CAT activity, which indicated the presence of at least three distinct CAT isoenzymes (Fig. 4). The total CAT activity did not appear to change after 6 and 9 h of CdCl_2 exposure, although a slight increase in total CAT activity was observed after 9 h in the 0.05 mM CdCl_2 treatment. An increase in total CAT activity was observed with increasing CdCl_2 treatment after 12 and 24 h, which was particularly apparent in the 0.05 mM CdCl_2 treatment. The increase in CAT activity was detected in all bands, but was particularly noticeable for isoenzyme I with the increase in CdCl_2 concentration.

Guaiacol peroxidase activity
An attempt to measure the activity of guaiacol peroxidase of A. nidulans following non-denaturing PAGE was carried out using the same extracts used for the
CAT and SOD activity stainings. Although several variations to the method were tested, guaiacol peroxidase activity was not detected in *A. nidulans* under the experimental conditions employed. To confirm this result, plant extracts which were applied simultaneously to the gels to serve as a control exhibited the enzyme activity, which in the case of maize roots, allowed the identification of seven guaiacol peroxidase bands (Data not shown).

**Glutathione reductase activity**

The activity of GR in the mycelium of *A. nidulans* was determined for all CdCl₂ treatments. A considerable variation in GR activity was observed over the time course of the experiment and with increasing CdCl₂ concentration (Fig. 5). A statistical analysis was carried out, which showed that there was a significant difference in activity with respect to time (prob. >*F* = 0.0111), concentration of CdCl₂ (prob. >*F* = 0.00001) and the interaction of both (prob. >*F* = 0.0015). By fixing the concentration factor and analyzing GR activity at the 6, 9, 12, and 24 h time points (Table 3), a significant increase in GR activity at 6 h of exposure was observed, which was maintained with the increase in CdCl₂ concentration. Although with 9 h exposure an increase in GR activity was observed concomitant with the increase in CdCl₂ concentration, with 12 and 24 h of CdCl₂ exposure a negative effect on GR activity was observed (Table 3). A clear effect on GR activity was only observed in the 0.05 mM CdCl₂ treatment for 12 and 24 h of exposure (Table 3).

**Discussion**

**Dry weight**

An increase in mycelium dry weight was observed, following the addition of 0.005 mM CdCl₂ to the cultures of *A. nidulans* (Fig. 1A) confirming the results previously obtained for *A. nidulans* (Cooley et al., 1986b) and also for sugar cane cell cultures (Formazier et al., 2002a). It has been suggested that a low concentration of Cd can stimulate growth by forming complexes with constituents of the culture medium, which could allow essential trace elements such as Cu to become

![Graph of GR specific activity vs Cd concentration](image)

**Fig. 5.** The specific activity of glutathione reductase (μmol min⁻¹ (mg protein⁻¹)) in *A. nidulans* mycelium grown for 6, 9, 12, and 24 h in the presence of 0, 0.005, 0.01, 0.025, and 0.05 mM CdCl₂. Data represent means of 3 measurements ± SE.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Regression (Prob. &gt;<em>F</em>)</th>
<th>Equation</th>
<th>R² (%)</th>
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<tbody>
<tr>
<td>Time (h)</td>
<td>6</td>
<td>0.0335</td>
<td><em>y</em> = 2.13 + 0.108x - 0.0018x</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.0112</td>
<td><em>y</em> = 2.21 + 0.030x</td>
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<tr>
<td></td>
<td>12</td>
<td>0.0005</td>
<td><em>y</em> = 2.48 - 0.048x</td>
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<tr>
<td></td>
<td>24</td>
<td>0.00001</td>
<td><em>y</em> = 1.83 - 0.102x</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0488</td>
<td><em>y</em> = 2.71 - 0.64x + 0.105x² - 0.004x³ + 5×10⁻⁵x⁴</td>
</tr>
<tr>
<td>Concentration (μM CdCl₂)</td>
<td>0</td>
<td>n.s.</td>
<td>(1st and 2nd grades)</td>
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<tr>
<td></td>
<td>5</td>
<td>n.s.</td>
<td><em>y</em> = 16.476 - 3.988x + 0.342x² - 0.008x³</td>
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<td></td>
<td>10</td>
<td>n.s.</td>
<td>(1st and 2nd grades)</td>
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<tr>
<td></td>
<td>25</td>
<td>n.s.</td>
<td><em>y</em> = 1.62 + 0.241x</td>
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<td>50</td>
<td>0.00001</td>
<td>(1st and 2nd grades)</td>
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Table 3. GR activity—effect of CdCl₂ exposure time and concentration—Polynomial regression.
available to the fungus (Cooley et al., 1986b). The first indication of an inhibitory effect on growth was obtained with 0.01 mM CdCl₂, which is a concentration lower than that required to reduce the growth rate of higher plant cells (Fornazier et al., 2002a). The higher concentrations of CdCl₂ (0.025 and 0.05 mM) completely prevented the growth of the A. nidulans and at later stages there was evidence of a loss of dry weight; this may be due to autolytic breakdown of the mycelium.

**Cd accumulation**

Cd accumulated to high concentrations in the mycelium of A. nidulans, and was readily detectable after 24 h of CdCl₂ treatment even at the lowest concentration tested (0.005 mM). The rapid accumulation of Cd has been shown previously in higher plants, mainly in the root system (Pereira et al., 2002; Vitória et al., 2001). Furthermore, the greatest effect on the activities of the antioxidant enzymes tested was observed at the higher concentrations of CdCl₂ tested, suggesting that the accumulation in the mycelium is required to induce the changes observed for the antioxidant enzymes.

**Protein content**

After 12 and 24 h of incubation, there was little evidence of any effect of concentrations of CdCl₂ up to 0.025 mM on the total protein or on the type of protein present. However, the highest concentration of 0.05 mM caused a reduction in the protein content and also a loss of the higher molecular mass proteins and subsequent increase in the lower molecular mass forms. These data also suggest that the higher concentrations of CdCl₂ induced autolysis of the A. nidulans mycelium, with subsequent proteolytic breakdown.

**Superoxide dismutase activity**

The identification of three Mn–SOD isoenzymes in A. nidulans is different from previous reports of other fungi, where Cu/Zn–SOD isoenzymes have also been identified (Chary et al., 1994; Lee et al., 2001; Park et al., 1998). In a range of Aspergillus sp. including A. nidulans, Cu/Zn–SOD isoenzymes have been identified, based on the inhibition by potassium cyanide and on the N-terminal sequences (Holdom et al., 1996). We have also tested inhibitors of Cu/Zn–SOD but the SOD isoenzymes observed were resistant to cyanide (Fig. 2), suggesting that only Mn–SOD isoenzymes were present. Furthermore, the cyanide test system has been confirmed using plant extracts. In C. albicans, two genes encoding Mn–SOD and one encoding Cu/Zn–SOD have been identified (Lamarre et al., 2001). Only the SOD isoenzymes of Rhizopogon roseolus have previously all been classified as Mn–SOD (Miszalski et al., 1996).

The clear increase in SOD activity, which was a general response and not due to a specific SOD isoenzyme, suggests that Cd is inducing the formation of superoxide and/or other ROS. In N. crassa Cd did not induce any significant changes in the activity of SOD (Kapoor et al., 1990), whilst in R. roseolus (Miszalski et al., 1996) and S. cerevisiae (Romandini et al., 1992) Cd caused reduction in SOD activity. Most interestingly, in two studies of gene expression (Momose and Iwahashi, 2001) and protein synthesis (Vido et al., 2001) in S. cerevisiae, an induction of Mn–SOD but not Cu–Zn SOD has been demonstrated following Cd addition. In plants, variations in response to Cd have been noted. SOD activity remained constant in soybean (Ferreira et al., 2002) and crotalaria (Pereira et al., 2002), or declined in pea (Sandalio et al., 2001), whereas in the marine dinoflagellate Gonyaulax polyedra (Okamoto and Colepicolo, 1990), sugar cane (Fornazier et al., 2002b) and radish (Vitória et al., 2001), increases in SOD activity have been observed.

**Catalase activity**

CAT along with peroxidases is involved in the detoxification of hydrogen peroxide in all aerobic organisms. By analysis of specific mutants, Kawasaki and Aguirre (2001) showed that the order of migration of the individual CAT proteins following PAGE (most electronegative first) was D, B, A, C. In this current work, we determined that there were three forms of CAT activity in the mycelia of A. nidulans that could be separated by native PAGE (Fig. 4). As the catA gene has been shown to be expressed only in conidia (Kawasaki et al., 1997), it is possible by comparing the results with Fig. 4 in Kawasaki and Aguirre (2001), to suggest that band II is equivalent to catD, band II is catB and band I is catC. However, definitive experiments with mutants would be needed to confirm this suggestion.

Increase in CAT activity in response to growth in the presence of Cd was demonstrated (Fig. 4), indicating that the metal may be generating H₂O₂ in the cell. Such an increase during the time course of the experiment was dependent upon the concentration of CdCl₂.
applied. However, in contrast to the isoenzyme-unspecific SOD activity increase, the CAT band I clearly exhibited the major response to Cd stress, particularly after 12 h of exposure, which may equate to catC of the classification proposed by Kawasaki and Aguirre (2001). Somewhat surprisingly catC, the gene encoding the catC is expressed constitutively in A. nidulans and unlike catB and catD, has not been shown to be upregulated by a range of environmental stresses.

Among three strains of S. cerevisiae that had been subjected to Cd treatment, one exhibited a significant increase in CAT activity when compared to the untreated control (Romandini et al., 1992). In recent analyses of gene expression (Momose and Iwashashi, 2001), and proteomic responses (Vido et al., 2001) of S. cerevisiae, the addition of Cd, did not indicate that there was any major effect on CAT. However, in higher plant species, the majority of the studies that have examined the effect of Cd on CAT, have reported a decrease in activity, although there was some variation over longer time courses (Ferreira et al., 2002; Sandalio et al., 2001; Vitória et al., 2001). We therefore conclude that in A. nidulans, there is an induction of CAT activity as part of a resistance mechanism to oxidative stress, caused by Cd, although which CAT gene is involved is still not clear.

Glutathione reductase activity

It has been demonstrated that glutathione is an important antioxidant in the fungi, when defective mutants for glutathione synthesis exhibited hyper-sensitivity to H$_2$O$_2$ and to superoxide anions, both in the exponential and stationary culture growth phases (Grant et al., 1998; Jamieson, 1998). The importance of glutathione in Cd detoxification was obtained from the proteomic analyses carried out by Vido et al. (2001) in S. cerevisiae.

GR is needed to maintain a high ratio of reduced glutathione to oxidized glutathione, as the latter has been shown to be toxic, and this key role has been demonstrated by analysis of fungal mutants lacking the enzyme (Grant et al., 1998; Jamieson, 1998). GR activity increased significantly in the A. nidulans mycelia after 24 h incubation with 0.05 mM CdCl$_2$. There is little evidence in the literature to indicate that Cd has any effect on GR in the fungi. It should also be noted that induction of GR was not detected following treatment with Cd, in either the DNA microarray expression (Momose and Iwashashi, 2001) or the proteome analyses (Vido et al., 2001) carried out on S. cerevisiae. However, reports concerning higher plants have shown that a major effect of Cd is the stimulation of GR activity, as GSH is required for the detoxification of ROS and the production of phytochelatins (Cobbett, 2000; Fornazier et al., 2002b; Pereira et al., 2002; Vitória et al., 2001; Xiang and Oliver, 1998).

Previous research with A. nidulans Cd-resistant mutants indicated that mechanisms other than reduced cellular uptake are involved in Cd resistance (Cooley et al., 1986a,b). Although other peroxidases may be involved in the response of fungi to Cd, the results obtained in this paper have demonstrated that the activities of SOD, CAT, and GR increased following the growth of A. nidulans in the presence of CdCl$_2$. This work indicates the importance of determining the responses of individual isoenzyme activities, in as wide a range of different organisms as possible. This information should provide a better insight into the metabolic and genetic regulation of the stress response, which will be useful when considering strategies and methodological improvement in fungal bioremediation processes.

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