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Effect of Cadmium Ions on the Fungal Strain *Aspergillus fumigatus* 3_2 Isolated fromMetal Polluted Soil

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Abstract

The fungal strain *Aspergillus fumigatus* 3_2 was isolated from metal polluted soil (around the tailing Vlajkov vrah, Bulgaria). Effect of enhanced concentrations of cadmium ions (redox-inactive metal) on the growth and morphology, as well as the participation of oxidative stress in cadmium-induced toxicity, was reported. *A. fumigatus* demonstrated a high tolerance to cadmium, exhibiting remarkable growth in liquid and agar media. High metal concentrations affected fungal morphology and caused oxidative stress events such as changes in the level of reserve carbohydrates and oxidative damaged proteins. A sharp increase in trehalose content and accelerated consumption of glycogen in the presence of cadmium ions at concentration above 5 μg/ml were detected. In addition, a decrease in carbonylated proteins was measured, particularly pronounced at high concentrations (70 and 100 μg/ml). Cadmium ions exposure with 5, 20, and 50 μg/ml resulted in enhanced superoxide dismutase (SOD) level, but the higher concentrations (70 and 100 μg/ml) significantly inhibited this activity. Continuous decrease was also observed for catalase (CAT) activity. Probably, at higher concentrations of cadmium ions antioxidant enzymatic defence does not seem to be a major mechanism of cadmium tolerance in the strain *A. fumigatus*.
Key words: heavy metals, filamentous fungi, oxidative stress, biomarkers, antioxidant enzymes

Резюме

Щам *Aspergillus fumigatus* 3_2 е изолиран от почви в района на медни мини Влайков връх (България), замърсени с тежки метали. Проучен е ефектът на повишаващи се концентрации кадмиеви йони върху растежа и морфологията на моделния организъм, както и участието на оксидативния стрес в метал-индуцираната токсичност. *A. fumigatus* проявява растеж в течна и агарова среда в присъствие на кадмиеви йони до 200 мкг/мл, което доказва неговата висока толерантност. Едновременно с това се наблюдават изменения в морфологията на колониите. Третирането на културата с повишаващи се концентрации метални йони предизвиква промени в нивата на резервните въглехидрати и оксидативно увредените белтъци, което е указание за проявата на оксидативен стрес. Установено е рязко увеличение в съдържанието на трехалозата и ускорено усвояване на гликогена в присъствието на кадмиеви йони в концентрация над 5 мкг/мл. Наблюдава се понижение в количеството на белтъците, съдържащи карбонилни групи, особено силно изразено при високите концентрации (70 и 100 мкг/мл). Докато във вариантите с 5, 20 и 50 мкг/мл се наблюдава повишена активност на ензима супероксид дисмутаза (СОД), то при концентрации 70 и 100 мкг/мл тази активност значително се понижава. Отбелязва се и понижаване активността на ензима каталаза във всички третирани култури. Вероятно, антиоксидантната ензимна защита не е част от основния механизъм за толерантност на *A. fumigatus* към високи концентрации на кадмиеви йони.

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Introduction

Heavy metals are present in soils as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates, and hydroxides, or they may form part of the structure of silicate materials (indigenous soil content) (Leyval *et al.,* 1997). Some metals that have received more attention are mercury, cadmium, and lead, because of their highly toxic properties and their effects on the environment and the living organisms (see Singare *et al.,* 2012).

Cadmium is a very toxic metal - a significant environmental pollutant. This heavy metal or a metal trace element, is dispersed in natural and agricultural environments principally through human activities such as mining, refining, municipal waste incinerators, and fossil fuel combustion sources (Wagner, 1993), as well as natural rock mineralization processes (Sanità di Toppi and Gabrielli, 1999). Cadmium presence into agricultural soils resulted in the application of phosphatic fertilizers (Williams and David, 1976; McLaughlin *et al*., 2000), soil amendments with municipal sewage sludges, and atmospheric deposition (Wagner, 1993; Weissenhorn and Leyval, 1995).

In naturally polluted environments, the microbe's response to heavy metals toxicity depends on the concentration and the availability of metals, and on the action of factors such as the type of metal, the nature of medium and microbial species (Hassen *et al.,* 1998). One of the major mechanisms behind heavy metal toxicity includes production of reactive oxygen species (ROS). These highly reactive ROS can interact with various cellular components leading to oxidative damage of all cellular macromolecules. To minimize the damaging effects of ROS, aerobic organisms evolved both non-enzymatic and enzymatic antioxidant defense systems. Microorganisms have been shown to possess an ability to survive by adapting or mutating at high concentrations of toxic heavy metals. Fungi are also known to tolerate heavy metals (Gavrilesca, 2004; Baldrian, 2003).

The response of microorganisms towards toxic heavy metals is significant due to the interest they represent in the reclamation of polluted sites. Living organisms exposed environmentally to high metal concentrations follow various mechanisms to counter potential toxicity. Recent research indicates that cadmium induces oxidative damage in cells, and alterations in the activities of antioxidant enzymes (Yildirim and Asma, 2010). Although it was described that heavy metals are extremely toxic to a lot of living cells, little is known about the defence responses to metal-induced oxidative stress at subcellular level. We are interested in the way the fungal cell counteracts cadmium toxicity. The aim of this recent study is to investigate the cell response of the filamentous fungus, isolated from metal polluted soils, against cadmium. Changes in the fungal morphology and physiology as a result of cadmium ions treatment were observed. The role of antioxidant enzyme system in the cell response against cadmium toxicity was investigated.

Materials and Methods

Microorganism and Culture Conditions

The investigation was performed with filamentous fungus *Aspergillus fumigatus* isolated from a soil sample taken from a metal polluted region with strong industrial activity in the tailing Vlajkov vrah near Pazardzhik (Bulgaria). Submerged cultivation was performed in 500 ml Erlenmeyer flasks for 72 hours. Composition of the seed and production media, and the culture conditions were as previously described (Krumova *et al.,* 2009). For investigation of the effect of different cadmium concentrations, cultures were incubated with different concentrations of cadmium chloride, in order to achieve 5, 20, 50, 70, and 100 μg/ml of cadmium ions, added at the beginning of cultivation. Results were evaluated from repeated experiments using three parallel runs.

Macroscopic Study

To monitor the metal-resistance of the fungal strain, conidiospores were cultivated in Petri dishes (d = 10 mm) with beer agar, supplemented with various concentrations of cadmium chloride in order to achieve 50, 100, and 200 μg/ml for 7 days at 28ºC. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production, etc.

Cell-Free Extract Preparation

The cell-free extract was prepared as described earlier (Angelova *et al.,* 1995). All steps were performed at 0-4°C.

Enzyme Activity Determination

SOD activity was measured in cell-free extracts (CFE) by nitro blue tetrazolium (NBT) reduction (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of

NBT by 50% (A_{560}) and was expressed as units per mg protein (U/mg protein). Catalase was assayed by the method of Beers and Sizer (1952) in which the decomposition of hydrogen peroxide was analysed spectrophotometrically at wavelength of 240 nm. One unit of catalase activity was defined as the amount of enzyme that decomposes 1 mmol hydrogen peroxide per minute at an initial hydrogen peroxide concentration of 30 mmol/L, at pH = 7.0 and 25°C. The specific activity is expressed in U/mg protein.

Measurement of Protein Carbonyl Content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content, using the dinitrophenyl hydrazine (DNPH) binding assay (Levine *et al.,* 1990), slightly modified by Adachi and Ishii (2000).

Determination of Reserve Carbohydrates

In order to determine glycogen and trehalose content, a procedure, previously described by Becker (1978) and Vandecamen *et al.* (1989), and then modified by Parrou *et al.* (1997), was used. Soluble reducing sugars were determined by the Somogyi-Nelson method (Somogyi, 1952).

Other Analytical Methods

Protein was estimated by the Lowry procedure (Lowry, 1952), using crystalline bovine albumin as a standard. Dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman filter (Clifton, USA No. 4). The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Results

Effects of Cadmium on the Colony Growth and Morphology

The effect of cadmium on the growth of *A. fumigatus* 3_2 consisted of three components: the reduction of radial growth rate, inhibition of formation of conidia, and changes in colony morphology in the presence of heavy metal of up to 200 µg/ml (Table 1). Concentrations of up to 20 µg/ml cadmium ions did not significantly affect the colony growth (data were not shown). However, the exposure to the enhanced concentrations of cadmium ions resulted in a significant reduction in colony diameter in a concentration-dependent manner. At the highest concentrations applied (200 µg/ml), the model strain formed microcolonies.

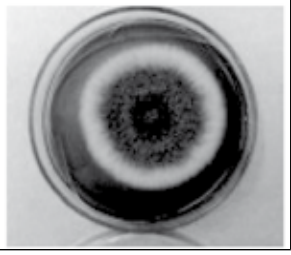
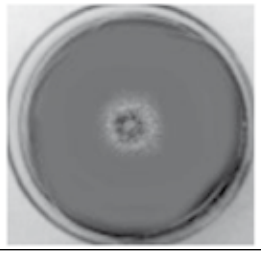
Table 1 also demonstrates the variability of morphological features in the presence of cadmium ions. Several authors have reported the formation of colorful mycelia in the presence of heavy metals on agar media (Darlington and Rauser, 1988). In the present study, changes in colony color from blue-green in the control variant, through yellow (at 50 and 100 µg/ml Cd^{2+}), to white (at 200 µg/ml) were observed.

Moreover, surface changes also occur in the presence of cadmium ions. The control variant demonstrated smooth velvety surface, gray-green aerial mycelium, and green substrate mycelium with white, jagged edge without fluid droplets. Presence of cadmium ions leads to forming of yellow velvet colony with streaked surface. With increasing metal concentrations, the culture showed delayed sporulation along with increase in content of cadmium ions.

Effect of Metal Concentration on Biomass Production under Submerged Cultivation

Growth of *A. fumigatus* was studied in relation to enhanced cadmium concentration (5, 20, 50, 70, and 100 µg/ml) under submerged conditions (Fig. 1). The results illustrated that the model strain can grow in the presence of a wide range of cadmium ion concentrations (5 - 100 µg/ml). However, the presence of cadmium ions influences the biomass production by decelerating the rate of growth

Table 1. Morphological changes in model strain in the presence of different concentration Cd ions

Cd^{2+} concentration [µg/ml]			
Control	50	100	200
			

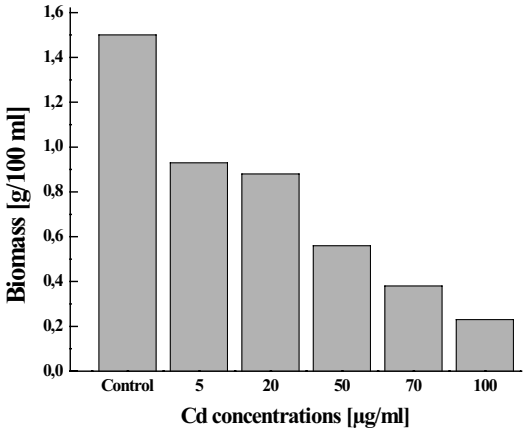


Fig. 1. Biomass content of *A. fumigatus* in presence of different Cd ions concentrations

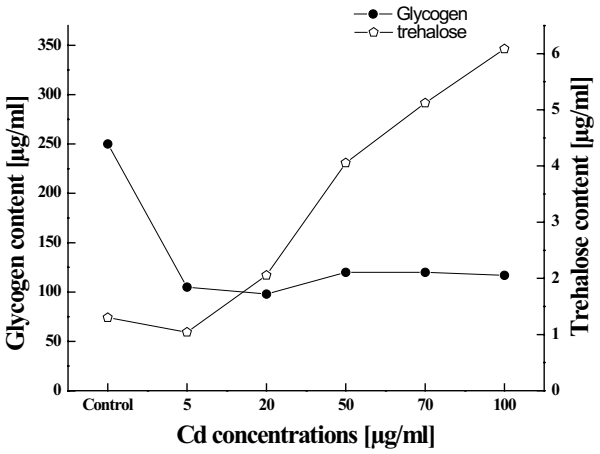


Fig. 2. Effect of enhanced concentrations of Cd ions on glycogen and trehalose accumulation

in comparison with the control medium. A clear trend of cadmium-induced decrease in biomass content in dose-dependent manner was observed. Concentrations of 5 µg/ml led to reduction in biomass by 38%, as compared with the control. Although the enhanced presence of cadmium ions in culture medium caused a significant decrease in dry mass content (about 80% of the control), the strain demonstrated mycelial growth even at 100 µg/ml.

Cadmium-Induced Toxicity and Oxidative Stress Biomarkers

Excess of heavy metals cause toxic effects in several ways, one of them being the excessive production of ROS, which disturb the cellular redox environment causing oxidative stress. To investigate whether cadmium ions interaction with *A. fumigatus* cells causes oxidative stress events, we evaluated the changes of reserve carbohydrate accumulation and oxidative damaged protein content after exposure to enhanced metal concentrations.

Figure 2 demonstrates the effect of cadmium ions on glycogen and trehalose level in the fungal cells. As the main source of carbon- and energy-storage in fungi, glycogen is an important factor for viability under stress conditions.

At low metal concentration (5 µg/ml), a abrupt reduction of the glycogen content was found (by 58% as compared with the control). The same glycogen level was determined in the cultures treated with higher concentrations (20 - 100 µg/ml). Thus, at each concentration, the glycogen accumulation in the fungal cells depends on presence of cadmium ions, but not on their content.

An opposite situation was observed for trehalose response to the metal-induced toxicity (Fig. 2). The interaction of fungal cell with 5 µg/ml cadmium ions did not cause significant change

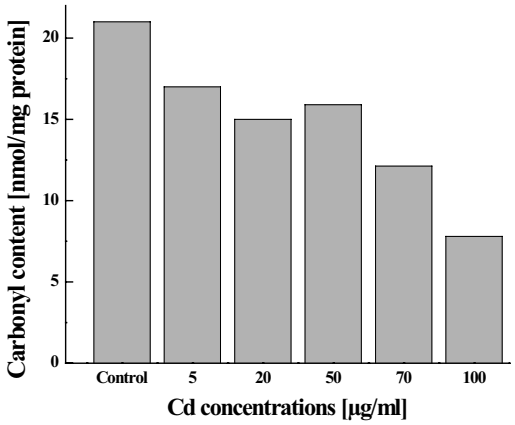


Fig. 3. Level of damaged proteins in the presence of different concentrations of Cd

in trehalose content, but the exposure to higher concentrations resulted in a sharp continuous increase. As seen in the figure, 2.5-fold increase in the trehalose level was found at application of 100 µg/ml cadmium ions.

The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relatively early formation and the relative stability of carbonylated proteins. The carbonyl content in the total proteins extracted from *A. fumigatus* cultures treated by enhanced concentrations of cadmium ions is presented in Fig. 3.

Unexpectedly, a tendency for gradual reduction in protein carbonylation level can be seen. The presence of 5, 20, and 50 µg/ml cadmium ions resulted in about 20 - 28% decrease in oxidative damaged protein content compared to the control. The results showed that at higher concentrations (50 and 70 µg/ml), the protein carbonyl content continued to decrease by 42 - 63% compared to untreated cultures.

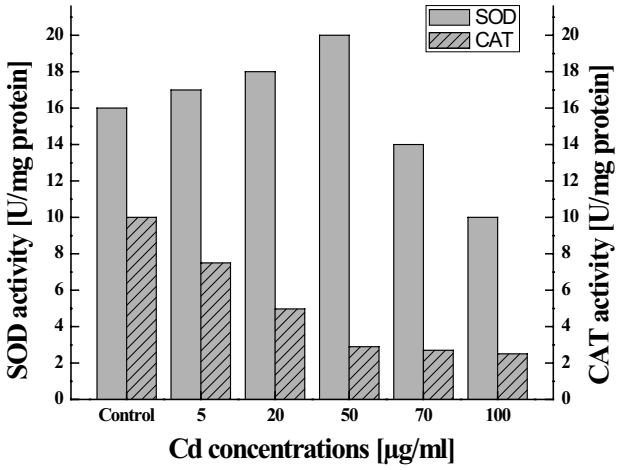


Fig. 4. Enzyme antioxidant activity in presence of Cd ions

Antioxidant Enzyme Activities in Presence of Cadmium Ions

The changes in activity of the main antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), in *A. fumigatus* cells, induced by cadmium exposure (from 5 to 100 µg/ml) are presented in Fig. 4.

As compared with the untreated cultures, SOD activity - responsible for the elimination of superoxide radicals in cells - gradually increased after exposure to the concentration of 5, 20, and 50 µg/ml. At cadmium doses of 70 and 100 µg/ml the SOD activity in the fungal cells decreased from 16.22 to 14.16 and 9.97 U/mg protein, respectively. In contrast, CAT activity in treated cultures was significantly lower than that in the control cells. In the presence of 50, 70, and 100 µg/ml cadmium ions only 25% of control activity was measured.

Discussion

Present data confirm that the fungus *A. fumigatus* 3_2 showed a higher tolerance to cadmium ions in comparison with other fungi, reported in the literature (Guelfi *et al.,* 2003; Todorova *et al.,* 2008). This strain exhibited growth at 100 and 200 µg/ml in liquid and agar media, respectively. Similar remarkable potential of growth in agar medium containing cadmium has been published for *Aspergillus versicolor*, *Pisolithus tinctorius*, *Terichoderma* sp., etc. (Mohammadian Fazli *et al.,* 2015; Carrillo-Gonzalez *et al.,* 2012; Joshi *et al.,* 2011). On the other hand, inhibition of growth was also found in both types of cultivation, under conditions of cadmium stress. Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrial, 2010). Ramsay *et al.* (1999) have found that the colony extension rates of several fungal species decreased under conditions of cadmium toxicity. Gadd *et al.* (2001) also reported a decrease in radial expansion of *Trichoderma viridae* and *Rhizopus arrhizus* in the presence of heavy metals, including cadmium. The authors noted the importance of glucose concentrations for metal-induced toxicity. *A. fumigatus* 3_2 demonstrated biomass formation under submerged cultivation in the presence of 100 µg/ml cadmium ions that were 20% of the control dry weight. The same concentrations (100 parts per million = 100 µg/ml) inhibited the dry weight of *Fusarium oxisporum* by 85% in comparison with the control (Golubović-Ćurguz *et al.,* 2010). Hassn *et al.* (2014) and Todorova *et al.* (2008) reported a similar trend in growth in the presence of cadmium ions of *Isaria javanica* and *Aspergillus niger* B77, respectively.

Besides the growth, cadmium, in concentration between 50 and 200 µg/ml, causes pronounced morphological aberrations in the *A. fumigatus* cells. This may be direct or indirect result of cadmium effects on cell division, protein synthesis, and cellular organelles such as mitochondria (Trevors *et al.,* 1986). According to Zafar *et al.* (2007), the morphological changes may be due to the vast detoxification/tolerance mechanisms that the fungus uses. Significant differences in pigmentation in *A. fumigatus* colonies were also observed in metal-treated colonies in comparison with the control mycelia. Similar decoloration occurred by increasing the cadmium concentration in medial growth for *A. versicolor*, *Cladosporium* sp., and *Paecilomyces* sp. (Mohammadian Fazli *et al.,* 2015). As it has been previously suggested, the production of pigments in fungal cell is accompanied by precipitation of metal ions on the cell wall (Gruhn and Miller, 1991). Opposite results were reported by Baldrian and Gabriel (2002) about the effect of cadmium on the morphology of *Piptoporus betulinus*. They established that 50, 100, and 250 mM cadmium ions did not cause color changes.

Many studies showed significant increase in ROS content during cadmium exposure that leads to oxidative stress. Cadmium itself is unable to generate free radicals directly, however, indirect formation of ROS involving the superoxide radical and hydroxyl radical has been reported (Aflaine *et al.,* 2015). In addition, the generation of non-radical hydrogen peroxide, which itself, in turn, maybe a significant source of radicals *via* Fenton chemistry, has to be noted. In this mechanism,

cadmium can replace iron and copper in various cytoplasmic and membrane proteins, thus increasing the amount of unbound free or chelated copper and iron ions participating in oxidative stress and cadmium-induced toxicity. Our results confirm the enhanced ROS generation, demonstrating changes of the oxidative stress biomarkers, such as reserve carbohydrates and oxidative damaged proteins. Glycogen and trehalose are the two major reserve carbohydrates in the fungi and can represent up to 25% of the dry cell mass, depending on the environmental conditions (Scebba et al., 2006). The changes in reserve carbohydrate content in fungal cells have been demonstrated for different adverse conditions (Parrou and Francois, 1997b; Ocón *et al.,* 2007; Kostadinova *et al.,* 2012). In the present study, there was a sharp increase in trehalose content of cells, grown in presence of cadmium ions in concentration above 5 μg/ml. This increase is of similar magnitude to the one observed in *Corollospora lacera* and *Monodictys pelagica* after exposure to cadmium ions (Taboski *et al.,* 2005) and *Candida albicans* under conditions of heat shock (Scebba *et al.,* 2006). The accumulation of trehalose has been correlated with higher tolerance to a variety of abiotic stresses (Elbein *et al.*, 2003). Previous research studies have reported that trehalose is necessary for the growth and stress adaptation. It is directly involved in the synthesis of other compounds, energy production, and membrane stabilization, acting as regulators of gene expression and sugar-sensing signal system (see Xie *et al.,* 2014). By rapidly reacting with ROS, trehalose would prevent their reaction with proteins and other cellular constituents (*e.g.* DNA, RNA, or lipids) (Benaroudj *et al.,*2001; Luo *et al*., 2008). Furthermore, Benaroudj *et al.* (2001) demonstrated the ability of trehalose to reduce oxidant-induced modifications of proteins and its capacity to prevent protein aggregation.

Glycogen is also known as useful biomarker since its changes are not transient or sensitive to non-toxicant stress. In contrast to the reported increase in glycogen level under stress conditions, our results revealed that cadmium exposure significantly reduced its content in the *A. fumigatus* cells. A similar decrease has also been highlighted in *A. niger* and *Trichosporon cutaneum* exposed to cadmium (Todorova *et al.,* 2008; Lazarova *et al.,* 2014). The decrease in energy content in cells, exposed to cadmium, could be explained by the energetic cost of tolerance, offsetting the stress produced by the toxicant (Sornom *et al.,* 2012). During stress conditions, the cell must regulate the flux of glucose into trehalose generation, glycogen synthesis, pentose phosphate shunt, and glycolysis. The cell may buffer its intracellular glucose levels by the continued flux into and out of its glycogen stores. A progressive glycogenolysis (breakdown of glycogen(n) to glucose-1-phosphate and glycogen(n-1)) in the cells has been found (Emad *et al.,* 2005).

Generally, the level of oxidatively damaged (carbonylated) proteins increases during oxidative stress. This situation indicates that the quantity of generated ROS exceeded the capacity of the antioxidant defensive system. Published data demonstrated that the exposure of different aerobic cells to cadmium ions caused a remarkable increase in carbonyl formation, indicating that cadmium promoted a high protein oxidation (Emad *et al.*, 2005; Aflanie *et al.,* 2015). In contrast, present study illustrated a decrease of carbonylated proteins content in *A. fumigatus* cells treated with cadmium. Similar data have been found for *Trichosporon cutaneum* R57 treated with 5 and 10 mM cadmium sulfate (Lazarova *et al.,* 2014). One possible explanation could be based on both the enhanced degradation of proteins by proteases and aggregation of heavily oxidized proteins. Oxidized proteins serve as better substrates for proteolytic digestion. It has been suggested that protein oxidation could predispose it to ubiquitination, which, in turn, would be a target for proteasomal degradation (Cabiscol *et al.*, 2000). At the same time, degradation of oxidized proteins removes potential toxic fragments and provides aminoacids for new protein synthesis (Pena *et al.,* 2008).

The fungal response to cadmium-induced cells includes activation of antioxidant enzymes. SOD and CAT are important components of defensive mechanisms in fungi. SOD catalyzes the dismutation of the superoxide radicals into either ordinary molecular oxygen or hydrogen peroxide. CAT is the most important enzyme for the regulation of intracellular hydrogen peroxide levels (Blokhina *et al.,* 2003). Some researchers have indicated the activity of antioxidative enzymes is elevated in fungal cells under cadmium stress (Ott *et al.*, 2002; Todorova *et al.,* 2008; Lazarova *et al,* 2014). A cadmium-stimulated increase in SOD and CAT activity was also observed in *Aspergillus nidulans* (Guelfi *et al., 2003).* But, our present results showed that the concentrations of cadmium ions above 50 μg/ml resulted in about 20 or 38% decrease in SOD activity. A significant decrease was also observed for CAT activity. According to Ott *et al.* (2003), the addition of cadmium

resulted in antioxidant enzyme fluctuations relative to controls in fungal cultures. In *Neurospora crassa* cadmium did not induce any changes in SOD level, whilst in *Rhizopogon roseolus* and *S. cerevisiae* cadmium caused reduction in SOD activity (see Guelfi *et al.,* 2003). Similar results have been reported on cadmium-stress response in different plants (Scebba *et al.,* 2006; Zhang *et al.,* 2015). Probably, besides the detoxification function, antioxidant enzyme molecules may also be sensitive targets of cadmium toxicity (Gallego *et al.,* 1996). The reduction in SOD activity can be attributed to the inhibition of enzyme activity by excess hydrogen peroxide content that is a product of accelerated superoxide dismutation (Malar *et al.,* 2014). At the same time, CAT inhibition could be explained by the increased accumulation of hydrogen peroxide. Moreover, induction of stress proteins and/or non-enzymatic antioxidant formation can be included in the defence system (Radhakrishnan, 2010).

Conclusion

Altogether, our results show that *A. fumigatus* 3_2, isolated from polluted soil, contain different mechanisms to cope with toxicity of cadmium ions. High metal concentrations affected the fungal growth and morphology. In addition, exposure to cadmium ions clearly resulted in oxidative stress events such as changes in the level of reserve carbohydrates and oxidative damaged proteins. Our study showed a dose-dependent alteration in the *A. fumigatus* antioxidant status. While at low cadmium concentrations, SOD and CAT provide defence against metal toxicity, the higher cadmium level caused significant inhibition of the activities of both enzymes. Apparently, scavenging of the produced ROS by antioxidative enzymes does not seem to be a major mechanism of cadmium tolerance in the model strain under extreme metal concentrations.

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