Egypt. J. Microbiol. **49**, pp. 99 - 117 (2014)

The Response of *Fusarium solani* to Cd(II) and Cu(II) in Pure Culture

Manal T. El-Sayed
Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt .

F*USARIUM solani* was isolated from soil receiving long term application of sewage and industrial effluents as irrigates and identified according to morphological characteristics and DNA sequence analysis. Its selection was based on the fact that work was done on Cd(II) and Cu(II) toxicity on *F. solani*. The minimum inhibitory concentration (MIC) values of *F. solani* for Cd(II) and Cu(II) were 900 mg/l and 600 mg/l, respectively. Scanning electron microscopy (SEM) showed that the cell surface morphology and surface area/volume ratio changed after Cd(II) and Cu(II) stress. Transmission electron microscopy (TEM) revealed that the cell wall thickness doubled, an increase in the number of intracytoplasmic vesicles and some cells were completely lysed after exposure to Cd(II) stress. Also, cell wall was outlined by Cu(II) particles and cells attracted Cu(II) deposits. The presence of Cd(II) and Cu(II) was confirmed by energy dispersive X-ray (EDX) microanalysis. The effects of Cd(II) and Cu(II) on radial growth, biomass production, protein content, total antioxidant and total thiol were investigated. Activities of polyphenol oxidase (PPO), glutathione reductase (GR) and peroxidase (POD) after Cd(II) and Cu(II) stress were determined.

Keywords: *F. solani,* Growth response, Cd(II), Cu(II) , Stress.

Heavy metals are a group of metals with density greater than 5 g/cm^3. They persist in nature and consequently tend to accumulate in food chain (Lima e Silva *et al.,* 2012). Heavy metals are stable and persistent environmental contaminants since they can not be degraded or destroyed. Therefore their toxicity poses major environmental and health problems and requires a constant search for efficient, cost-effective technologies for detoxification of metal-contaminated sites. Some heavy metals such as nickel, iron, copper and zinc are essential microelements in life cycle of prokaryotes and eukaryotes. Conversely, the high concentration of these metal species might be a great threat to all organisms including human (Ahmad & Kibret, 2013). Other heavy metals such as Cd(II), Pb(II) and Hg(II) are nonessential and are known to cause severe damage in organisms even at very low concentrations (Kaplan, 2013). Toxic effects may be caused by a number of mechanisms: (a) The blocking of functional groups of biologically important

molecules (*e.g.,* enzymes and transport systems for essential nutrients and ions); (b) The displacement and/or substitution of essential metal ions form functional cellular units; and (c) Induction of cellular generation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radical. High levels of ROS can oxidize proteins, lipids, and nucleic acids. This may result in modification and inactivation of enzymes as well as disruption of cellular and organellar integrity (Sharma & Dietz, 2009). Some microorganisms possess a variety of mechanisms to maintain metal homeostasis and prevent poisoning. Heavy metal resistance in microorganisms may result from the ability to prevent uptake (avoidance). This is achieved by adsorption of toxic metal ions to cell-associated materials and/or cell wall components (De Philippis & Micheletti, 2009) or secretion of metal binding organic compounds to the surrounding environment (Levy *et al.*, 2008). Metal resistance may also result from the ability to cope with high amounts of heavy metals inside the tissues (tolerance), an active process that involves the uptake (absorption) and accumulation of the metal ions inside the cell (Kaplan, 2013). Smirnoff (1993) has divided antioxidative mechanisms into two categories: one that interacts with active forms of O_2 and maintains them at low levels. Some of the enzymes involved here are superoxide dimustases (SODs), catalases (CATs), and ascorbate peroxidases (APXs). In other system, oxidized antioxidants like glutathiones (GSHs), glutathione reductases (GRs), ascorbate and mono-and dihydroascorbate reductase are regenerated. Todorova *et al.,* (2008) showed that Cd(II) stress induced reactive oxygen species generation in *A. niger* B77 and tolerance of the strain being highly correlated to the efficiency of its antioxidative defense system.

Responses of several fungi to heavy metal toxicity have been extensively studied but less work was done on Cd(II) and Cu(II) toxicity on *Fusarium solani*. Several microorganisms have been shown to remove toxic metals from solution. To select a particular species for bioremediation of toxic metals, it is very important to identify the effects of those metals on that particular species and cellular responses elicited by that species to counteract such toxic environment and thus understanding mechanisms involved in tolerance or detoxification of toxic metal stress.

This study was undertaken to examine the responses evoked by *F. solani* to counter the toxicity of Cd(II) and Cu(II).

Materials and Methods

Isolation and morphological identification

The fungal strain used in the present study was isolated from soil receiving long term application of sewage and industrial effluents as irrigates. The isolated fungus was identified on the basis of its morphological characteristics and microscopical examination (Seifert, 1996). *Fusarium solani* was selected based on the fact that less work was done on Cd(II) and Cu(II) toxicity on *F. solani*.

DNA extraction PCR amplification and sequencing

PCR amplification

The fungal isolate was identified based on its rDNA sequence (18S-28S rRNA, flanking ITS 1, 5.8S rRNA, and ITS 2). Two sets of primers were used: Fw 18S rRNA 5'-GTAACAAGGTTTCCG TAGGT-3', Fw ITS1 5'-AGGATCATTACCG AGTGCG-3', Rev ITS2 5'-CCTGGAAAAAAAG TATGCTTAAGTTCGGCCG-3'. GC-3', and Rev 28S rRNA 5'- The PCR reaction contained 5-10 mg DNA, 5 µl of a 10× reaction buffer, 1.25 U Taq polymerase, 200 µM dTNP, and 0.2 mM of each primer in a total volume of 50 µl. The designed PCR protocol included 35 cycles of denaturation at 95 °C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 1 min. The PCR amplicon (Fermentas, Germany) prior to DNA sequencing. The purified PCR products were sequenced using an ABI 377 DNA Autosequencer (PerkinElmer, Applied Biosystems Div., Waltham State, USA) based on the same primers as mentioned above. The retrieved sequence (18S-28SrRNA) was deposited in the GenBank and BLAST-submitted for phylogenetic tree construction (http:// www.geno me.HP/tools/.clustals) (Thompson *et al.*, 1994).

The strain was routinely mentioned on potato glucose agar (PDA) slants and stored at 4^{o}C for the development of the strain, it was grown for 72 h at 30^{o}C on PDA.

Materials

Oxidized glutathione, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and nicotineamide adenine dinucleotide phosphate (NADPH) were purshed from Sigma-Aldrich (St Louis, MO, USA). Folin reagent was obtained from El-Gomhoryah Company, Egypt. All other chemicals were of analytical grade.

Stock metal solutions were prepared by dissolving appropriate quantities of $CdCl_2$ and $CuSO_4.4H_2O$ salts in double distilled water. The stock solutions were diluted further with deionized distilled water to obtain working solutions of different concentrations.

Determination of minimum inhibitory concentration (MIC)

A sterilized solutions of $CdCl_2$ and $CuSO_4.4H_2O$ were aseptically added to the sterilized PDA medium to get the final concentrations ranged from 0 to 1000 mg/l for mineral salt formulations. The plates were centrally inoculated with 5 mm fungal plugs from 4 days old fungal colonies in three replicates and were incubated at 30°C for 6 days. MIC was identified as the minimum concentration of metal ions that inhibited visible growth of *F. solani.*

Scanning electron microscopy investigation (SEM)

Fusarium solani cells were fixed in 2.5% glutaraldehyde at 4°C, for 24 h and then post-fixed in 1.0% osmium tetraoxide at room temperature for 1 h (Harely & Ferguson, 1990). Samples were then dehydrated in acetone, coated with gold and examined using a Jeol scanning electron microscope (JEM-1200XII) (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Morphometric analysis

Dimensions of macroconidia were directly measured from the SEM photographs to calculate cell volume (v) and surface area (A) by the following equation, normal and stressed yeast

$$V\ (\pi m^3) = \pi\ r^2 h$$

$$A\ (\pi m^2) = 2\pi\ r^2 + 2\pi\ r\ h$$

where r and h are radius and length of a macroconidium in µm (Neumann *et al.*, 2005). Mean cell dimension of the *F. solani* were measured. Average cellular volume and surface area were calculated from normal cells. Cells showing deformations/depression were not considered.

Transmission electron microscopy investigation (TEM)

Fusarium solani (native, 500mg Cu/l-treated and 800 mg Cd/l-treated) cells were fixed in 2.5% glutaraldehyde for 3 h (Gupta & Berridge, 1966), washed twice with 0.2 M phosphate buffer of pH 7.4 for 30 min, then post-fixed in 1.0% osmium tetraoxide for 2 h (Palade, 1952). After that, the cells were washed with phosphate buffer for 30 min. All the previous steps of fixation were carried out at 4°C. Samples were dehydrated in a graded ethanol series (50%-100%). They were passed through three changes of acetone: ethanol (1:2, 1:1 and 2:0) for 10 min each and embedded in epoxy medium (Epon 812) (Luft, 1961). Blocks were sectioned with a diamond knife (ultramicrotome RMC USA) into ultrathin section about 70 nm. These ultrathin sections were constructed with uranyl acetate (Sptempack & Ward, 1969) followed by lead citrate (Reynolds, 1963) each for 30 min. Transmission and photographing were done using a JEOL-1010 electron microscope (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Energy dispersive X-ray microanalysis (EDX)

Cu(II) and Cd(II)-loaded (0.5 mg Cu/ml and 0.8 mg Cd/ml) biomass were used for energy dispersive X-ray microanalysis using X-ray micro analyzer (Model Oxford 6587 INCA x-sight) attached to JEOL JSM-5500 LV scanning electron microscope at Regional Center of Mycology and Biotechnology, Cairo, Egypt

Effect of Cd(II) and Cu(II) stress on F. solani growth

To investigate the heavy metal stress response in *F. solani*, the changes in radial growth, biomass production, the concentration of protein content, total antioxidant and total thiols and the activity of peroxidase (POD), polyphenol oxidase (PPO) and glutathione reductase (GR) were determined.

To study the effect of Cd(II) and Cu(II) on radial growth, the metal ions treated plates were inoculated as mentioned before. The diameters of the fungal colonies were measured in millimeters (mm) at certain intervals.

To study the effect of metal ions on the biomass production of *F. solani*, the fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of potato glucose broth (PD). A sterilized solutions of metal ions were aseptically added to the sterilized medium to get the final concentration ranging from 0-1000 µg/ml.

Then incubated at 30°C for 6 days on a rotary shaker at 125 rpm. The biomass was harvested and dried to a constant weight at 60°C.

Mycelium extract

The effects of Cd(II) and Cu(II) on the concentration of total soluble protein, thiol contents and total antioxidant and on the activity of POD, GR and PPO were determined. The fungal mycelia were ground using a cold mortar in an ice bath with 50 mM cold phosphate buffer (pH 7.0) of 50 mM EDTA. The cell suspensions were centrifuged at 3824 g for 15 min at 4°C. The supernatants of cell homogenate after centrifugation were used to elucidate the tolerance mechanism of *F. solani* to Cd(II) and Cu(II).

Polyphenol oxidase

Polyphenol oxidase (PPO) activity was determined according to Bergmeyer *et al.* (1974). Briefly, the reaction mixture contained 200 µl of enzyme preparation in 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mM guaiacol, 5 U/ml horseradish peroxidase and 10 mM catechol as substrate, in a total volume of 1 ml. The reaction was incubated for 60 min at 30°C. The reaction was frozen for 10 min, the developed color was measured at 436 nm. One unit of the enzyme was expressed by the amount of enzyme that released 1 µmol H_2O_2 per min under optimal assay conditions.

Glutathione reductase

Glutathione reductase (GR) activity was determined spectrophotometrically according to the method of Barata *et al.* (2000). The reaction mixture contained 3ml of 100mM potassium phosphate buffer (pH 7.5) containing 1 mM 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 reduction of oxidized glutathione were monitored at 412nm for 2 min.

Peroxidase assay

Briefly, the reaction mixture contained 200 µl of enzyme preparation in 0.1 M potassium phosphate buffer (pH 7.0), 2.5 mM Gauiacol and 1 mM H_2O_2 in a total volume of 1 ml. The reaction was incubated for 60 min at 30°C. After stopping the reaction, the developed color was measured at 510 nm (Bergmeyer, 1974). Activity was calculated as described above. One unit of POX was expressed by the amount of enzyme decomposition of one µM of hydrogen peroxide per minute at 25°C, and pH 7.0 under standard assay conditions.

Total antioxidant

Concentration of the total antioxidant of the crude fungal extract was determined by the ferric-thiocyanate method (Gupta *et al.*, 2004) with slight modifications. In brief, 1 ml of the crude enzyme preparation was mixed with 0.2 ml of 20 mM ferrous chloride and 0.2 ml ammonium thiocyanate (30%). After incubation for 10 min, the developed red color was measured at 500 nm. Ascorbic acid was used as standard.

Protein measurement

The method of Lowry *et al.* (1951) was used to measure extracellular and intracellular protein.

Assay of total thiol content

For total thiol assay, Ellman (1959) method was followed. Three milliliters of samples was mixed with 2 ml phosphate buffer (pH 7.0) and 5.0 ml distilled water and they were mixed well to get a 10 ml reaction mixture. Twenty micro liters of 0.01 M DTNB solution was added to 3 ml of the reaction mixture, shaken well and absorbance was recorded at 412 nm.

Results and Discussion

Molecular identification of the isolate

The sequence data of the isolate was deposited to GenBank with accession number of KJ623702.

Heavy metal tolerance

Metals and their compounds interact with fungi in various ways depending on metal species, organism and environment. In addition, fungal metabolic activity can influence metal speciation and mobility (Ahmed *et al.,* 2011). Metal toxicity can be indicated by the minimum inhibitory concentration (MIC). Results showed a higher fungal tolerance to Cd(II) (up to 900 mg/l) than to Cu(II) (600 mg /l). MIC of Cd (II) is 180000 fold the maximum admissible Cd(II) concentration in drinking water (0.005 mg /l) and that of Cu(II) is 6.0 fold the maximum admissible Cu(II) concentration in drinking water (0.1 g/l) (WHO, 2008). There was no pigmentation on PDA plates amended with either 50 mg/l concentration of Cd(II) as well as these of Cu(II). On the other hand, there was a greenish yellow pigment turned dark brown (at the end of incubation period) in PDA plates amended with 100-900 mg/l concentration of Cd(II). Gabriel *et al.* (1996) reported that mycelia pellets of *Daedalea quercina* harvested from 1 mmol/l Cd(II) had a brownish-yellow color while the control samples remained white. This pigmentation may be due to Cd(II) stress. The nature of these changes can‘t be discussed without a detailed chemical analysis. Comparable findings were obtained by Hefnawy & Razab (1998) . Ezzouhri *et al.* (2009) mentioned that the blue color of *Fusarium.* sp. mycelia on agar media amended with Cu(II) may be due to binding of Cu(II) ions to the fungal cell wall. Ban *et al.* (2012) reported that the colony of *Gaeumannomyces cylindrosporus* turned dark or brown on agar medium under Pb(II) concentrations. Color changes of mycelia exposed to metals may be an indicator of metal complexation (Machuca *et al.,* 2001). Comparable results were obtained by Huang *et al.* (2010). The reasons for color changes of mycelia are complex and need further studies.

Effect of Cd(II) and Cu(II) on F. solani radial growth and biomass production

Heavy metal toxicity influences several aspects of the fungal growth as the lag phase of the fungi, the growth rate, the density of the mycelium and the biomass

production (Jones & Hutchinson, 1988). A preliminary experiments were carried out to establish the range of $CdCl_2$ and $CuSO_4$. $4H_2O$ concentrations and exposure time that were able to inhibit mycelium growth (data not shown). It was observed that Cd(II) concentrations of 900 mg/l (solid media) and 700 mg/l (liquid media), respectively inhibited the growth of *F. solani* completely. The lower concentrations of Cd(II) tested caused a slight stimulation of growth by forming complexes with constituents of the culture medium, which could allow essential trace elements to become available to the fungus (Cooley *et al.,* 1986). The higher concentration of Cd (II) inhibited the growth of *F. solani* could be due to autolytic break down of the mycelium (Guelfi *et al.,* 2003). An increase in the length of the lag phase was evident in concentrations 400 and 500 mg/l of Cu(II). A complete inhibition in the radial growth and biomass production was observed at 600 mg/l (solid media) and 400 mg/l (liquid media), respectively. Copper is toxic to most microorganisms at elevated concentrations, largely through enzyme inhibition, the oxidation of membrane components, which might be related to the ability of copper to generate toxic hydroxyl radicals (Melo *et al.,* 2004). In liquid medium, the metal toxicity becomes more evident when the medium is supplemented with lower concentrations than agar plates. This appearant abnormality is due to the more intimate contact between the cells and the ions and also due to the absence of the protective chelating effect of the agar (Ruta *et al.,* 2010).

Electron microscopy analysis

SEM analysis was carried out to observe the differences in the surface morphology after Cu(II) and Cd(II) exposure. Prior to heavy metal stress, cells of *F. solani* had smooth, turgid and uniform cell surface with macroconidia with about 11 µm length (Fig. 1a and b). Macroconidia from Cd(II)-stressed cultures were extremely elongated (20 µm length), became flaccid (Fig. 1c). Severe inhibition in conidiation was noticed also rupture and extrusion of cellular contents occurred (Fig. 1d). Formation of chlamydospores was observed (Fig. 1e). On the other hand, exposure to Cu(II) had no noticeable effect on conidiation. Slightly wavy surface of macroconidia with a decrease in turgidity (Fig.1f). Severe flaccidity, rupture and extrusion of cellular contents were noticeable on mycelia (Fig. 1g) than on conidia. The disruption of cellular and organelle membranes are among the toxic effects of heavy metals (Gadd, 1992). Average dimension of native cells was 1.1-1.2 µm by 9.34-9.5 µm and the surface area\volume ratio (A\V) was 2.43. In Cu(II)-stressed cells average dimension was 1.67-1.75 µm by 8.0-8.12 µm and A\V was 1.39. In Cd(II)-stressed cells average dimension was 1.9-2.1 µm by 20-20.13 µm and A\V was 1.15. In conclusion, the reduction in A\V due to Cu(II) and Cd(II) stress was 42.72 and 52.55%, respectively. The relative decrease in cell volume plays a key role in the consequent reduction in attachment /uptake sites on the cell surface for the heavy metals and lowering the toxic effects of environmental stress factors (El-Sayed, 2013).

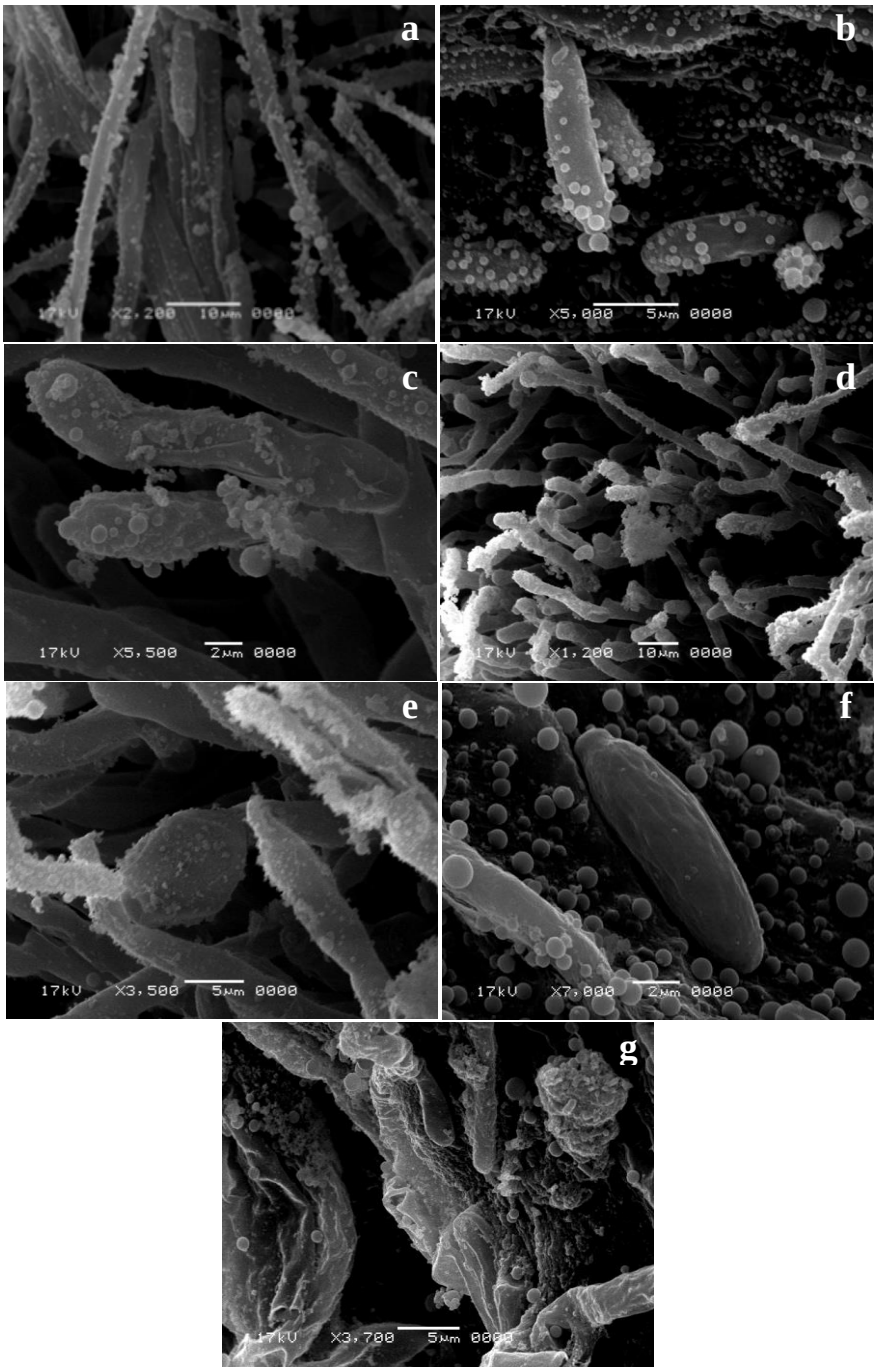


Fig. 1. SEM of *F. solani* (a and b) native cells, (c-e) Cd(II)-stressed cells, (f and g) Cu(II)-stressed cells

The presence of Cd(II) and Cu(II) was confirmed by EDX microanalysis which revealed the presence of Cd(II) and Cu(II) signals along with other ions (Fig. 2a and b).

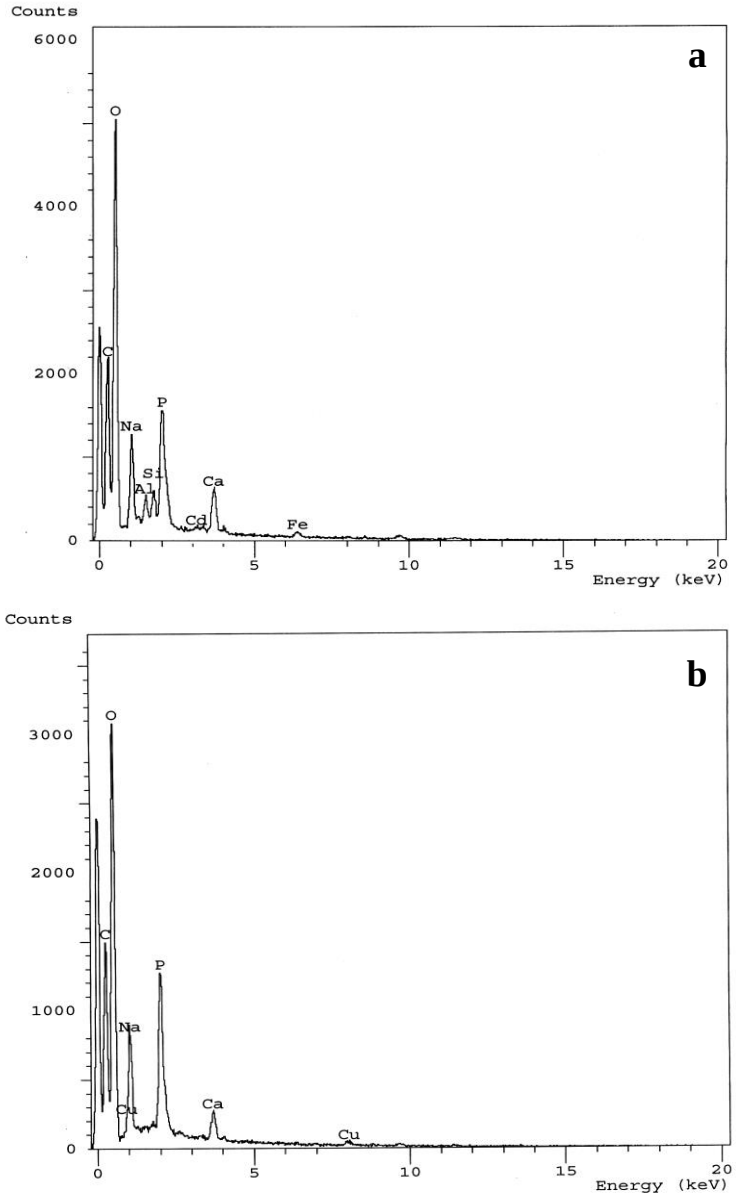


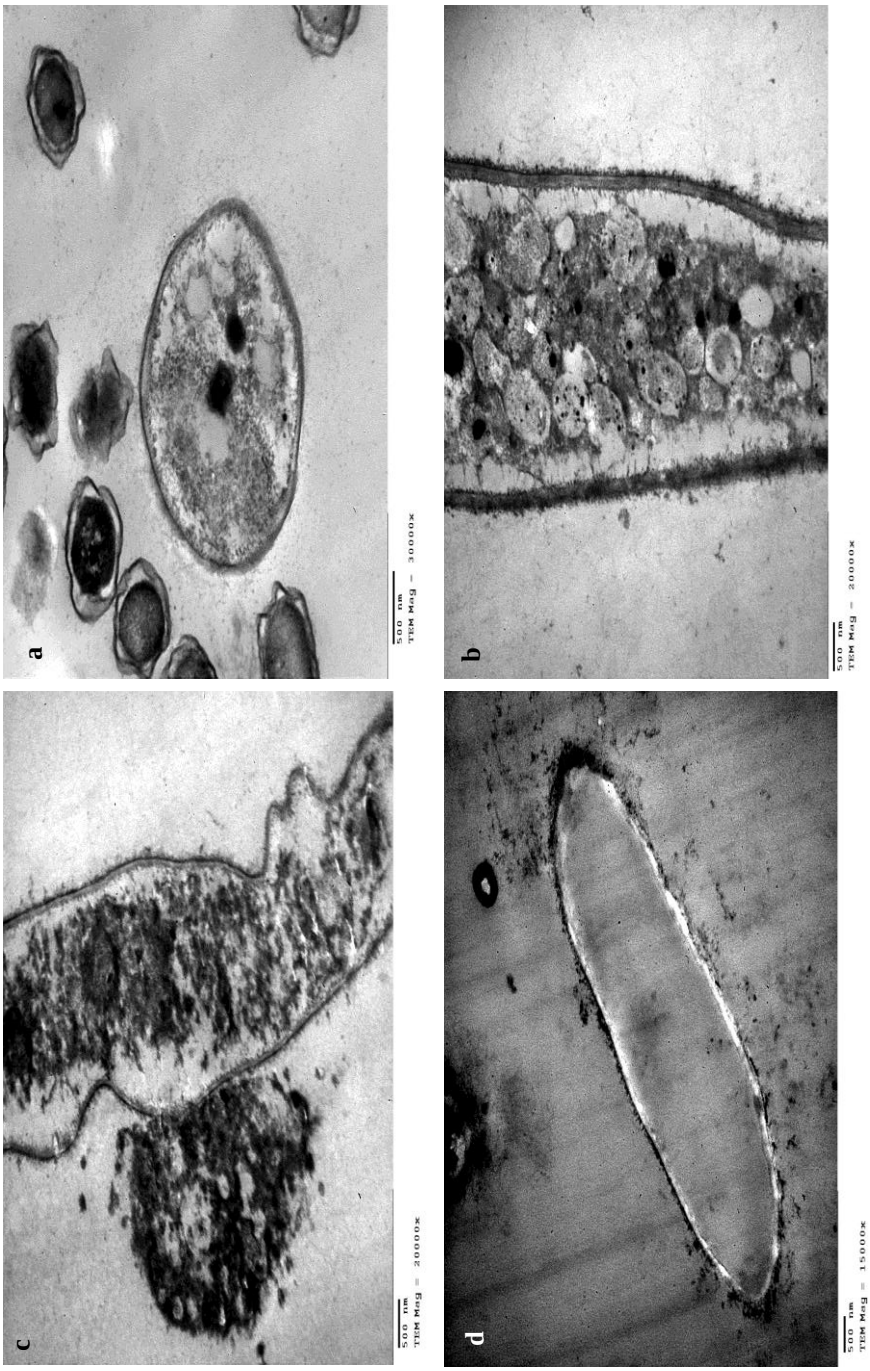
Fig. 2. EDX of *F. solani* (a) Cd(II)-exposed cells, (b) Cu(II)-exposed cells .

TEM observations of native, Cu(II) and Cd(II)-stressed cells (500 and 800 mg/l, respectively) were represented in Fig. 3a-h. Ultrathin sections of metal-less cells revealed a distinct cell wall (107 nm in thickness), clear cytoplasm with few electron dense areas probably representing the genetic material and cytoplasmic deposits and few number of intracellular vesicles (Fig. 3a). TEM micrographs of Cd(II)-loaded cells showed that the cell wall thickness of some cells doubled (2oonm). Cd(II) particles dotted on the cell wall and intracellularly with an increase in the number of intracytoplasmic vesicles (Fig. 3b). In this connection, Lima e Silva *et al.* (2012) found that a considerable increase in the number of intracellular vesicles could be related to an increase in the synthesis of lipid compounds that usually do not have metal affinity but which play an important role as a carbon reserve in microorganism under stress. Decreasing the bioavailability of the toxic metals/metalloids can be managed through extracellular complexation, precipitation and binding to cell wall constituents (Pòesi, 2011). In some cells, the invagination and undulation in cell wall can be observed. Some cellular contents without either cell wall or plasma membrane are found (Fig. 3c). Empty cells are showed as a result of the complete lyses of the cellular contents and only Cd(II) precipitates appear either on cell wall or intracellularly (Fig. 3d and e, respectively). After Cu(II)-stress, cell wall outlined by Cu(II) particles and cells appear as a magnet that attracts Cu(II) deposits and intracellular contents are entirely converted into single large vesicle (Fig. 3f), Cu(II) precipitates are observed extracellularly (Fig. 3g) and rupture of cell wall and extrusion of cellular contents (Fig. 3h).

Changes of antioxidant substances in F. solani under Cd(II) and Cu(II) stress

The contents of soluble protein, total antioxidants and activities of GR, PPO and POD were measured to detect the responses of antioxidant substances to Cd(II) and Cu(II). Figure 4 showed that Cd(II) treatments caused a sharp increase in soluble protein content. At 700 mg/l Cd(II), protein content increased to 366% of the control. A significant increase in protein content occurred at 50 mg/l Cu(II) (305%, with respect to control) and a subsequent decrease in protein with respect to control was observed. In most fungi, the presence of bivalent heavy metals like Cd(II) and Cu(II) induces the production of intracellular binding compounds. These can be divided into two main groups; metal binding oligopeptides containing cysteine-glutathione (GSH), phytochelatins and related compounds and proteins such as metallothioneins. Both types of compounds can be produced simultaneously (Baldrian, 2003).

Antioxidants (both enzymatic and nonenzymatic) provide a protection against deleterious metal-mediated free readical attacks (Volka *et al.,* 2013). Thus , the influence of Cd(II) and Cu(II) on nonenzymatic antioxidant production was determined (Fig. 5). Except for 200 mg Cd/l concentration and 300 mg Cu/l concentration, all nonenzymatic levels seem to be within the same range.


a
b
c
d

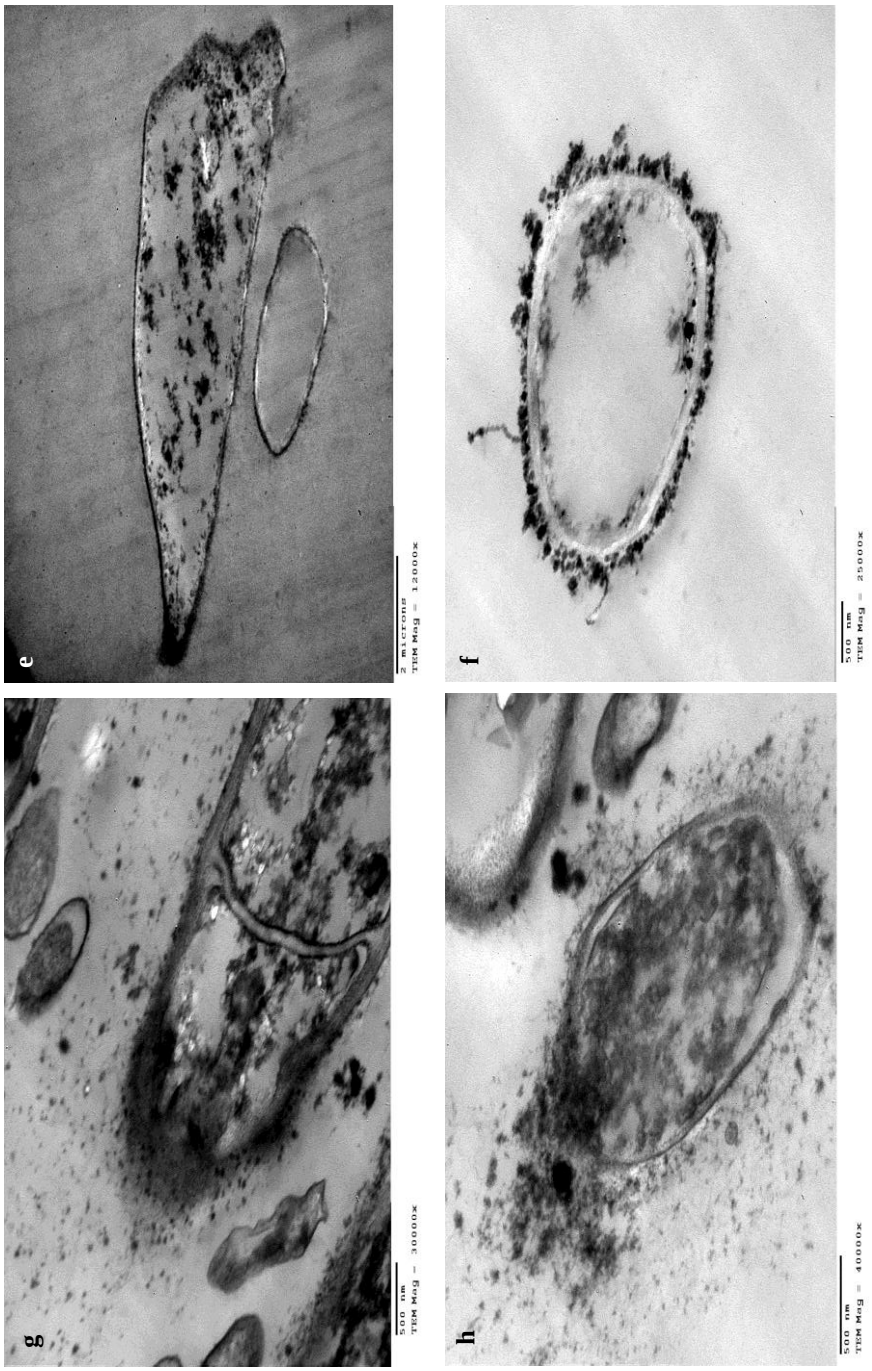


Fig. 3. TEM of *F. solani* (a) native cells, (b-e) 800 mg Cd(II)/l stressed cells, (f-h) 500 mg Cu(II)/l stressed cells

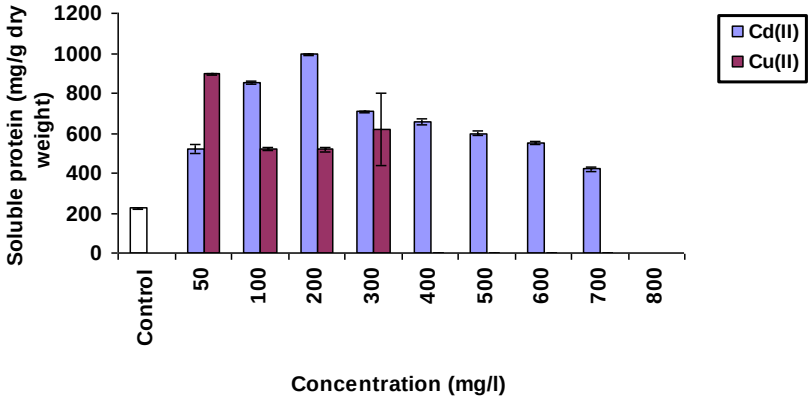


Fig. 4. Intracellular protein content in *F. solani* after 6 days of growth in presence of different Cd(II) and Cu(II) concentrations. Data represent the mean of three measurements ± SE.

It seems logic to hypothesize that cells could adapt to oxidative stress by increasing their antioxidant capacity. Since antioxidant enzymes represent the first line of defense against ROS, unsurprisingly their activities are often observed increased during adaptation. The balance between ROS production and antioxidant defense could be disturbed in favor of the former, by the addition of oxidant species, or sudden abundance of oxygen (Li *et al.*, 2009).

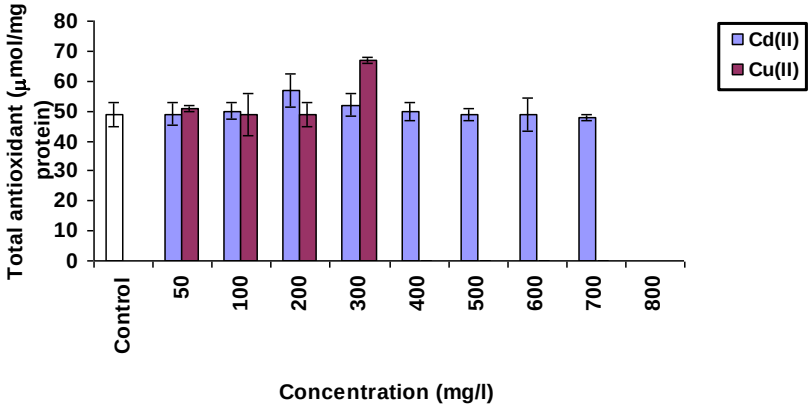


Fig. 5. Total antioxidant content of *F. solani* was measured after 6 days of growth in presence of different Cd(II) and Cu(II) concentrations. Data represent the mean of three measurements ± SE.

Glutathione reductase (GR) is a FAD-containing protein that reduces oxidized glutathione (GSSG) using NADPH as an electron donor (Sato *et al.*, 2009). Only the Cd-treated cells had increased GR activity, whereas Cu-treatment decreased the activity with the exception of 300 mg Cu/l (Fig. 6). This increase indicating higher enzyme activity either in response to the superoxide anions formed due to Cd(II) and Cu(II) toxicity or for more synthesis of glutathione to trap intracellular Cd(II) and Cu(II). A gradual decrease in enzyme activity was observed along with increase in Cd(II) concentration. This decrease in enzyme activity is indicative of drastic stress of higher Cd(II) concentration on *F. solani* .

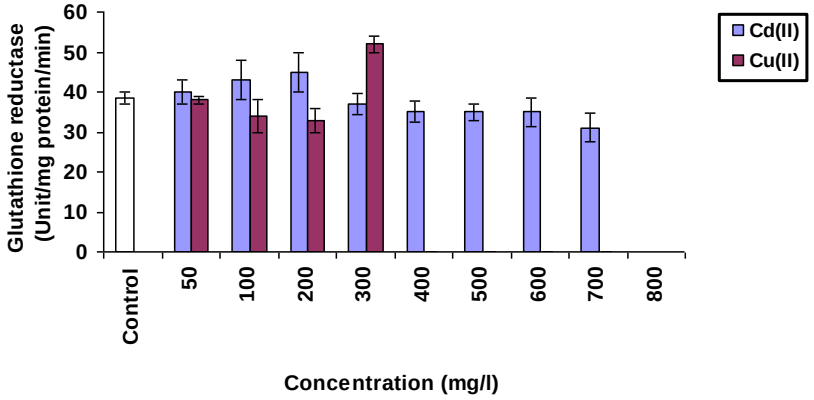


Fig. 6. Glutathione reductase (GR) activity in *F. solani* was determined in mycelium extract after 6 days of growth in presence of different Cd(II) and Cu(II) concentrations Data represent the mean of three measurements ± SE.

Peroxidase (POD) activity was found to be increased at 200 mg/ l and 50 mg/l concentrations of Cd(II) and Cu(II), respectively (Fig. 7). A gradual decrease was followed. An initial increase in POD activity suggest the fact that Cd(II) and Cu(II) induced antioxidative response in *F. solani* and there was an effort on part of fungus to minimize the hazardous effect of ROS. Decrease in POD activity at high heavy metal concentrations suggest that ROS generated at these concentrations provoked the inhibition of POD activity.

Polyphenol oxidase (PPO) gradually increased with increased Cd (II) stress and was found to be maximum at 200 mg/l Cd(II) treatment after which it was reduced (Fig. 8). On the other hand, Cu(II) stress reduced PPO activity. Hossain & Kermasha (1998) reported that metallothioneins (MTs.) bind selectively large amounts of heavy metal ions such as Hg(II) Cu(II), Cd(II) and Zn(II). Copper-metallothioneins (Cu-T) by donating copper, could act as an inhibitor for the PPO activity.

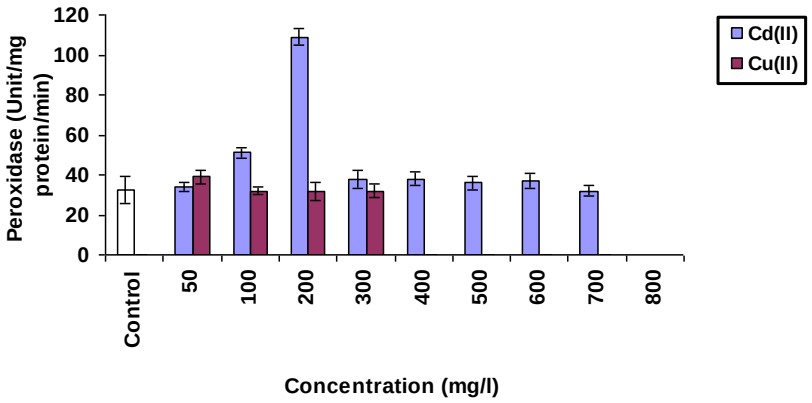


Fig. 7. Peroxidase (POD) activity in *F. solani* was determined in mycelium extract after 6 days of growth in presence of different Cd(II) and Cu(II) concentrations. Data represent the mean of three measurements ± SE .

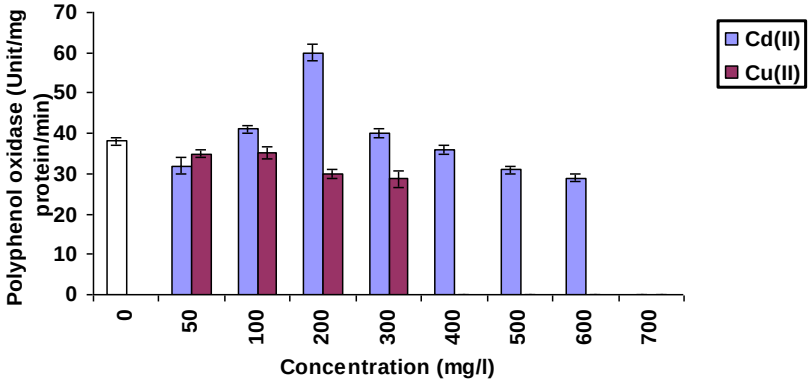


Fig. 8. Polyphenol oxidase activity in *F. solani* was determined in mycelium extract after 6 days of growth in presence of different Cd(II) and Cu(II) concentrations Data represent the mean of three measurements ± SE.

References

Ahmad, I., Imran, M., Ansari, M.I., Malik, A. and Pichtel, J. (2011) Metal tolerance and biosorption potential of soil fungi: Applications for a green and clean water treatment technology. In: *"Microbes and Microbial Technology"* I. Ahmad *et al.* (Ed.) pp 321-361, Agricultural and Environmental Applications. Springer Science + Business Media LLC.

Ahmad, M. and Kibret, M. (2013) Recent trends in microbial biosorption of heavy metals. A review. *Biochem. & Mol. Biol.* **1** (1), 19 - 26.

Baldrian, P. (2003) Interactions of heavy metals with white-rot fungi: *Enzyme Microb. Tech.* **32**,78-91.

Ban, Y., Tang, M., Chen, H., Xu, Z., Zhang, H. and Yang, Y. (2012) The response of dark septate endophytes (DSE) to heavy metals in pure culture. *PloS ONE.* **7**(10), e47968.

Barata, R.M., Chaparro, A., Chabregas, S. M., Gonzalez, R., Labate, C. A., Azevedo, R. A., Sarath, G., Lea, P. J. and Silva-Filho, M. C. (2000) Targeting of the soybean leghemoglobin to tobacco chloroplasts : Effects on aerobic metabolism in transgenic plants. *Plant Sci.* **155**,193-202.

Bergmeyer, H.V., Gaulehn, K. and Grassl, M. (1974) "*Methods of Enzymatic Analysis"*. Bergmeyer, H. V. (Ed.), **1**: pp. 495-496. 2nd ed., Academic Press, New York.

Cooley, R.N., Hasiock, H. R. and Tomsett, A.B. (1986) Isolation and characterization of cadmium-resistant mutants of *Aspergillus nidulans*. *Curr. Microbiol*.**13**, 265 – 268.

De Philippis, R. and Micheletti, E. (2009) Heavy metal removal with expolysaccharide-producing cyanobacteria. In: *"Heavy Metals in the Environment"* L. K. Wang, J.P. Chen, Y.T. Hung & N.K Shammas (Ed.), pp.89-122.CRC press, Boca Raton, Fl.

Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys*, **82**,70-77.

El-Sayed, M. T. (2013) Removal of lead (II) by *Saccharomyces cerevisiae* AUMC 3875. *Ann. Microbiol.* **63**, 1459-1470.

Ezzouhri, L., Castro, E., Moya, M., Espinola, F. and Lairini, K. (2009) Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier , Morocco. *Afri. J. Microbiol. Res.* **3**, 35-48.

Gabriel, J., Kofronovà, O., Rychlovsky, P. and Krenzelok, M. (1996) Accumulating effect of cadmium in the wood – rotting basidiomycete *Daedalea querclina*. *Bull . Environ. Contam. Toxicol.* **57**, 383-390.

Gadd, G.H. (1992) Metals and microorganisms: a problem of definition. *FEMS Microbiol. Lett.* **100**,197-204.

Guelfi, A., Azevedo, R.A., Lea, P.J. and Molina, S.M.G. (2003) Growth inhibition of the filamentous fungus *Apergillus nidulans* by cadmium: an antioxidant enzyme approach. *J. Gen. Appl. Microbiol.* **49**, 63-73.

Gupta, B.L. and Berridge, M.J. (1966) Fine structural organization of the rectum in the blowfly *Calliphora* yellow pine. IRG/ WP/O1- 10388. International Research Group on Wood Protection Stockholm, Sweden.

Gupta, M., Mazumdar, U.K., Gomathi, P. and Kumar, R.S. (2004) Antioxidant and free radical scavenging activities of *Ervatamia coronaria* Stapf leaves. *Iran. J. Pharm. Res.* **2**,119-126.

Harley, M.M. and Ferguson, I.K. (1990) The role of SEM in pollen morphology and plant systematic. In: *"Electron Microscopy in Taxonomy and Functional Morphology"*. Claugher D.(Ed.). pp.45-68. Sys. Assoc. 41: Clarendon Press, Oxford.

Hefnawy, M.A. and Razab, A.A. (1998) Alteration of cell-wall composition of *Fusarium oxysporum* by Copper stress. *Folia Microbiologica*, **43**(5), 453 – 458.

Hossain, A. and Kermasha, S. (1998) Inhibition of polyphenol oxidase activity by copper metallothionein, in polyphenol communications, *XIXth International Electronic Conference on Polyphenols*, Lille, France, v. **2**: pp. 285-286.

Huang, D. L., Zeng, G. M., Feng, C. L., Hu S., Zhao, M. H., Zhao, M. H., Lai, C., Zhang, Y., Jiang, X. Y. and Liu, HL. (2010) Mycelial growth and solid- state fermentation on lignocellulosic waste by white-rot fungus *Phanerochate chrysosporium* under lead stress. *Chemosphere*, **81**,1091-1097.

Jones, M.D. and Hutchinson, T.C. (1988) The effects of nickel and copper on the axenic growth of ectomycorrhizal fungi. *Can. J. Bot.* **66**,119-124.

Kaplan, D. (2013) Absorption and adsorption of heavy metals by microalgae. *"Handbook of Microalgal Culture: Applied Phycology and Biotechnology"*. Second edition. Edited by Amos Richmond and Qiang Hu. pp. 602-611. John Willy & Sons, Ltd.

Levy, J.L., Angel, B.M., Stauber, J. L., Poon, W.L., Simpson, S.L., Cheng, S.H. and Jolley, D.F. (2008) Uptake and internalization of copper by three marine microalgae: Comparison of copper-sensitive and copper-tolerant species. *Aquat. Toxicol*. **89**, 82-93.

Li, Q., Harvey, L.M. and McNeil, B. (2009) Oxidative stress in industrial fungi. *Crit. Rev. Biotechnol.* **29**(3),199-213.

Lima e Silva, A.A.L., Carvalho, M. A.R., Souza, S.A.L., Dias, P.M.T., Silva Filho, R.G., Meirelles Saramago, C.S., Melo, Bento C.A. and Hofer, E. (2012) Heavy metal tolerance (Cr, Ag and Hg) in bacteria isolated from sewage. *Braz. J. Microbiol*. **43**(4), 1620 – 1631.

Lowry, O. H., Rosebrough, H.J., Farr, A.L. and Radnall, R. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **193**, 263-275.

Luft, J.H. (1961) Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**, 409-414.

Machuca, A., Napoleào, D. and Milagres, A. M. F. (2001) Detection of metal- chelating compounds from wood-rooting fungi *Trametes versicolor* and *Wolfiporia cocos*. *World J. Microbiol. Biotechnol.* **17**, 687-690.

Melo, R.G., Leitao, A.C. and Pàdula, M. (2004) Role of OGGI and NTGZ in the repair of oxidative DNA damage and mutagenesis induced by hydrogen peroxide in *Saccharomyces cerevisiae*: relationships with transition metals iron and copper. *Yeast* **21**, 991-1003.

Neumann, G., Veeranagouda, Y., Karegoudar, T.B., Sahin, Ö., Mausezahl, I., Kabelitz, N., Kappelmeyer, U. and Heipieper, H.J. (2005) Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. *Extremophiles* **9**, 163-168.

Palade, G.E. (1952) A study of fixation for electron microscopy. *J. Exp. Med.* **95**(3), 285-298.

Pòesi, I. (2011) Toxicmetal/metalloids tolerance in fungi- A biotechnology oriented approach In: *"Cellular Effects of Heavy Metals"*, Bànflavi, G. (Ed.) pp. 31–58. Springer Science+Business Media BV, Netherlands.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell. Biol.* **17**, 208-212.

Ruta, L., Paraschivescu, C., Matache, M., Avramescu, S. and Farcasanu, H.C. (2010) Removing of heavy metals from synthetic effluents using "Kamikaze" *Saccharomyces cerevisiae* cells. *Appl. Microbiol. Biotechnol.* **85**, 763-771.

Sato, I., Shimizn, M., Hoshino, T. and Takaya, N. (2009) The glutathione system of *Aspergillus nidulans* involves a fungus glutathione S- transferase. *J. Biol. Chem.* **284**, 8042-8053.

Seifert, K. (1996) *Fusarium* interactive key. *Agriculture and Agri. Food Canada.* pp. 1 – 65.

Sharma, S.S. and Dietz, K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci.* **14**, 43- 50.

Smirnoff, N. (1993) The role of active oxygen in the response of plants to water deficit and desiccation (Tansleg Review No. 52). *New Phytol.* **125,** 27 - 58.

Sptempack, J.G. and Ward, R.T. (1969) An improved staining for electron microscopy. *J. Cell Biol*. **22**, 679-701.

Thompson, J.D., Higgins, D.G., Gibson, T.J. and Clustal, W. (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice. *Nucleic Acid Res*. **22**, 4673-4680.

Todorova, D., Nedeva, D., Abrashev, R. and Tsekova, K. (2008) Cd (II) stress response during the growth of *Aspergillus niger* B77. *J. Appl. Microbiol.* **104**, 1541- 1547.

Volka, M., Morris, H. and Cronin, M.T.D. (2013) Metals toxicity and oxidative stress. *Curr. Med. Chem.* **13** (10), 1161-1208.

WHO (2008) Guidelines for drinking water quality, *World Health Organization,* Geneva.

(*Received* 8/4/2014;
accepted 22/6/2014)

استجابة فطرة *فيوزاريوم سولانى* لأيونات عنصرى الكادميوم والنحاس فى مزرعة نقيه

منال توفيق السيد
قسم النبات – كلية العلوم – جامعة الزقازيق – الزقازيق – مصر .

تم عزل فطرة *فيوزاريوم سولانى* من تربة زراعية يتم ريها لمياه الصرف الصحى والصناعى، وتم التعريف على أساس الشكل الظاهرى وباستخدام الحامض النووى الديوكسى ريبوز. اختيار الفطره كان على أساس أنه لا يوجد دراسات كثيرة سابقة على سمية أيونات عنصرى النحاس والكادميوم عليها. قيمة أقل درجة تثبيط للنمو كانت عند 900 مللى جرام/لتر و 600 مللى جرام/لتر لأيونات عنصرى الكادميوم والنحاس على التوالي. الفحص بالميكروسكوب الالكتروني الماسح أكد وجود تغيرات على الشكل الظاهرى للسطح ونسبة مساحة السطح/الحجم. الميكروسكوب الالكتروني النافذ كشف أن سمك الجدار الخلوى قد تضاعف مع زيادة فى عدد الفجوات الداخل خلوية وبعض الخلايا تحللت بالكامل بعد التعرض للإجهاد بأيونات الكادميوم. أيضا الجدار الخلوى تحديده بأيونات النحاس والخلايا جذبت ترسيبات النحاس. التأكد من وجود أيونات العنصرين تم باستخدام EDX. تأثيرات أيونات العنصرين على النمو والمحتوى البروتينى ومضادات الأكسدة الكلية والثيولات الكلية تم دراستهم. تم تقدير أنشطة إنزيمات البولى فينول أوكسيديز والجلوتاثيون ريدكستيز والبروأوكسيديز بعد التعرض لإجهاد من أيونات عنصرى النحاس والكادميوم .