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# ORIGINAL ARTICLES

## Morphological and Metabolic Response of *Aspergillus nidulans* and *Fusarium oxysporum* to Heavy Metal Stress

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**ABSTRACT**

*Aspergillus nidulans* and *Fusarium oxysporum*, which isolated from soil long term treated with sewage water, are able to grow on Cezapek – Dox media amended individually with a different concentration of tested heavy metals. *A.nidulans* and *F. oysporum* failed completely to grow on $Zn^{+2}$ , $Pb^{+2}$ , $Co^{+2}$ , $Ni^{+2}$ and Hg at concentration of 5000 , 1000 , 700 , 200 and 400 ppm respectively and 6000 , 5000 , 800 , 800 and 400 ppm, respectively . Cobalt , nickel and mercury were mostly suppressed the growth rate and morphology of both fungi than zinc and lead, they caused malformation of conidiophores , mycelia and conidia with accumulation of heavy metal ions on the surface of the cell wall. Whereas, total protein, lipid and carbohydrates in the mycelium of both fungi where slightly increased up to 500 , 800 , 500 , 500 and 100 ppm of $Zn^{+2}$ , $Pb^{+2}$ , $Co^{+2}$ , $Ni^{+2}$ and Hg , respectively, for *F.oxysporum* , but it was 500 , 400 , 300 , 50 and 100 ppm of $Zn^{+2}$ , $Pb^{+2}$ , $Co^{+2}$ , $Ni^{+2}$ and Hg , respectively, for *A.nidulans* , and decreased above these concentrations .

***Key words:*** Fungi, protein, lipids, carbohydrates, heavy metal.

### Introduction

In recent years, heavy metal pollution has become one of the most serious environmental problems. Presence of heavy metals even in traces is toxic and detrmental to both flora and fauna (Volesky, 1990).

The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in the microbial communities (Vadkertiova and Slavikova, 2006). Toxic effects of heavy metals include the blocking of functional groups of biologically important molecules (e. g. enzymes and transport system for nutrients), denaturation and inactivation of enzymes and disruption of cellular organelle membrane integrity (Ochiai, 1987).

Bio materials like fungi have been proven more efficient and economical for removal of toxic metals from dilute aqueous solutions because of their filamentous morphology and high percentage of cell walls (Lacina *et al.*, 2003).

The microorganisms respond to these heavy metals by several processes, including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Veglio *et al.*, 1997).

Mercury is one of the major pollutants in the environment which is highly toxic to human (Meyer-Baron *et al.*, 2002). Besides human beings, plants, animal and microorganism also affected (Iwahori *et al.*, 2000) Sujoy *et al.*, (2007) found that the uniform distribution of metal ions of mercury on the mycelial surface of *Aspergillus versicolor* caused morphological changes in mycelial structures.

Sarita and Tabitha (2008) reported that lead was the most easily tolerated heavy metals tested caused variations in cultural and morphological characteristics of *Penicillium.*

Nickel is released into the environment by large number of industrial processes, and the concentration level of nickel in the environment widely varies. Ni (II) is more toxic against all living system (Shankar *et al.*, 2007). Also it can act as a co-factor for several microbial enzymes (Gikas, 2008). Ni at a concentration of 69 mg (Kg soil)$^{-1}$ Ni negatively influenced soil microbial communities (Baath *et al.*, 1998).

Parameswari *et al.*, (2010) found that *A. niger* which isolated from sewage contaminated soil has ability to accumulate and biosorped Ni and Cr. The conidia of *Pandorina neoaphidis* were unable to germinate in the presence of Cr, Cu, Pb and Zn ions in the medium at a concentration that was 100-times higher than the mean one.

Co is toxic to the cells as it inhibits cellular respiration and enzymes of citric acid cycle. Also, Co exhibited spare mycelia and conidiation to *A. nidulanse* but secreted higher amount of melanin (Pushplata and Sheela, 2007).Also Matazanke, (1994) reported that cobalt is toxicity to *A. flavus* and *U. chlamydosporum* at 2000

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µg/ml may be due to the lack of metabolization of iron by the fungi which in turn inhibits mycelial heam synthesis.

Zn is essential element required for physiological function and growth of organisms. Also it modified the fungal morphology is relation to the range of Zn concentration and fungal strain (Lanfranco *et al.*, 2002). Also Zn caused a significant reduction of conidial germination of fungi at high concentration (Thaczuk, 2005).

The aim of these studies was to determine the effects of five heavy metal ions ($Zn^{+2}$, $Pb^{+2}$, $Co^{+2}$, $Ni^{+2}$ and Hg) on the growth and certain biological activities of *A. nidulans* and *F. oxysporum*

## Materials and Methods

*Samples:*

Soil samples were collected from El-Gabal El-Asfar site, 25-30 Km from Cairo-Egypt. These sites were irrigated with sewage water for long time. The physiochemical characteristics of contaminated soil were identified in previous work (Elkhawaga, 2010).

*Isolation of heavy metal-tolerant fungi:*

0.1 g of aseptically collected soil was used to isolate metal tolerant fungi by spread plate method. Cezapek Dox-medium supplemented individually with 1g/l $NiCl_2$, $H_2O$, $CoCl_2$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$ and $HgCl_2$ was used the Dox medium plates in duplicate, incubated at 28± 2C for 6 days. The fungus isolated and purified. Identification of the most tolerant fungi was done in the Regional Center for Mycology and Biotechnology (Egypt).

*Determination of minimum inhibitory concentrations (MICs):*

The tolerance of the selected isolates to $Ni^{2+}$, $Pb^{2+}$, $Co^{2+}$, $Zn^{2+}$ and Hg was determined by dilution method. Metal ions were added separately on Dox-medium at concentration from (1 to 6000 ppm). The plates were inoculated with 8 mm agar plugs of young fungal colonies, pre-grown on Dox-medium for 7 days. Three replicates of each concentration and controls without metal were used. The inoculated plates were incubated at 28±2 °C for 7-10 days. The effects of the heavy metals on the growth were estimated by measuring the radius of the colony extension (mm) against the control and the determination of the index of tolerance. The (MIC) is defined as the lowest concentration of metal that inhibit visible growth of isolates.

*Effects of heavy metal ions concentration on fungal mycelial dry weight:*

Inoculum (8 mm disk) of isolated fungi was inoculated in 250 ml Erlenmeyer flasks containing 100 ml of liquid Dox media containing different concentration of each heavy metal ions individually. Each salts treatment had three replicates. All flasks were incubated at 28 ±2 °C for 7 days. The mycelium was harvested, washed several times by distilled water and transferred to pre weight filter paper, oven-dried at 80Cº for 24 hours and weighted.

*Determination of cellular contents of protein, sugar and lipid:*

*Preparation of cell-free extracts:*

The fungi were cultured on Dox liquid medium amended with different concentrations of heavy metals. All treatments were performed in 250 ml Erlenmeyer flasks and replicated 3 times. The flasks were incubated at 28 ±2 °C for 7 days. Mycelia of the growing fungi were harvested by filtration using filter paper, the fresh weight of the mycelia were determined in miligram.The harvested mycelia were ground with an approximately equal weight of clean cold sand in mortars and extracted with 70% (V/V) ethyl alcohol. The obtained slurry was centrifuged at 6000 rpm for 10 minutes. The supernatant was decanted and used for different analysis.

Total protein was determined by the method of (Lowery *et al.*, 1951). Sugar determination was carried out using the anthron technique as described by (Umbriet *et al.*, 1959). While, total lipid was determined using phosphovanillin reagent after extraction by chloroform methanol mixture (Barnes and Blackstock, 1973).

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*Scanning electron microscopy:*

Coated specimens of each fungal isolate were examined using high vacuum mode of JOEL JSM-5500LV Scanning Electron Microscope at Regional Center for Mycology and Biotechnology (RCMB) at AL-Azhar University.

**Results and Discussion**

In present investigation various filamentous fungi were isolated from agricultural soil receiving long-term application of waste water. Soil fungi able to grow in the presence of 1g/l heavy metal. *A. nidulans* and *F. oxysporum* which were the most tolerance fungal isolates to heavy metals were selected and subjected to further studies from these fungi. These isolates are tested to determine the Minimum Inhibitory Concentration (MIC) for the metals. The order of toxicity of the metals to *A. nidulans* was Ni > Hg > Co > Pb > Zn, but it was Hg > Ni > Co > Pb > Zn for *F. oxysporum* (Table 1). Thus, high level of tolerance to heavy metals was detected in selected isolates through plate tests. The occurrence of various fungi such as *Aspergillus*, *Rhizopus*, *Penicillium*, *Fusarium*, *Chaetomium* species in soil polluted with heavy metals (Cu, Cd, Pb, As, and Zn) has also been reported (Gad, 1993). The variation in the metal tolerance might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different fungi (Parameswari *et al*., 2010).

**Table.1:** Minimum inhibitory concentration (MIC) of heavy metals against test Fungi.

| Tested heavy Metals / Fungal isolates | $Co^{+2}$ | $Pb^{+2}$ | $Zn^{+2}$ | $Ni^{+2}$ | Hg |
|---|---|---|---|---|---|
| | MIC (ppm) | | | | |
| *A.nidulans* | 700 | 1000 | 5000 | 400 | 400 |
| *F.oxysporum* | 800 | 5000 | 5000 | 600 | 400 |

*Effect of different concentrations Co, Ni, Pb, Zn and Hg on mycelial dry weight:*

The growth of *A. nidulans* and *F. oxysporum* markedly decreased with increasing metal concentration (Fig 1 and 2). The dry mass of *A. nidulans* decreased to approximately more than 55% at 10, 10, 200, 800 and 400 ppm of Hg, Ni, Co, Zn and Pb, respectively, and failed completely at 400, 400, 700, 1000 and 5000 ppm of Hg, Ni, Co, Pb and Zn respectively . Also mycelial dry weight of *F. oxysporum* sharply decreased by about 60% when it grown at 50, 200, 400, 1000 and 3000 ppm of Hg, Ni, Co, Zn and Pb respectively, and completely inhibited at 400,800,800and 6000 ppm for Hg,Ni,Co and Zn ,respectively, but the fungus still grow after 5000pppm of lead .

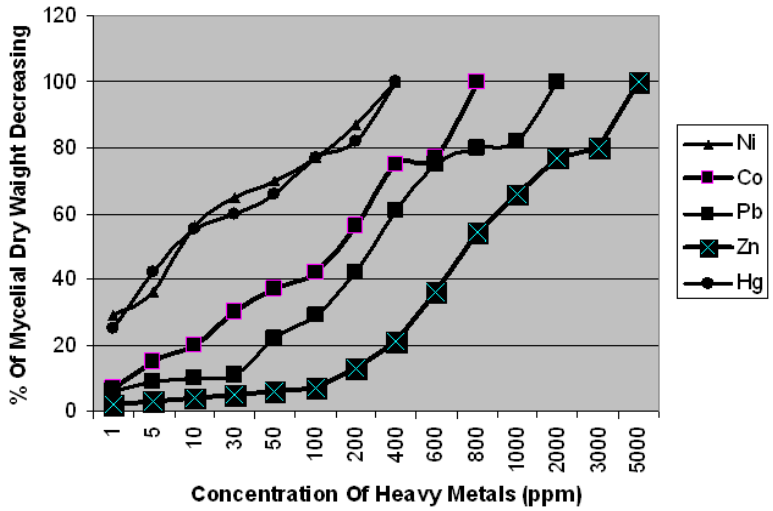


**Fig. 1:** Effect of different concentrations Co, Ni, Pb, Zn and Hg on mycelial dry weight of *A.nidulans.*

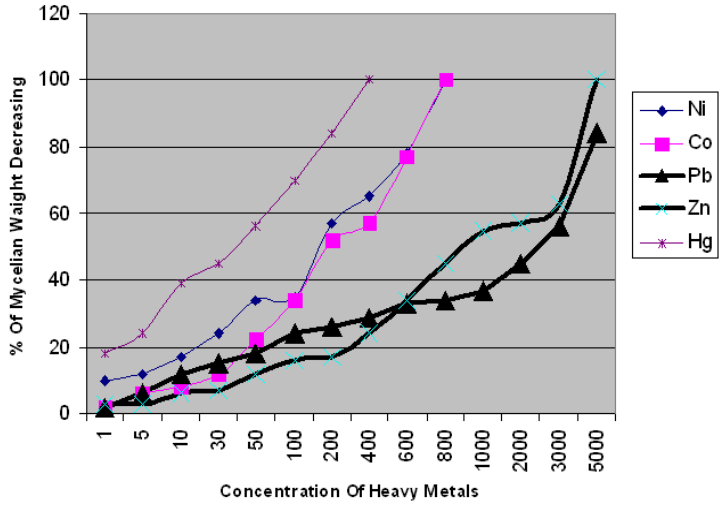


**Fig. 2:** Effect of different concentrations Co, Ni, Pb, Zn and Hg on mycelial dry weight of *F.oxysporun.*

Shazia *et al*. (2002) reported some isolates of G +ve and G-ve bacteria, which tolerate Hg until 450 µg $ml^{-1}$. The resistance mechanisms to Hg involves: the uptake of $Hg^{+2}$ into the cytoplasm, reduction of ionic mercury $Hg^{+2}$ by mercury reductase, encoded by mer A gene, to Hg and release of Hg from the cell by diffusion through cell membrane (Hobman and Brown, 1997).

Al-Obaid and Hashem (1996) indicated that higher concentration of Zn and Cu (3000 µg/ml) allowed the growth of *Aspergillus candidus* and *Drechslera rostrata*. While higher concentrations of cobalt and lead inhibited growth of *Fusarium moniliforme* and *Ulochladium atrum* (3000 µg/ml).

Nickel toxicity also reported against *Agaricus bisporus*, *Agrocybe praecox*, *Gymnopus peronatus* and *Gymnopilus spineus*. Low concentration of Ni (20 mg $1^{-1}$) inhibited the growth of all fungi on humus (Lankinen,*et.al*., 2011).

Mycelial dry weight of *Chaetomium globosum* and *Stachybotrys chartarum* decreased with increased co-concentration up to 800 mg/L and failed completely to grow at 1000 mg/L (Hefinway *et al.*, 2009).

Zn and Ni affect the growth and sporulation of *Heliscus submerses* and *Tricladium chaetocladium* (Azevedo and Cassio, 2010).

*Effect of heavy metals on morphology of F. oxysporum and A. nidulans:*

*Fusarium oxysporum* not strongly affected under Zn stress up to 1000 ppm (Fig,3,B&C) except elongation in both hyphae and macroconidia. This elongation may be due to disturbance of septation or thickening of hyphal and conidial cell wall. On the other hand Zn has poorly effect on conidial formation of *A. nidulans* but cause increasing in hyphal branching, swelling and septation until 1000 ppm (Fig 4B&C). This may be due to zinc is essential element required for physiological function and growth of organisms. Zn modified the fungal morphology is relation to the range of Zn concentration and fungal strain The presences of high concentrations of Zinc modify the hyphal morphology of ericoid fungi which cause increase in hyphal branching, swelling and septation (Lanfranco *et al.*, 2002).

Alternatiely toxic effect of Zn showed on the growth of *Schizochytrium*, where it cause significant reduction in growth at concentration (0.5 – 8 ppm), but showed shrinkage and general deformation of cell structure and eventually, cell death at high concentrations (256-384 ppm) (Chen *et al.*, 2010).

On the other hand Pb cause high positively malformation effect on both fungi, when *F. oxysporum* grown on 500 ppm Pb both hyphae and macroconidia become elongated (Fig 3D) compared to control. The curved shape of macroconidia disappear with increasing Pb concentration up to 1000 ppm , also increasing malformation of hyphae, macro and microconidia and accumulation of large amount of metal ions on cell wall layer also detected (Fig 3E ). On the other hand pb has strongly effect on *A. nidulanse* where it caused hyphal malformations with many twisted and swollen like shape at 200 ppm, This malformations increased with metal ion increased(Fig 4D,E).

Seratia and Tabitha (2008) indicated that lead was the most easily tolerated of the heavy metals tested and caused the least variations in cultural and morphological characteristics on *Penicillium* sp. large amount of lead granules accumulated in the cell as well as on the outer layer of the cell wall when *Penicillium* sp. grown in the presence of 24 mM Pb $(NO_3)_2$. (Sun and Shao, 2007).

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On the other hand, Co, Hg and Ni had high toxic effect of both fungi. *F. oxysporm*, showed high malformation in hyphae and increase thickening of macro and microconidia as grown on these metals (Fig 3.F, G,H,I,Jand K ). At high concentrations it showed very little macrocoidia, hyphal elongation and precipitation of metal ions on the outer surface of hyphae.

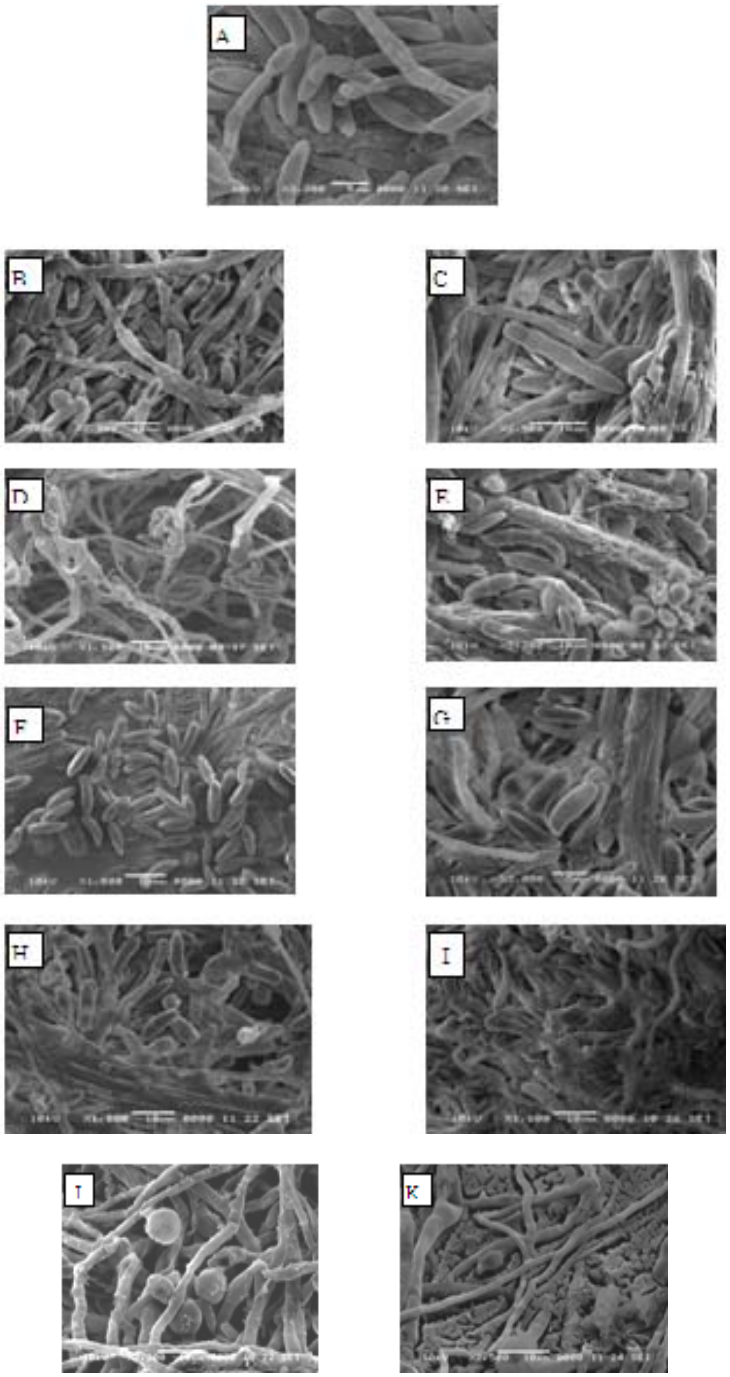



**Fig. 3:** Effect of heavy metal on morphology of *Fusarium oxysporum* ,(A) control ,(B) 500 ppm Zn ,(C)1000 ppm Zn, (D)500ppm pb ,(E)1000ppm pb, (F)100 ppm Co, (G) 1000 ppm Co, (H)100 ppm Ni, (I)600 ppm Ni, (J)50 ppm Hgand (K)200 ppm Hg.

Similar result was for *A. nidulans* where the mycelium becomes malformed with many vesicles and swollen cells formed (Fig 4F, G, H, I, J and K). Also reduced and distorted the conidial formation was observed. Precipitation of heavy metals also appeared on the surface of mycelium due to increase of metal concentration.

The same result showed in *Aspergillus nidulans* ($Co^R$ mutant) at concentration up to 2.5 mM in the medium and exhibited sparse mycelia and conidiation but secreted higher amount of melanin (Tripathi and Srivastiva, 2007).

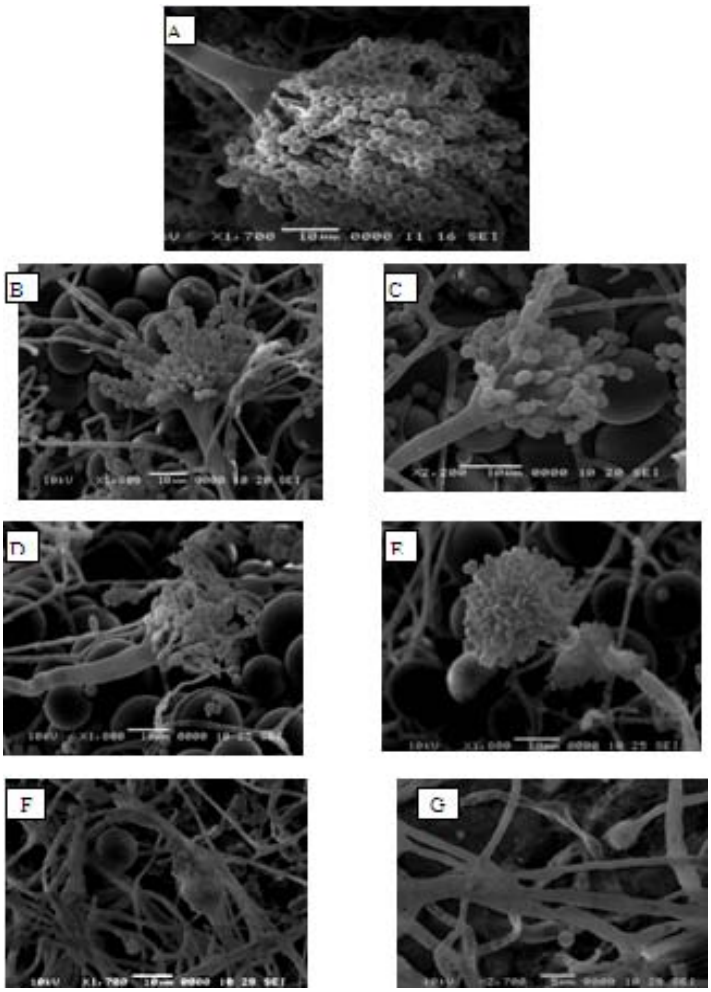
The toxic effect of Ni and Hg indicated by Petr, (2003) when caused inhibition on the growth of Basidiomycetes and also cause morphological and physiological changes and affect the reproduction. Also Hg caused changes in mycelial morphology and induced fruit body formation in *Pycnoporus cinnabarinus* (Mandal *et al*., 1998).

The precipitation of Hg on the cell wall surface of *Aspergillus versicolor* indicated by Sujoy *et al.,* (2007). Also quite similar observation was found by precipitation of Ni on the surface of *A. niger* (Magyarosy *et al.*, 2002).

Data cited in table (2) revealed that in case of *fusarium oxysporum*, increasing the concentration of Zn, Pb, Co, Ni and Hg heavy metals from (200-500), (500-800), (100-500), (100-500) and (50-100) ppm respectively, increased total soluble proteins , lipid and carbohydrate , and then decrease at 1000, 2000,1000, 600 and 200 ppm of Zn, Pb, Co, Ni, and Hg respectively, for the tested metabolic activities. (Table. 2.)

The same result obtained with *A. nidulanse* total protein, lipid and carbohydrates increased with increasing concentration of ( 200-500), (200-400), (100-300), (30-50), and (50-100) ppm Zn, Pb, Co, Ni and Hg then decreased at concentration at 1000, 600, 500, 100 and 200 of Zn Pb, Co, Ni and Hg respectively (table 3). The fungus might alter its metabolic activities to overcome the presence of high concentrations of the heavy metals in the environment or loss its ability to regulate the permeability of ions in to the cells. The increasing of protein, lipid and carbohydrates of *F.moniliforme* with tellurium reported by Amer *et al*, (2005) who explained that the increase in such metabolic product might be due to osmotic equilibration as well as an activation of the biosynthesis of several adsorbents within the fungal cells. The same result reported by Shadia. *et al*., (1988),who found that the total protein, lipid and carbohydrate of *A carbonarium* increased in the presence of Cd, and then decreased when increased Cd concentration up to 2.5% (w/v).

Effect of heavy metals on fungal proteins, carbohydrates and lipids



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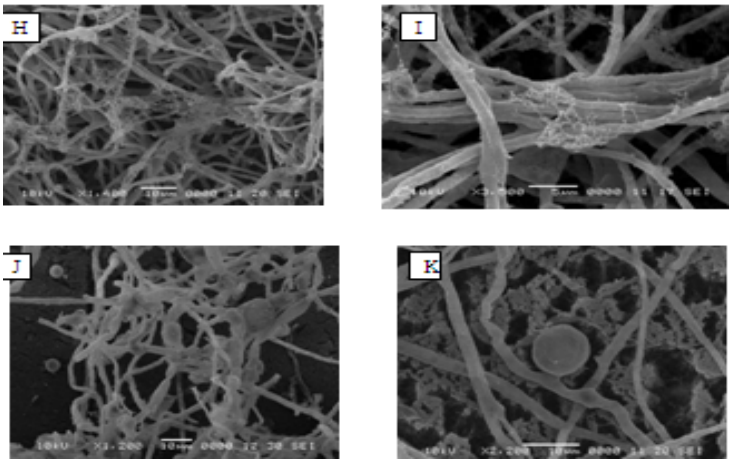


**Fig. 4:** Effect of heavy metal on morphology of *Aspergillus nidulans* ,(A) control ,(B) 500 ppm Zn ,(C)1000 ppm Zn, (D)200 ppm pb ,(E)600 ppm pb, (F)100 ppm Co, (G) 500 ppm Co, (H)50 ppm Ni, (I)100 ppm Ni, (J)50 ppm Hg and (K)200 ppm Hg.

**Table. 2:** Total protein, lipid , carbohydrates µg/ml ±SE in the cell free extract of *Fusarium oxysporum* cultivated on Czapeks –Dox medium amended with different concentrations of heavy metal .

| Heavy Metal Ions | Concentration (ppm) | Protein (µg/ml) | Lipid (µg/ml) | Carbohydrate (µg/ml) |
|---|---|---|---|---|
| control | 0.0 | 970±0.577 | 1900±1.15 | 4110±0.577 |
| $Zn^{+2}$ | 200 | 1200±0.577 | 2500±0.577 | 6210±1.15 |
| | 500 | 1600±0.577 | 3000±1.15 | 8310±1.15 |
| | 1000 | 1000±0.577 | 1600±0.577 | 4551±1.15 |
| $Pb^{+2}$ | 500 | 1400±0.577 | 2800±1.15 | 7522±0.577 |
| | 800 | 1800±0.577 | 3200±0.577 | 8911±1.15 |
| | 2000 | 1200±0.577 | 2120±1.15 | 4151±0.577 |
| $Co^{+2}$ | 100 | 1100±0.577 | 2300±0.577 | 6921±1.15 |
| | 500 | 1500±0.577 | 2900±1.15 | 8820±0.577 |
| | 1000 | 1000±0.577 | 1000±0.577 | 4121±0.577 |
| $Ni^{+2}$ | 100 | 1200±0.577 | 2200±1.15 | 5980±1.15 |
| | 500 | 1500±0.88 | 3000±0.577 | 6610±0.577 |
| | 600 | 1100±1.15 | 1224±0.577 | 4211±1.15 |
| Hg | 50 | 1200±1.15 | 2124±1.15 | 5019±0.577 |
| | 100 | 1400±0.577 | 2611±0.577 | 6117±1.15 |
| | 200 | 800±1.15 | 1112±1.15 | 3910±0.577 |

**Table. 3:** Total protein, lipid, carbohydrate, µg/ml ±SE in cell free exract of *A nidulans* cultivated in Cezapeks-Dox medium with different concentration of heavy metals .

| Heavy Metal Ions | Concentration (ppm) | Protein (µg/ml) | Lipid (µg/ml) | Carbohydrate (µg/ml) |
|---|---|---|---|---|
| control | 0.0 | 1100±0.577 | 2197±0.577 | 2974±1.15 |
| $Zn^{+2}$ | 200 | 1700±1.15 | 3500±1.15 | 4638±0.577 |
| | 500 | 2151±0.577 | 3911±0.577 | 6911±1.15 |
| | 1000 | 1560±1.15 | 2654±1.15 | 3100±0.577 |
| $Pb^{+2}$ | 200 | 2112±0.577 | 3221±0.577 | 6959±1.15 |
| | 400 | 2500±1.15 | 4152±1.15 | 4871±0.577 |
| | 600 | 1650±0.577 | 2500±0.577 | 3102±1.15 |
| $Co^{+2}$ | 100 | 2000±0.677 | 2912±1.15 | 3100±0.577 |
| | 300 | 2841±0.577 | 3100±0.577 | 3515±1.15 |
| | 500 | 1105±1.15 | 1521±1.15 | 2000±0.577 |
| $Ni^{+2}$ | 30 | 1800±0.577 | 3100±0.577 | 3300±1.15 |
| | 50 | 2400±1.15 | 3741±1.15 | 3690±0.577 |
| | 100 | 1214±0.577 | 2000±0.577 | 2100±1.15 |
| Hg | 50 | 1541±1.43 | 2400±1.15 | 3000±0.577 |
| | 100 | 2000±0.577 | 2951±0.577 | 3200±1.15 |
| | 200 | 1000±1.15 | 1100±1.15 | 1801±0.577 |

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