

## **Influence of Copper and Cobalt Stress on Morphology and Ultra-structure of *Chaetomium Globosum* and *Stachybotrys Chartarum***

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**Abstract:** The growth of *Chaetomium globosum* and *Stachybotrys chartarum* markedly decreased with increasing Cu and Co ion concentrations in the medium upto 800 mg/L while, they failed completely to grow at 1000 mg/L. Cu and Co uptake by the fungal mycelium was increased by increasing their concentrations in the medium. Whereas, total protein, lipid and carbohydrates in the mycelium were slightly increased at 200 and 400 mg/L and decreased above this concentration. The perithecia of *C. globosum* and sporulation of *S. chartarum* markedly decreased with increasing both Cu and Co concentration in the growth medium. High damage in the perithecial seta of *C. globosum*, conidiophores and phialides of *S. chartarum* was observed with elevated concentrations of Cu and Co. Transmission electron microscopic study revealed that the cell wall of both organisms increased in its diameter at 400 and 800 mg/L Cu or Co except that *S. chartarum* exhibited a rupture in its wall and damage in the cells at 800 mg Co/L. It was also observed that fat bodies or more likely oil droplets were found in the cytoplasm. Moreover, black dense electron areas were also found in the cytoplasm, which may be aggregates of metal complex that deposited in the form of granules or crystals.

**Key words:** Copper and Cobalt Stress, Morphology and Ultra-Structure, *Chaetomium globosum* and *Stachybotrys chartarum*

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### **INTRODUCTION**

Heavy metal contamination caused either by natural process or by human activities is one of the most important environmental problems (Reedy, L.H., M.N.V. Prasad, 1990). Toxic effects 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, E.I., 1987).

Heavy metal uptake by fungi is of fundamental importance to organisms growing in polluted habitats since tolerance may be determined by the ability to prevent cellular entry of a potentially toxic metal or the ability to compartmentalize or detoxify it within the cell. Heavy metals accumulated mainly in the fungal cell wall and in the vacuoles of *Glomus intraradices*, while minor changes in metal concentrations were detected in the cytoplasm (Gonzalez, G.M. *et al.* 2008). The cell walls of some microbes appear to have a greater and more selective ability to accumulate some metals. A large portion of the Cu<sup>2+</sup> taken up by *Penicillium ochro-chloron* was accumulated in the cell walls (Motohiro, F. *et al.* 1983).

Localized silver deposition around cell walls and within vacuoles has observed in *Cryptococcus albidus*, mercury precipitation in electron-dense bodies occurs in mercury-exposed hyphae of *Chrysosporium pannorum* and similar bodies, presumed to contain zinc, occurred in *Neurospora vasinfecta* after Zn<sup>2+</sup> influx (Simmons, P. *et al.* 1995).

Heavy metals were deposited in the form of granules or crystals on the cell wall and in the cytoplasm or vacuoles of fungal or bacterial cells when grown in vitro under high metal stress. Analytical electron microscopy of thin sections of *Aspergillus niger* hyphae revealed that nickel was localized in the form of rectangular crystals of nickel oxalate dehydrate with the cell wall and also inside the cell (Magyarosy, A. *et al.* 2002). A lead resistant *Penicillium* spp. accumulated a large amount of lead granules in the cell, as well as adsorbed on the outer layer of the cell wall, as observed under a transmission electron microscope. These granules were either in the vicinity of the cytoplasm membrane or in the vacuoles (Sun, F., Z. Shao, 2007). It was also observed that a heavy metal resistant *Cladosporium cladosporioides* deposited intracellular crystals of manganese phosphate when the hyphae grown in a Mn-rich medium (Shao, Z., F. Sun, 2007).

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Bacterial cells exhibited also heavy metal complex deposition in cell walls and cell membrane. Transmission electron microscopy of a heavy metal resistant *Bacillus* spp. showed a large amount of electron dense granules of lead found mainly in cell walls and cell membrane (Kim, S.U. *et al.* 2007). In this study, two fungal species tolerant to heavy metals were tested for their ability to tolerate copper and cobalt in the growth medium and subsequent morphological, ultrastructure and metal deposition in the fungal cells were studied.

## MATERIAL AND METHODS

**Organisms and culture conditions.** *Chaetomium globosum* and *Stachybotrys chartarum* were isolated from industrial polluted area of Helwan Egypt. They were isolated on modified Dox agar medium containing 200mg/L Cu SO<sub>4</sub> or Co SO<sub>4</sub> and identified according to (Domsch, K.H *et al.* 1980). The Dox agar medium contains (g/L) sucrose, 30; NaNO<sub>3</sub>, 2; KH<sub>2</sub>PO<sub>4</sub>, 1; MgSO<sub>4</sub> ·7H<sub>2</sub>O, 0.5; KCl, 0.5; FeSO<sub>4</sub>·7H<sub>2</sub>O, trace; agar, 20; and 1 L distilled water. The pH was adjusted to 6.5.

**Growth, metal content and biochemical analysis.** *C. globosum* and *S. chartarum* were grown on Dox liquid medium supplemented with different concentrations of Cu and Co (0.0, 200, 400, 600, 800 & 1000 mg/L). Both fungi were grown in 250-ml Erlenmyer flasks containing 100mL of the medium (pH 6.5). The flasks were incubated for 7 d. at 28 °C ±2; the mycelium was harvested, washed several times by distilled water and dried at 85 °C until a constant weight and the mycelium dry weight was determined. The dry mass (0.2g) was treated with 10 mL of 0.5mol/L HCl at 50 °C for 30 min with occasional shaking, after centrifugation the supernatant was analyzed by atomic absorption spectrophotometer, Perkin Elmer As-60 apparatus (Venkateswerlu, G., G. Stotzky, 1989). Sugar determination was carried out using the anthrone technique as described by (Umbriet, W.W. *et al.* 1959). Total protein was determined by the method of (Lowery, O.H. *et al.* 1951) While, total lipid was determined using phosphovanillin reagent after extraction by chloroform methanol mixture (Barnes, H., J. Blackstock, 1973).

**Morphological and sporogenesis studies.** The test organisms were grown on Dox agar medium in the presence of different concentrations of Cu and Co. The morphological features of both organisms were studied under the light microscope and light micrographs were obtained. 1cm<sup>2</sup> of the agar colony was cut and suspended in 9mL sterile distilled water. The spores or cleithothecia was counted per mL of the suspension using a Haemocytometer.

**Electron microscopy.** Hyphal tips obtained from mycelium grown in the presence of different concentrations of copper and cobalt ions were fixed separately for 1 h. in 0.05% sodium phosphate buffer (pH 6.9) containing 1% formaldehyde, 3% glutaraldehyde and 0.05% tannic acid and then washed with 0.1 mol/L sodium phosphate buffer (pH 6.9) for 10 min. Mycelial pieces were treated with 0.5% osmium tetroxide in 0.1 mol/L sodium phosphate buffer (pH 6.9) and then left over night at 4 °C. Fixed mycelia were dehydrated in an ethanol series and transferred to 2-methyloxirane for ½ h. and embedded in Spurr's resin. Thin sections were cut with glass knife and collected on uncoated copper grids, stained with concentrated uranyl acetate for 5 min followed by lead citrate for 10 min and observed with a Jeol JEM 100SX electron microscope.

## RESULTS AND DISCUSSION

### Results:

*Chaetomium globosum* and *Stachybotrys chartarum* were able to tolerate Cu and Co metal ions in the growth medium upto 800 mg/L and failed completely to grow at 1000mg/L. Their growth markedly decreased with increasing metal concentrations in the medium (Table I and 2). 50% growth inhibition of *C. globosum* occurred approximately at 400 mg/L Cu or Co. while it was between 400-600mg/L Cu or Co for *S. chartarum*. The dry mass of *C. globosum* sharply decreased to approximately 87.5 and 90.2% when it was grown at 800 mg/L Cu or Co respectively. On the other hand the growth of *S. chartarum* at 800 mg/L Cu or Co markedly decreased to approximately 85.6 and 87.4% respectively. Metal content in the mycelium of both fungi was subsequently increased with increasing metal concentrations in the medium and represented high affinity to absorb Cu more than Co. On the other hand, total protein, lipid and carbohydrates showed also a slight increase in the mycelium with elevated Cu and Co concentrations up to 400 mg/L and decreased above this concentration.

**Table 1:** Dry mass, metal, protein, lipid and carbohydrate content of *Chaetomium globosum* grown in presence of different concentrations

of copper and cobalt.

Metal conc. (mg/L)	Dry mass		Metal content		protein		lipid		Carbohydrate	
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	Cu	Co	Cu	Co	Cu	Co	Cu	Co	Cu	Co
0.0	256±6.2	260±6.6	0.0	0.0	44±0.6	45±0.6	53±0.8	52±1.2	172±4.4	172±4.2
200	175±5.4	184±4.2	34±0.6	30±0.2	45±0.4	50±1.2	54±1.1	55±0.8	175±4.8	174±5.4
400	120±3.3	142±4.2	40±0.8	35±0.4	51±0.8	60±1.4	56±0.8	58±1.4	186±5.4	182±4.0
600	52±1.2	62±1.4	46±1.2	41±0.8	43±0.4	42±0.8	44±1.6	43±0.6	171±3.3	165±3.4
800	32±1.4	25±1.2	56±0.8	44±1.4	32±0.6	31±0.4	41±0.6	41±0.4	143±4.4	140±3.6

Data are expressed as mg per 100 mL liquid medium and µg metal, protein, lipid and carbohydrate per mg dry mycelium ±SE of three investigations.

**Table 2:** Dry mass, metal, protein, lipid and carbohydrate content of *Stachybotrys chartarum* grown in presence of different concentrations of copper and cobalt.

Metal conc. (mg/L)	Dry mass		Metal content		protein		lipid		Carbohydrate	
	-----		-----		-----		-----		-----	
	Cu	Co	Cu	Co	Cu	Co	Cu	Co	Cu	Co
0.0	167±3.4	167±3.4	0.0	0.0	43±0.4	45±1.6	50±1.8	51±1.7	146±3.4	147±5.2
200	145±4.2	149±3.2	28±0.4	25±0.4	47±1.2	49±1.2	51±1.1	52±0.8	149±2.8	155±6.4
400	112±4.1	131±2.4	33±0.8	28±0.4	47±0.8	50±1.3	52±1.6	55±1.6	150±5.6	157±4.4
600	50±2.4	57±1.4	40±1.4	38±0.6	43±0.4	39±0.8	48±0.6	43±0.8	143±3.6	140±3.8
800	24±0.5	21±0.2	47±1.2	40±1.4	30±0.5	30±0.6	40±0.8	35±0.6	134±2.4	130±3.5

Data are expressed as mg per 100 mL liquid medium and µg metal, protein, lipid and carbohydrate per mg dry mycelium ±SE of three investigations.

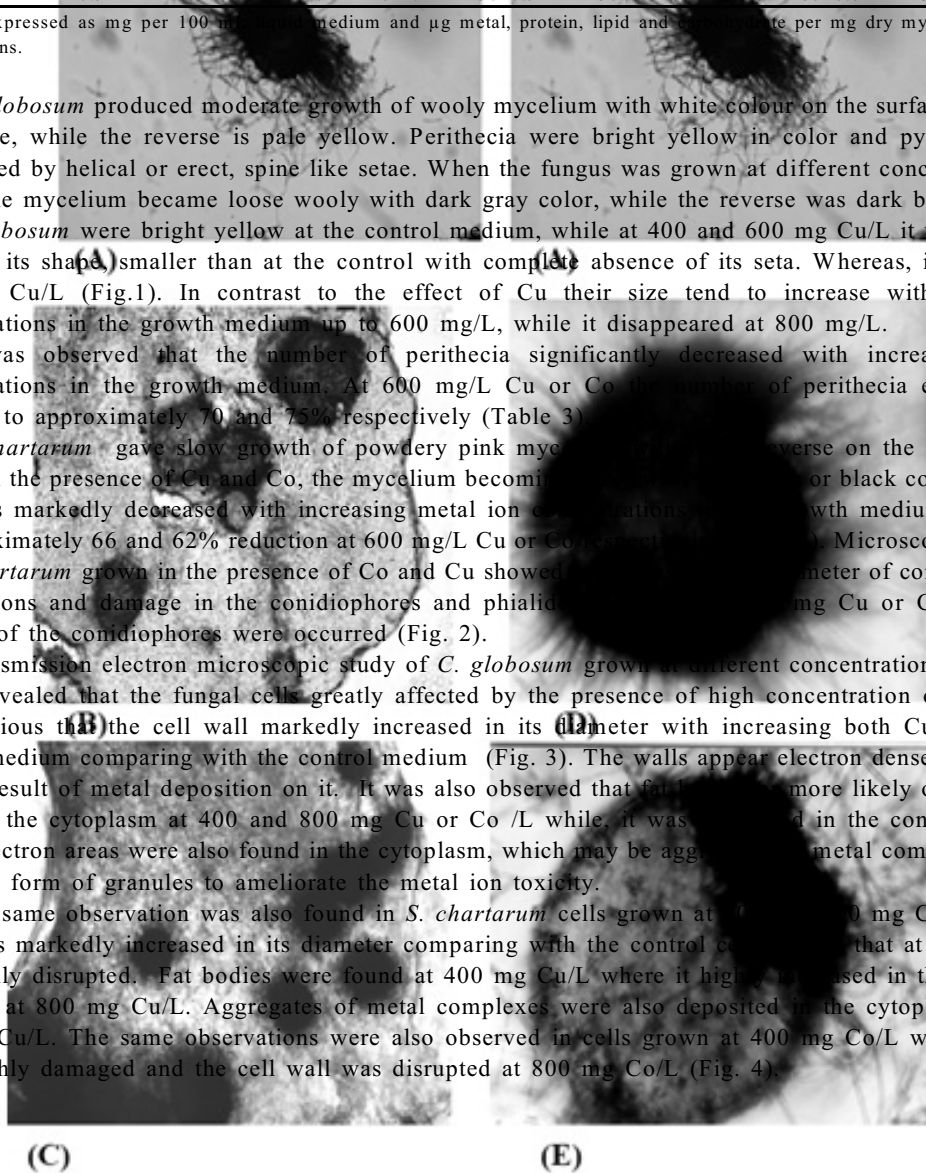
*C. globosum* produced moderate growth of woolly mycelium with white colour on the surface of the control agar plate, while the reverse is pale yellow. Perithecia were bright yellow in color and pyriform in shape, surrounded by helical or erect, spine like setae. When the fungus was grown at different concentrations of Cu or Co, the mycelium became loose woolly with dark gray color, while the reverse was dark brown. Perithecia of *C. globosum* were bright yellow at the control medium, while at 400 and 600 mg Cu/L it was dark brown, round in its shape, smaller than at the control with complete absence of its seta. Whereas, it disappeared at 800 mg Cu/L (Fig.1). In contrast to the effect of Cu their size tend to increase with increasing Co concentrations in the growth medium up to 600 mg/L, while it disappeared at 800 mg/L.

It was observed that the number of perithecia significantly decreased with increasing metal ion concentrations in the growth medium. At 600 mg/L Cu or Co the number of perithecia exhibited a high decrease to approximately 70 and 75% respectively (Table 3).

*S. chartarum* gave slow growth of powdery pink mycelium on the surface of the control medium, Where in the presence of Cu and Co, the mycelium becoming black or black color. The number of spores markedly decreased with increasing metal ion concentrations in the growth medium till it reached to approximately 66 and 62% reduction at 600 mg/L Cu or Co respectively (Table 3). Microscopic examination of *S. chartarum* grown in the presence of Co and Cu showed that the diameter of conidiophores with constrictions and damage in the conidiophores and phialides. At 400 mg Cu or Co/L a complete absence of the conidiophores were occurred (Fig. 2).

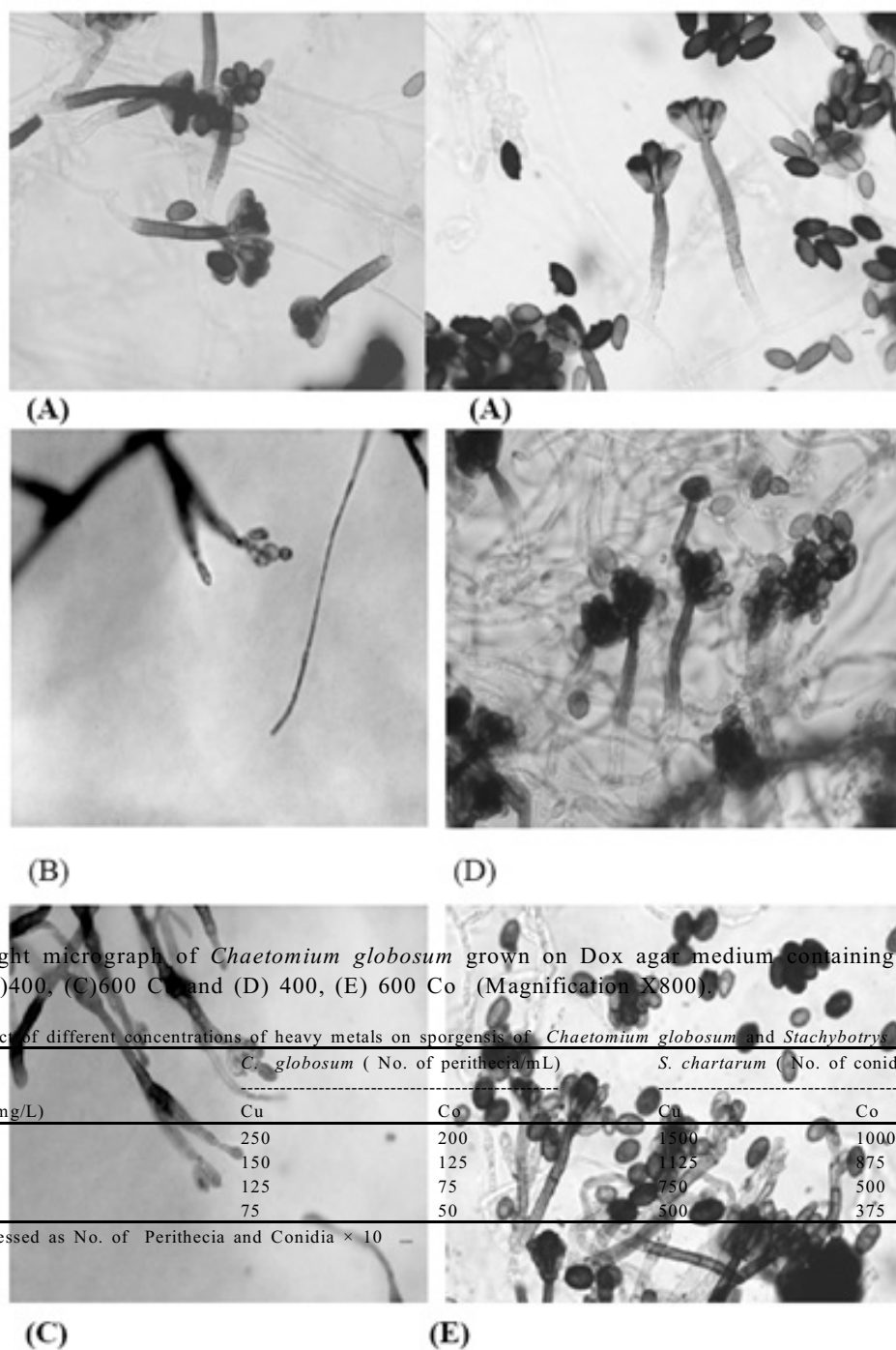
Transmission electron microscopic study of *C. globosum* grown at different concentrations of copper and cobalt revealed that the fungal cells greatly affected by the presence of high concentration of heavy metals. It is obvious that the cell wall markedly increased in its diameter with increasing both Cu and Co in the growth medium comparing with the control medium (Fig. 3). The walls appear electron dense black, this may be as a result of metal deposition on it. It was also observed that fat bodies or more likely oil droplets were found in the cytoplasm at 400 and 800 mg Cu or Co /L while, it was not observed in the control cells. Black dense electron areas were also found in the cytoplasm, which may be aggregates of metal complex, which may be in the form of granules to ameliorate the metal ion toxicity.

The same observation was also found in *S. chartarum* cells grown at different concentrations of Cu or Co/L. The cell walls markedly increased in its diameter comparing with the control cells. It was observed that at 800 mg Co/L it was highly disrupted. Fat bodies were found at 400 mg Cu/L where it highly increased in their number and diameter at 800 mg Cu/L. Aggregates of metal complexes were also deposited in the cytoplasm at 400 and 800 mg Cu/L. The same observations were also observed in cells grown at 400 mg Co/L whereas, the cells were highly damaged and the cell wall was disrupted at 800 mg Co/L (Fig. 4).



(C)

(E)

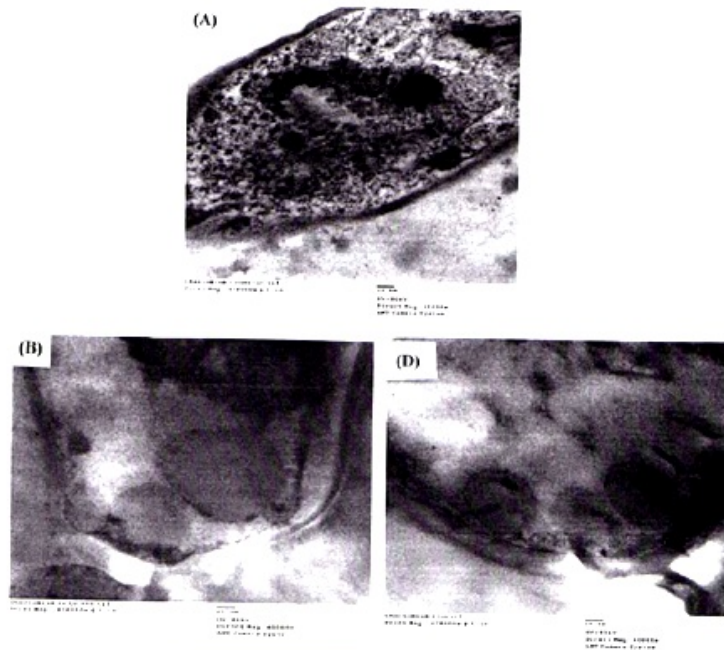


**Fig. 1:** Light micrograph of *Chaetomium globosum* grown on Dox agar medium containing mg/L (A)0.0, (B)400, (C)600 Cu and (D) 400, (E) 600 Co (Magnification X800).

**Table 3:** Effect of different concentrations of heavy metals on sporogenesis of *Chaetomium globosum* and *Stachybotrys chartarum*.

Species	<i>C. globosum</i> ( No. of perithecia/mL)		<i>S. chartarum</i> (No. of conidia/ mL)	
	Cu	Co	Cu	Co
Metal conc. (mg/L)				
0.0	250	200	1500	1000
200	150	125	1125	875
400	125	75	750	500
600	75	50	500	375

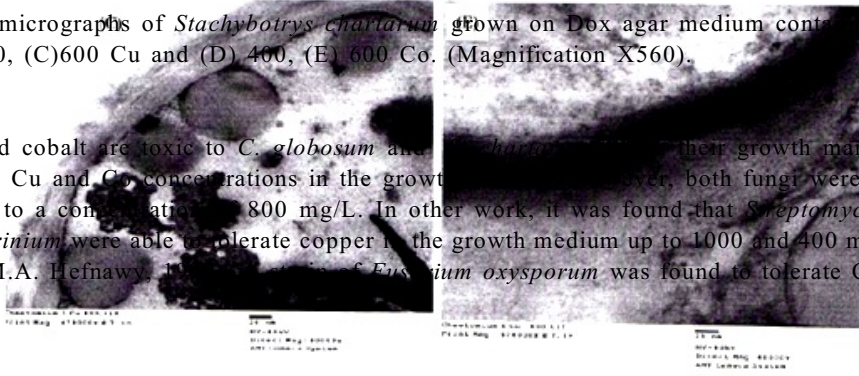
Data are expressed as No. of Perithecia and Conidia  $\times 10^{-4}$



**Fig. 2:** Light micrographs of *Stachybotrys chartarum* grown on Dox agar medium containing mg/L (A)0.0, (B)400, (C)600 Cu and (D) 400, (E) 600 Co. (Magnification X560).

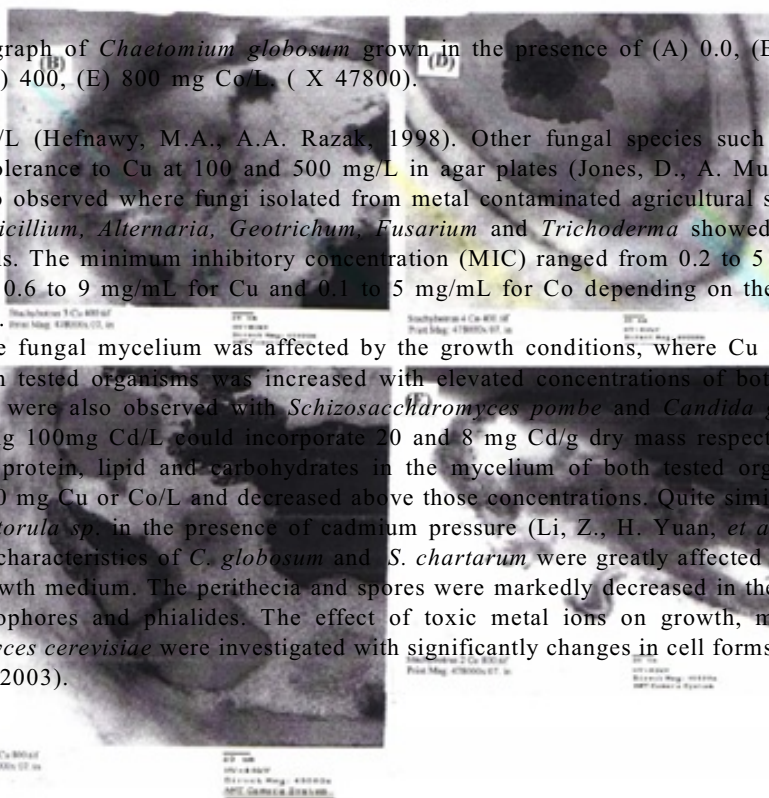
**Discussion:**

Copper and cobalt are toxic to *C. globosum* and *S. chartarum* as their growth markedly decreased with increasing Cu and Co concentrations in the growth medium. However, both fungi were able to tolerate Cu and Co up to a concentration of 800 mg/L. In other work, it was found that *Streptomyces anulatus* and *Penicillium citrinum* were able to tolerate copper in the growth medium up to 1000 and 400 mg/L respectively (Azab, A.A., M.A. Hefnawy, 1997). A strain of *Fusarium oxysporum* was found to tolerate Cu in the growth





**Fig. 3:** Electron micrograph of *Chaetomium globosum* grown in the presence of (A) 0.0, (B) 400, (C) 800 mgCu/L and (D) 400, (E) 800 mg Co/L. ( X 47800).



medium up to 600 mg/L (Hefnawy, M.A., A.A. Razak, 1998). Other fungal species such as *Thelephora terrestris* proved high tolerance to Cu at 100 and 500 mg/L in agar plates (Jones, D., A. Muehlchen, 1994). Similar results were also observed where fungi isolated from metal contaminated agricultural soil belonged to genera *Aspergillus*, *Penicillium*, *Alternaria*, *Geotrichum*, *Fusarium* and *Trichoderma* showed a significantly tolerance to heavy metals. The minimum inhibitory concentration (MIC) ranged from 0.2 to 5 mg/mL for Cd, 0.3 to 7 mg/mL for Cr, 0.6 to 9 mg/mL for Cu and 0.1 to 5 mg/mL for Co depending on the isolate (Zafar, S., F. Aqil, *et al.* 2007).

Metal uptake by the fungal mycelium was affected by the growth conditions, where Cu and Co content in the mycelium of both tested organisms was increased with elevated concentrations of both metals in the medium. Similar results were also observed with *Schizosaccharomyces pombe* and *Candida glabrata* grown in the medium containing 100mg Cd/L could incorporate 20 and 8 mg Cd/g dry mass respectively (Krumov, N. *et al.* 2007). Total protein, lipid and carbohydrates in the mycelium of both tested organisms slightly increased at 200 and 400 mg Cu or Co/L and decreased above those concentrations. Quite similar results were also observed in *Rhodotorula sp.* in the presence of cadmium pressure (Li, Z., H. Yuan, *et al.* 2008).

The morphological characteristics of *C. globosum* and *S. chartarum* were greatly affected by the presence of Cu and Co in the growth medium. The perithecia and spores were markedly decreased in their number with damage of seta, conidiophores and phialides. The effect of toxic metal ions on growth, morphology and functions of *Saccharomyces cerevisiae* were investigated with significantly changes in cell forms and its activity (Yang, H.C., L.L. Pon, 2003).

**Fig. 4:** Electron micrograph of *Stachybotrus chartarum* grown in the presence of (A) 0.0, (B) 400, (C) 800 mgCu/L and (D) 400, (E) 800 mg Co/L. ( X 47800).

*C. globosum* and *S. chartarum* could accumulate Cu and Co either on the cell wall or in the cytoplasm, where black dense granules were observed by transmission electron microscope. These may be a deposition of metal complexes as a mean of compartmentation of these ions inside the cell. Quite similar observations were also found by *Aspergillus niger*, where analytical electron microscopy revealed that nickel was localized in the form of small rectangular crystals associated with the cell wall and inside the cell (Magyarosy, A. *et al.* 2002). Ultrastructural localization of heavy metals in the mycelium of *Glomus intraradices* revealed that metals were accumulated mainly in the cell wall and in the vacuoles. These results suggest that is essential for metal detoxification (Gonzalez, G.M. *et al.* 2008).

Another study, which may sustain our results, showed that *Cladosporium cladosporioides* could sequester manganese in the form of intra-cellular crystals as shown by transmission electron microscope (Shao, Z., F. Sun, 2007). *Penicillium spp.* Was also accumulated a large amount of lead granules in the cell as well as on the outer layer of the cell wall when grown in the presence of 24 mM Pb (NO<sub>3</sub>)<sub>2</sub> as observed by electron microscope (Sun, F., Z. Shao, 2007).

Large bodies were also observed in the cytoplasm of *Chaetomium globosum* and *Stachybotrys chartarum*. These bodies may be fat bodies or oil droplets and might be formed as a result of some metabolic changes due to the presence of toxic metal ions. Similar bodies were also found in the cytoplasm of *Fusarium oxysporum* cells grown under copper stress (Hefnawy, M.A., 1996).

In conclusion, both tested organisms confront the toxic effect of metal ions by binding it on the cell wall or by transform it into metal complex granules which may deposit on the wall or sequestered in the cytoplasm to ameliorate its toxicity.

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