Morphological and Ultra-Structure Changes of *A.niger* and *A.terreus* Grown Under Chromium and Silver Stress

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Abstract

The growth of *A.niger* and *A.terreus* were markedly decreased with increasing Cr ion concentrations in the growth medium up to 2000 mg/L. Whereas, *A.niger* tolerated Ag up to 800mg/L and *A.terreus* tolerated it up to 200 mg/L. The conidiophores of *A.niger* and *A.terreus* exhibited a constrictions and distortion in the conidial heads and phialides with elevated Cr in the growth medium. Except that at 2000mgCr/L, *A.terreus* failed to form conidiophores and the mycelia seemed to be distorted with appearance of intercalary spores. A constriction in the conidiophores and distortions in the conidial heads and phialides were also observed when *A.niger* and *A.terreus* were grown in elevated concentrations of silver. Electron microscopic study of *A.niger* and *A.terreus* cells grown with elevated Cr and Ag concentrations, revealed that the cytoplasm of both organisms aggregated in the form of black dense area inside the cells and also the cell wall increased in its diameter and appeared electron dense black, which may be due to deposition of metals in the form of granules or crystals on the cell wall or in the vacuoles.

Key words: Heavy Metals, Light Microscope, Electron Microscope (TEM), Fungi, Morphological characteristics.

INTRODUCTION

Heavy metal contamination occurred as a natural process or as a result of human activities is a great environmental problem. The morphological changes induced by heavy metals are common among all groups of fungi. Some reports are available on the effect of heavy metals on morphogenesis of the vegetative hyphae (**Gabriel *et al.*, 1996 and Baldrain 2003**), sporulation (**Duarte *et al.*, 2004**).

The morphology of *Fusarium moniliforme* was completely distorted in the presence of 3.0% (w/v) of sodium tellurite (**Amer *et al.*, 2005**). The heavy metals Cu, CO, Hg, Zn and Cd at concentration between 0.5 and 3.0 mM decreased the mycelia area and radial extension of *Achlya bisexualis* and displayed spiral growth at 3.0 mM Hg (**Lundy *et al.*, 2001**). Lead has strongly effect on *Aspergillus nidulans* where it caused hyphal malformation with many twisted and swollen like shape at 200 ppm, this malformation increased with increasing metal ion concentration (**Elkhawage, 2011**).

Heavy metals were accumulated or deposited in the form of granules or crystals on the cell wall, cytoplasm or vacuoles of fungal or bacterial cells when grown in the presence of high metal ion stress. Transmission electron microscopic study showed that the cell wall of *Chaetomium globosum* and *Stachybotrys chartarum* increased in its diameter at 400 and 800 mg/L Cu or Co. Furthermore, black dense electron areas were also found in the cytoplasm, which may be aggregates of metals complex that deposited in the form of granules or crystals (**Hefnawy *et al.* 2009**).

Localized silver deposition a round cell wall and within vacuoles has observed in *Cryptococcus albidus*. Mercury precipitation in electron dense bodies occurs in

mercury exposed hyphae of *Chrysoporium pannprum* and similar bodies , presumed to contain Zinc, occurred in *Neurospora vasinfecta* after Zn^{+3} influx (**Simmons *et al.*, 1995**). 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 *et al*., 2002**).

A lead resistant *Pencillium sp.* accumulated a large amount of lead granules in the cell and the outer layer of the cell wall, as observed under a transmission electron microscope. Theses granules were either in the vicinity of the cytoplasm membrane or in the vacuole (**Sun and Shao, 2007**). *Pencillium brevicompactum* grown at 50 ppm cobalt exhibited the presence of more dense electron deposits in the vacuoles and on the outer layer of the cell wall(**Rasha , 2009**). This work aims to investigate the tolerance of two studied fungal species to chromium and silver stress with subsequent morphological, ultra-structure changes metal deposition in the fungal cells.

MATERIALS and METHODS

Organisms and culture conditions: *A. niger* and *A. terreus* were isolated from industrial polluted area of Quesna, Egypt on Czapek's –Dox agar medium containing 100 mg/L chromium or silver and identified according to (**Raper and Fennell, 1965**).

Growth and metal tolerance: Both organisms were cultivated on Czapek's –Dox liqued medium containing different concentrations of chromium sulphate (0.0, 100, 200, 400, 800, and 2000mg/L) and silver sulphate(0.0, 10, 20, 30, 50, 100, 200,

400, 600, and 800mg/L) separately. They were grown on 250 ml Erlenmyer flasks containing 50 ml of the medium (pH 6.5). The flasks were inoculated with 7 mm disc from the margin of 5 days old colony of tested fungi, incubated at 27±1° C in an orbital shaker at 120rpm for 7 days. After incubation period, the cultures were filtered and the fungal pellets were dried in an oven at 80°C till constant weight (**Hefnawy and Azab, 2000**), and their weights were determined. Three replicates were made for each treatment and the dry mass was determined.

Morphological examinations: Both organisms were grown on Czpek's -Dox agar medium supplemented with different concentrations of chromium sulphate (800 and 2000 mg/L), the most effective concentrations, for *A.niger* and *A.terreus* separately and silver sulphate (200and 600 mg/L) for *A.niger,* (100 and 200mg/L) for *A.terreus.* The plates were incubated at 27±1° C for 2-3 days after inoculation. The morphological features of both organisms were examined by light microscope and photographed.

Ultrathin section examination: *A.niger* and *A.terreus* were grown separetely on Czapek's-Dox agar plates containing different concentrations of Cr (0.0 and 800mg/L) for both fungi and Ag (0.0, 600mg/L) for *A.niger* while, (0.0 and 50mg/L) for *A.terreus*. After growth for 3 days at 27±1°C. The hyphal tips of the fungal mycelia were cut, fixed separately for 1 h. in 0.05% sodium phosphate buffer (pH 6.9) containing 1% formaldehyde, 3% glutraldehyde 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 sodium phosphate buffer (pH 6.9) and then left over night at 4 ^{0}C. Fixed mycelia were dehydrated in an ethanol series (**Zain, 1998**), and transferred to 2-methyloxiran for half an hour

and embedded in Spurr's resin. Thin sections were cut and collected on coated carbo, stained with concentrated uranyl acetate for 5 min followed with lead citrate for 10 min and examined with a JEOL 1010 Transmission Electron Microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

RESULTS and DISCUSSION

Growth and metal tolerance: *A.niger* and *A.terreus* were able to tolerate Cr ions in the growth medium up to 2000 mg/L. Their growth showed a slight increase at 200 mg Cr/L while, it was markedly decreased by increasing chromium concentrations above this concentration (**Table 1**). At 2000 mg Cr/L, *A.niger and A.terreus* growth decreased to approximately 32% and 49% respectively.

A.terreus was able to grow on Cazpek's- Dox liquid medium supplemented with different silver concentrations up to 200mg/L. While, *A.niger* could grow in the presence of silver up to 800mg/L. The growth of both fungi was decreased by increasing silver concentrations in the growth medium. The growth of *A.niger* slightly decreased at 50mgAg/L while, the dry mass markedly decreased to approximately 81.5% at 800 mg Ag/L. Whereas, *A.terreus* was able to grow in the presence of silver up to 200mg/L. At this concentration its growth was markedly decreased to approximately 84%. The difference between fungal species in tolerance to heavy metals may be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different fungi as explained by **Zafar *et al* ., (2007)**.

In this study, the growth of *A.niger* and *A.terreus* were decreased with increasing silver and chromium concentrations in the growth media and this may be due to the toxic effect of heavy metals on the fungal cell. Many studies revealed that the presence of toxic metals in the growth medium inhibited fungal growth, **Al-Kadeeb (2007)** found that by increasing Cu and Pb concentration in the growth medium, the growth and colony diameter of eighteen fungal isolates decreased. (**Hefnawy *et al.*, 2010**) mentioned that, the dry mass of *Stachybotrys chartarum* was markedly decreased with increasing Cu and Co in the growth medium.

Morphological characteristics examination: *A.niger* produce colonies covering the whole Petri dish with white mycelium covered by black conidial heads with reverse pale to bright yellow. Conidiophores borne from hyphae with heavy smooth walls, with spherical heads or vesicles bearing closely packed phialides over the whole surface of conidial heads. The presence of 800 mg Cr/L in the growth medium leads to increase in the length of conidiophores with appearance of some constrictions in the conidiophores and distortion in the conidial heads. Whereas, in the presence of 2000 mgCr/L the number of conidiophores highly decreased with the presence of distorted short conidiophores (**Fig.1**). On the other hand, the morphology of *A.niger* in the presence of 200mgAg/L exhibited constrictions in the conidiophores and distortions in the conidial heads and phialides. Whereas, at 600mgAg/L, the conidiophores were very short with complete absence of phialides and spores (**Fig. 2**).

A.terreus produces quite dense velutinous colonies with white mycelium, heavy brown conidia, reverse dull brown or yellow brown. Conidiophores borne from surface hyphae long, smooth walled with hemispherical conidial heads and dense

phialides. Growing of *A.terreus* in the presence of 800mgCr/L causing some constrictions in the conidiophores and complete distortions in the phialides with appearance of intercalary spores. Whereas, at the presence of 2000mgCr/L the fungus failed to form conidiophores and the mycelia seemed to be distorted with appearance of intercalary spores (**Fig. 3**). On the other hand, at 100mg Ag/L the conidiophores showed some constrictions and also distortion in the conidial heads and phialides while, at 200mgAg/L the fungus failed to form a vesicles with complete distortion in the conidial heads and phialides (**Fig. 4**).

The effect of toxic heavy metals on the morphological characterisitic of filamentous fungi have been studied, cobalt, nickel and mercury were suppressed the growth and morphology of *A.nidulans* and *Fusarium oxysporium* and caused malformation of conidiophores (**Elkhawaga, 2011**).

It has been also observed that heavy metals exhibited high effect on the reproduction of fungi. The perithecia of *Chaetomium globosum* and sporulation of *Stachybotrys chartarum* markedly decreased with increasing both Cu and Co concentration in the growth medium and also high damage in the perithecial seta of *C.globosum*, conidiophores and phialides of *S.chartarum* was also observed at high concentrations of Cu and Co (**Hefnawy *et al.*, 2009**).

Also similar results were also obtained, tellurite and selenite caused obvious malformation in the morphology of *Aspergillus parasiticus var. globosus* under the presence of different levels of selenite or tellurite (**Zohari *et al.*, 1997**). Another studies showed also morphological distortions of *A.fumigatus*, *A.terreus, A.niger* and *A. tamarii* in the presence of tellurite in the growing medium (**Aboul- Dahab, 1991 and Razak *et al.*, 1990**).

Ultra thin section examination: Electron micrographs of thin sections of *A.terreus* cells grown in the control medium revealed the presence of electron transparent cytoplasm with numerous vacuoles and spherical mitochondria. The cytoplasm filled the cell with the presence of several vacuoles and also hyaline cell wall and plasma membrane were appeared. Whereas, when *A.terreus* grown in the presence of 800mgCr/L the cytoplasm was aggregated in the cells and appeared electron dense and also the cell wall which may be due to binding of Cr to the cell wall and cytoplasm. The same finding was also observed in the cells grown in the presence of 50mgAg/L (**Fig. 5**).

The cytoplasm of *A.niger* cells grown in the presence of 800 mg Cr/L was aggregated and separated from the cell wall and appeared black dense which may be due to the presence of Cr in it. Whereas, at 600 mg Ag/L the cell walls were increased in its diameter and appeared electron dense black which may be due to precipitation of Ag on the cell wall and also the cytoplasm was aggregated inside the cell (**Fig. 6**).

In this respect the cell wall of *Chaetomium globosum* and *Stachybotrys chartarum* increased in it's diameter in the presence of 400 and 800 mg/L Cu or Co and also black dense electron areas were found in the cytoplasm as a result of metal deposition in the form of granules or crystals (**Hefnawy *et al.*, 2009**). It was also observed that localized silver deposition around cell walls and within the vacuoles has found in *Cryptococcus albidus*, mercury precipitation in electron dense bodies were occurred in mercury exposed hyphae of *Chrysosporium pannorum* (**Simmons *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 under high metal stress. *A. niger* localized nickel in the form of rectangular crystals of nickel oxalate dehydrate with the cell wall and also inside the cell (**Magyarosy *et al.*, 2002**). Another work, which may sustain this study, showed that *Cladosporium cladosporioides* could sequester manganese in the form of intra-cellular crystals. *Penicillium sp.* was also accumulated 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 $pb(NO3)_2$ (**Shao and Sun, 2007**). Large bodies which may be fat bodies or oil droplets might be formed in the cytoplasm of *Fusarium oxysporium* grown in the presence of high concentrations of $CuSO_4$ (**Hefnawy,1996**). *Penicililum brevicompactum* highly tolerated cobalt concentrations up to 1000 ppm through cell wall and intracellular sequestration (**Rasha, 2009**).

In conclusion, both tested organisms were able to tolerate high concentrations of Cr and Ag in the growth medium through sequestering or immobilization of these toxic metals in the cytoplasm and cell wall. Finally, *A.niger* and *A.terreus* could be used as a module for purification of heavy metal polluted soil and also waste water by sequestering or accumulation of these toxic metals.

Table 1. Mycelial dry weight of *A.niger* and *A.terreus* grown on Czapek's -Dox liquid medium supplemented with different chromium concentrations.

Chromium concentrations mg/L	Dry mass (mg)	
	A.niger	***A.terreus***
0	626.5±1.25	442.8±0.98
200	642.2±0.25	520.2±1.02
400	544.2±0.25	444.7±0.99
800	507.1±1.03	372.3±0.98
2000	427.6±0.25	229±1.01

Table 2. Mycelial dry weight of *A.niger* and *A.terreus* grown on Czapek's-Dox liquid medium supplemented with different silver concentrations.

Silver concentrations mg/L	Dry mass (mg)	Silver concentrations mg/L	Dry mass (mg)
	A.niger		***A.terreus***
0	616.2±1.06	**0**	550.8±1.02
50	529.2±1.22	**10**	452.3±1.005
100	490.6±0.98	**20**	254.7±0.85
200	254.2±1.02	**30**	201.3±0.54
400	198.8±0.58	**50**	164.9±1.03
600	145.7±0.86	**100**	153.1±0.23
800	114±0.76	**200**	90±0.23

Fig. 1. Light micrographs of *A.niger* grown on Czapek's Dox agar (a) control, (b) 800 mg/L chromium, and (c) 2000 mg/L chromium.(Magnification, x560)

Fig. 2. Light micrographs of *A.niger* grown on Czapek's Dox agar medium (a) control, (b) 200 mg/L silver, and (c) 600 mg/L silver.(Magnification, x650)

Fig. 3. Light micrographs of *A.terreus* grown on Czapek's Dox agar medium (a)control, (b) 800 mg/L chromium, and (c) 2000 mg/L chromium. (Magnification, x650)

Fig. 4. Light micrographs of *A.terreus* grown on Czapek's Dox agar medium (a) control, (b) 100 mg Ag/L, and (c) 200 mg Ag/L. (Magnification, x650)

Fig.5. Transmission electron micrograph of thin section of cells of *A.niger* after growth for 3 days on Czapek's- Dox agar medium (a) control, (b) 800 mg/L chromium, and (c) 600 mg/L silver

Fig. 6. Transmission electron micrograph of thin section of cells of *A. terreus* after grown on Czapek's -Dox agar medium (a) control, (b) 800 mg/L chromium, and (c) of 50 mg/L silver.

REFERENCES

Aboul-Dahab, N.F. (1991). Response of certain fungal communities towards environmental pollutants. **MSc Thesis, *Botany Department, Fac Sci, Al-Azhar Univ., Cairo, Egypt*.**

Al-Kadeeb, A. Siham (2007). Effect of lead and copper on the growth of heavy metal resistance fungi isolated from second industrial city in Riyadh, Saudi Arabia. ***J. of Appl. Sci.*, 7(7): 1019-1024.**

Amer,S.M.;El-Refai,I.M. and Yehia A.G. (2005). Morphological and Metabolic response of *Fusarium moniliforme* to tellurium stress. ***Pakistan Journal of Biol. Sci.*,8(3): 379-383.**

Baldrain, P. (2003). Interactions of heavy metals with white-rot fungi. ***Enzyme and Microbial Technol.*, 32(1-2): 78-91.**

Duarte, S.; Pascoal, C. and Acassio, F. (2004). Effects of zinc on leaf decomposition by fungi in streams: studies in microcosm. ***Microb.Ecol*.93:366-374.**

Elkhawaga, M.A. (2011): Morphological and Metabolic response of *Aspergillus nidulans* and *Fusarium oxysporium* to heavy metal stress. ***J. of app.sci. Res.*, 7(11):1737-1745.**

Gabriel, J.; Kofronova, O.; Rychlovky, P.O. and Krenzeluk, M. (1996). Accumulation and effect of cadmium in the wood-rotting basidiomycete *Daedalea quercina*. ***Bull.Environ. Contam. Toxicol.* 57:383-390.**

Hefnawy, M.A. (1996). Phospholipids and fatty acids composition in copper tolerant *Fusarium oxysporum*. **Proceedings of the First International Conference on Fungi: Hopes & Challenges Cairo, 2-5 Sept. (1):21-30.**

Hefnawy, M.A. and Azab, E.A.(2000)**.** Cupper induced cell wall changes and metal-binding capacity in Pencillium citrinum and Streptomyces anulatus. **Al-Azhar J. Microbiol., 49:161-174.**

Hefnawy, M.A.; Ali, M. and Abdul- Ghany, S. (2009). Influence of copper and cobalt stress on Morphology and Ultra-structure of *Chaetomium globosum* and *Stachybotrys Chartarum*. ***Australian J. of Basic and Appl. Sci.*, 3(4):3158-3165.**

Hefnawy, M.A.; Ali, M. and Abdul- Ghany, S. (2010). Influence of copper and cobalt stress on membrane fluidity of *Stachybotrys chartarum*. ***SENRA academic publishers, Burnaby, British Columbia*, 4(1):1003-1009.**

Lundy, S.D.; Payne, R.P.; Giles, K. and Garrill, A. (2001). Heavy metals have different effects on mycelia morphology of *Achlya bisexualis* as determined by fractal geometry. ***FEMS Microbiol. Lett,* 201:259-263.**

Magyarosy, A.; Laidlaw, R. D.; Kilaas, R.; Echer, C.; Clarck, D. S. and Keasling, J. D. (2002). Nickel accumulation and nickel oxalate precipitation by *Aspergillus niger*. ***Appl. Microbiol. Biotechnol*., 59(2-3):382-388.**

Raper, K.B. and Fennel, D.I. (1965). The Genus *Aspergillus*. ***The Williams and Wilkins Co.* Baltimore, Pp. 686.**

Rasha, M. Farag (2009). Ultrastructure, Glutathione and low Molecule weight Proteins of *Pencillium brevicompactum* in response to Cobalt. ***J. of Microbiol.*, 58(4): 327-338.**

Razak, A.A.; Ramadan, S.E.; Ragab, A.M. and El-Meleigy, M.A. (1990) Telurium volatilization by *A.fumigatus*, *A.terreus* and *P.chrysogenum*. ***The fourth interional mycological congress*, 28Aug- 3rd Sept, Regensburg, W Germany.**

Shao, Z. and Sun, F. (2007). Intracellular sequestration of manganese and phosphorus in a metal-resistant fungus *Cladosporium cladosporioides* from deep-sea sediment. ***Extrimophiles*, 11(3): 435-443.**

Simmons, P.; Tobin, J. M. and Singleton, I. (1995). Consideration of the use of commercially available yeast biomass for the treatment of metal containing effluents. ***J. Indust. Microbiol.*, 14:240-246.**

Sun, F. and Shao, Z. (2007). Biosorption and bioaccumulation of lead by *Penicillium sp. Psf-2* isolated from the deep sea sediment of Pacific Ocean. ***Extremophiles*, 11(6):853:858.**

Zafar, S.; Aqil, F. and Ahmed, I. (2007). Metal tolerance and biosorption potential of Filamentous fungi isolated from metal contaminated agricultural soil. ***Bioreso. Technol.*, 98:2557-2561.**

Zain, M.E., (1998). Modern approaches to the taxonomy of fungi. **Ph.D. thesis, Bot. and Microb. Dept., Fac. of Sci. for Boys, Al-Azhar Univ., Cairo, Egypt.**

Zohri, A. A.; Saber, S.M. and Mostafa, M.EA. (1997). Effect of selenite and tellurite on he morphological growth and toxin production of *Aspergillus parasiticus var. glubosus IMI 120920*. ***Mycopathol.*, 139:51-57.**

التغيرات المورفولوجية والتركيبية فى فطرة اسبيرجلس نيجر واسبرجلس تيريس النامية تحت إجهاد الكروميوم والفضة

فى هذا البحث لوحظ أن نموفطرتى *الأسبرجيليس نيجر وأسبيرجيليس تيرس* يقل نحوهما مع زيادة تركيزات أيونات الكروميوم حتى تركيز 2000 ملجم / لتر بينما يتحمل *أسبرجيليس نيجر* النمو فى وجود أيونات الفضة حتى تركيز800 ملجم / لتر وكذلك *أسبيرجليس تيرس* حتى 200 ملجم / لتر وبدراسة تأثير كل من أيونات الكروميوم والفضة على الشكل الظاهرى للفطرتين قيد الدراسة وجد أن الحامل الكونيدى لهما يظهرا بعض التشوهات والأختناقات فى الحوصلة والزنيبات للفطرتين مع التركيزات العالية فى بيئة النمو بينما عند تركيز200 ملج / لتر لا يتكون حامل كونيدى *للأسبرجيليس تيرس* ويظهر الغزل الفطرى مشوها مع ظهور بعض الجراثيم البينية وهذة الظاهرة تظهر أيضا مع *الأسبرجيليس نيجر* عند نموها فى التركيزات العالية من الفضة.

وبدراسة قطاع من خلايا *الأسبرجيليس نيجر والأسبرجيليس تيرس* النامية فى تركيزات عالية من الكروميوم والفضة بالميكروسكوب الألكترونى وجد تجمع السيتوبلازم على هيئة مساحات سوداء داخل الخلايا بينما حدث زيادة فى سمك الجدار الخلوى وذلك لإحتمال ترسيب عنصرى الكروميوم والفضة على شكل حبيبات فى الجدار الخلوى والفجوات الموجودة فى السيتوبلازم .

الكلمات المفتاحية : المعادن الثقيلة – الفحص بالميكروسكوب الضوئى – الفحص بالميكروسكوب الإلكترونى – الفطريات – الصفات الظاهرية.