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INFLUENCE OF COPPER AND COBALT STRESS ON MEMBRANE FLUIDITY OF *STACHYBOTRYS CHARTARUM*

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ABSTRACT

The growth of *S. chartarum* markedly decreased with elevated concentrations of Cu and Co in the growth medium. Total lipids and proteins in isolated plasma membrane were increased at 400mg Cu or Col^{-1} and decreased above this concentration while, carbohydrates markedly increased with elevated concentrations of both metals. The total amount of detected phospholipids in the membranes was decreased at 800mg l^{-1} of both metal ions. However, Phosphatidyl ethanolamine and phosphatidyl glycerol showed an increase at this concentration. Moreover, most of the detected fatty acids (C16:0, C16:1, C18:1 and C18:2) in the plasma membrane were increased with elevated concentrations of both metals to approximately 1.5-2 fold higher than in the control except C16:1 at 800mg Cu l^{-1} highly increased to 24.9 times higher than in the control. Whereas, C16:0 was the only fatty acid which decreased at 800mg Col^{-1}. The unsaturation index of fatty acids at 400mg l^{-1} exhibited a slight decrease while, at this concentration the fluorescence polarization value of DPH in the plasma membranes markedly increased. On the other hand, at 800mgl^{-1} the unsaturation index was increased while, fluorescence polarization value of DPH markedly decreased. This refers that the membrane at 400mgl^{-1} might be less fluid and at 800mgl^{-1} more fluid and cannot able to control the entry of toxic metals.

Keywords: *S. chartarum*, lipid composition, membrane fluidity, copper and cobalt stress.

INTRODUCTION

Living organisms are exposed in nature to heavy metals, commonly present in their ionized species. These ions exert diverse toxic effects on microorganisms. Metal exposure selects and maintains microbial variants able to tolerate their harmful effects. Varied and efficient metal resistance mechanisms have been identified in diverse species of bacteria, fungi and protists (Cervantes *et al*., 2006).

Fungal survival and tolerance to toxic metals depend on intrinsic biochemical and structural properties, physiological and genetical adaptation (Gadd and Griffiths, 1978; Gadd, 1993). Heavy metals (cobalt, nickel, cadmium and chromium) at different concentrations were toxic and inhibited the growth of *Rhizoctonia solani* in both solid and liquid media. Total inhibition was observed upon treatment with 1000ppm of each tested heavy metal. Nickel was the most toxic to the fungus, followed by cadmium, cobalt and chromium (Singh and Singh, 2002).

Metal resistant fungi belonged to the genera *Aspergillus, Penicillium, Alternaria, Geotrichum, Fusarium, Rhizopus, Monilia* and *Trichoderma* were isolated from wastewater-treated soil. The minimum inhibitory concentration (MIC) for Cd, Ni, Cr, Cu and Co was determined. The MIC ranged from 0.2-0.5mg l^{-1} for Cd, followed by Ni 0.1-4mg l^{-1}, Cr 0.3-7mg l^{-1}, Cu 0.6-9mg l^{-1} and Co 0.1-5mg l^{-1} (Zafar *et al*., 2007).

Much work has been done on the biosorbtion of heavy metals by fungal cell wall and also the chemical changes that have happened to cell wall in presence of toxic metals. However, there is spare information on changes in chemical composition of plasma membrane of fungi cultured in toxic concentrations of metals. The effect of some metals such as aluminum on membrane proteins and lipids of plants has been described (Haug and Caldwell, 1985). Total proteins and sugars in isolated plasma membrane of *Penicillium expansum* were slightly increased at 20mg Se l^{-1} and decreased above this concentration. Whereas, total lipids was increased at higher concentrations of selenium in the growth medium. The fungus responds to selenium stress by increasing the biosynthesis of phospholipids, fatty acids and unsaturation index of fatty acids in plasma membrane (Hefnawy, 2002). Addition of different levels of lead, zinc and barium acetates in the growth medium of *Neurospora indica* [*Tilletia indica*], revealed that lead acetate and zinc acetate inhibited the synthesis of both lipids and proteins in the membrane, while barium acetate only had a mild effect. Among phosphatides, phosphatidic acid, phosphatidyl inositol and phosphatidyl ethanolamine progressively accumulated while phosphatidyl choline declined by increasing the concentrations of all 3 metal salts (Sushma *et al*., 1996).

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The changes in phospholipids composition and its content may play a crucial role in fungal tolerance to toxic metals. It was observed that cells of *Microsporum gypseum* grown in the presence of Ca exhibited increased content of phospholipids and enhances its synthesis. The rise in the levels of phospholipids was found to be due to increased synthesis of Fatty acids. Moreover, the changes in the phospholipids composition increased the membrane fluidity (Giri *et al*., 1995).

Aluminum (10mM Al^{3+}) stimulated mycelial growth and increased membrane fluidity of *Lactarius piperatus* (Zel and Gogala, 1989). While, in *Amanita muscaria*, aluminum (10mM Al^{3+}) inhibited mycelial growth and decreased membrane fluidity (Zel *et al*., 1993). Hefnawy (1999) found that copper stress increased total lipids and proteins in plasma membrane of *Fusarium oxysporium* and decreased its fluidity.

This work aims at investigation of the changes in the plasma membrane composition as a response of toxic metals and also gives light on the role of the membrane in metal tolerance by fungal cells.

MATERIALS AND METHODS

Organism and culture conditions. *S. chartarum* was isolated from heavy metal contaminated Egyptian soil, on Dox agar medium amended with 400 mgl^{-1} $CuSO_4.5H_2O$ and $CoSO_4.7H_2O$ separately. The isolated fungus was identified according to Domsch *et al*. (1980).

The fungus was grown on Dox liquid medium supplemented separately with different concentrations of Cu^{2+} and Co^{2+} (0, 200, 400, 600, 800, 1000mgl^{-1}), incubating in an orbital incubating shaker (120 rpm) at 28ºC ± 2ºC for 7 days. The mycelial pellets were harvested, washed several times with distilled water and blotted with tissue paper prior to biochemical analysis. While, for dry mass determination the harvested pellets after washing were dried at 85ºC until constant weight was obtained.

Isolation of plasma membrane enriched fraction. The method was based on that described by Touze-Soluet *et al.* (1990) and Umura and Yoshida (1983). A known weight of fresh mycelia was ground using a Bead- Beater homogenizer with 0.5 mm diameter glass beads in 0.05M Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 1mM EDTA, 0.1 mM $MgCl_2$, chloramphenicol (200mgl^{-1}) and cycloheximide (200mgl^{-1}). The homogenate were subjected to differential centrifugation (1000g for 10 minutes, 6000g for 15minutes and 105000 g for 20minutes), using a Beckman centrifuge (L5 50 SW 28) Ultra-Centrifuge. The homogenate was fractionated, the supernatant was retained and re-centrifuged. After the final centrifugation the pellet containing the crude microsomal fraction was washed once with a 10mM phosphate buffer (pH 7.4) containing 0.25M sucrose and 30mM NaCl, and resedimented by recentrifugation at 105000g for 20minutes. The washed microsomes were subjected to phase partition for further purification by placing it on the top of polyethylene glycol (PEG 400) and dextran T500 (5.6:5.6% W/W), mixed and centrifuged at 400g for 3min. The upper layer was removed and placed on top of freshly prepared lower phase and centrifuged as before. The upper layer from the second centrifugation was plasma membrane enriched fraction.

Extraction and estimation of total lipids, proteins and carbohydrates. Lipids were extracted from plasma membrane enriched fraction by chloroform methanol mixture (2:1, v/v) at 40ºC with occasional manual stirring for 1h. The extract was filtered and the non-lipid materials were removed by adding 1ml of 0.88% (w/v) KCl (Hunter and Rose, 1972), the upper phase was removed and discarded. While, the lower phase was evaporated to dryness under nitrogen and the total amount of lipids were determined, using the method of Barnes and Blackstock (1973). Protein was determined according to the methods of Lowry *et al*. (1951), after extraction with 1 M NaOH. Sugar determination was carried out using the anthrone technique as described by Umbriet *et al*. (1959).

Phospholipids determination. Phospholipids were extracted from plasma membrane enriched fraction obtained from (1g fresh weight) according to the method of Bligh and Dayer (1959) as detailed by Kates (1972).

Polar lipids were resolved by thin layer chromatography (TLC) on silica gel plates or commercially prepared plates in two dimension chromatography according to the method described by Nichols (1964). The spots were revealed by iodine vapor and outlined with a pencil. The iodine was removed in a vacuo and the chromatogram sprayed with fine mist of cupric acetate (3% w/v) in sulphric acid (8% w/v). The Plates were sprayed until translucent and then heated to approximately 170ºC for 30min. Phospholipids spots appeared dark brown and were identified by comparing R_F values with standard phospholipids. The content of each spot was measured calorimetrically according to the method of Miller (1985).

Fatty acid methyl ester analysis. Lipids were extracted from plasma membrane enriched fraction obtained from 1 g fresh weight mycelium in chloroform/ methanol (2:1), the extract was removed by shaking with 0.2 volumes of distilled water. The chloroform layer was separated by centrifugation or allowing the tubes to stand over night, this layer was then removed and washed once with methanol / water (1:1, v/v). Butylated hydroxytoluene (0.005% w/v) was added as antioxidant. The chloroform was then evaporated under nitrogen. Lipids were converted into fatty acid methyl esters by adding 5 ml of

methylation reagent (conc. H_2SO_4: toluene: methanol 1: 10:20 by vol.) to each sample. The mixture was refluxed for 1 h at 90ºC. The resulting fatty acid methyl esters were extracted with hexane and analyzed by GLC using a (Chrompack CP 9000 with a column packed with CP-Sil-58 Support Chromsorb WHP). The degree of unsaturation was expressed as unsaturation index, defined by Kates and Hagen (1964) as : Δ mol^{-1} = 1x (% monoenes)/100 +2 x (% dienes)/100 + 3 x (% trienes)/100

Measurement of plasma membrane fluidity. To monitor the fluidity of lipid regions in the plasma membrane1, 6-diphenyl 1, 3, 5- hexatriene (DPH) was used as a probe (Shinitzky and Inbar, 1976; Shinitzky and Barenholz, 1978). A solution of 2mM DPH in tetrahydrofuran was diluted 1000 fold with 50mM Tris/ HCl pH (7.4) containing NaCl (0.9%, w/v). Freshly prepared membranes with 3 ml of diluted DPH were incubated for 30minutes at 30ºC. Fluorescence polarization measurements were made in a Fluorescence Spectrophlorometer F-2000 at 30ºC. DPH was excited at 340nm while the emission was measured at 440nm. The degree of fluorescence polarization (P), was calculated, according to the equation P = (I//- I$^{\perp}$) / (I//+ I$^{\perp}$), where I//and I$^{\perp}$ are the fluorescence emission intensities measured at right angles to the excitation beam, with the analyzer polarization axis parallel to, and perpendicular to, the polarization axis of the polarizer respectively.

RESULTS

Growth, Total carbohydrate, protein and lipid content in plasma membrane. *S. chartarum* was able to tolerate elevated concentrations of Cu and Co in the growth medium up to 800mg l^{-1}. The dry mass was markedly decreased with increasing both metal ion concentrations in the medium. At 800mg Cu and Co l^{-1} the growth was decreased to approximately 85%. Total carbohydrate content in isolated plasma membrane were markedly increased with elevate concentrations of both metal ions. Where, total protein and lipid were increased at 400mgl^{-1} and decreased at 800mg^{-1} comparing with the control (Table 1).

Polar phospholipids composition in plasma membranes. It is clear that phospholipid composition of *S. chartarum* membranes markedly changed in the presence of both Cu and Co in the growth medium. Some of the detected phospholipids slightly increased in the presence of 800mg Cu l^{-1} except that phosphatidyl coline, cardiolipin and phosphatidic acid showed a decrease at this concentration (Table 2 and Fig. 1). At 800mg Cu l^{-1}, phosphatidyl ethanolamine and phosphatidyl glycerol showed an increase to approximately 2.4, 1.1 fold

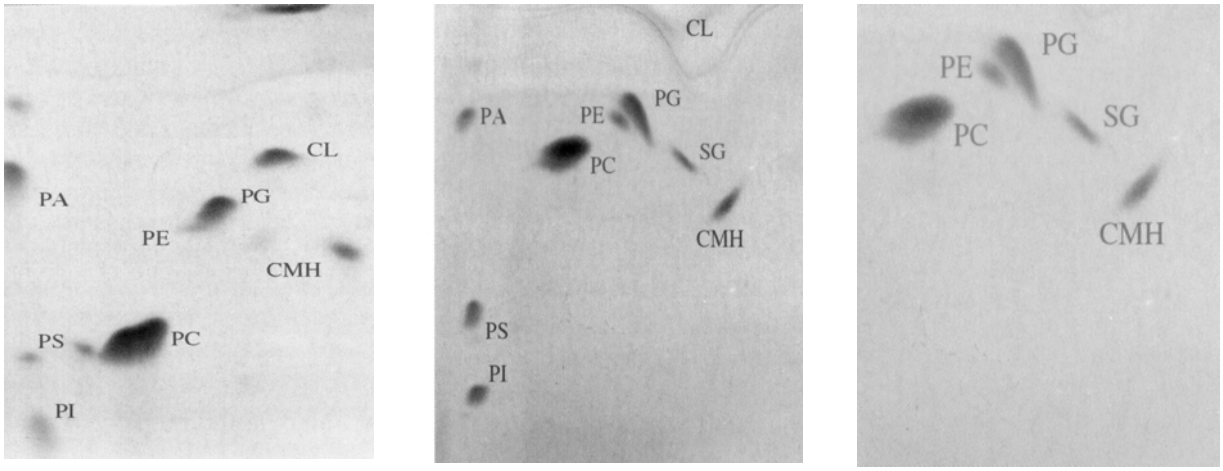
Table 1. Dry mass (mg/50ml culture medium), total carbohydrate, protein and lipid (mg g^{-1} fresh mycelium weight) in plasma membrane fraction obtained from 1 gm fresh weight of *Stachybotrys chartarum* grown in the presence of different concentrations of heavy metals. ± SE of three determinations.

Metals	Conc. (mgl^{-1})	Dry mass	Carbohydrate	Protein	Lipids
Cu	0.0	167±3.4	3.9±0.4	2.2±0.4	2.9±0.22
	400	112±4.1	4.4±0.5	3.3±0.2	3.9±0.4
	800	24±0.5	7.4±0.6	2.0±0.1	2.3±0.42
Co	0.0	160±4.2	3.4±0.4	2.4±0.2	2.8±0.2
	400	57±2.4	3.8±0.6	2.8±0.1	3.2±0.32
	800	21±0.2	6.3±0.5	1.4±0.12	2.2±0.4

Table 2. Phospholipid composition in plasma membrane fraction of *Stachybotrys chartarum* grown in absence and in the presence of 800mg Cu or Co l^{-1}.

Phospholipids	control	Cu	Co
PI	152.1±4.2	180.4±4.6	ND
PS	82.2±2.6	155.2±4.2	ND
PC	320.6±8.4	230.4±6.8	250.5±11.4
PE	45.2±2.2	110.4±8.2	87.7±4.2
PG	135.5±4.6	148.4±4.5	160.5±6.2
CL	175±5.6	trace	ND
CMH	170.5±8.4	165±4.4	175.6±4.8
PA	250.4±10.6	128.6±4.6	ND
Total	1331.5	1118.4	674.3

Data are expressed as nM of phospholipids/ gm of fresh weight mycelium ±SE of three investigations. PI, phosphtidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl choline; PE, phosphatidyl ethanol amine; PG, phosphatidyl glycerol; CL, cardiolipin; CMH, ceramide monohexoside; PA, phosphatidic acid. ND, not detected.



(A) (B) (C)

Fig. 1. Two-dimensional TLC plates of polar lipid classes of *Stachybotrys chartarum* (A) control; (B) 800mg Cu l^{-1} and (C) 800mg Co l^{-1}. (PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PI, Phosphatidyl inositol; PS, phosphatidyl serine; CMH, ceramide monohexoside; SG, steryl glycoside; CL, cardiolipin).

Table 3. Fatty acids composition of total lipids extracted from isolated plasma membrane fraction obtained from 1 g fresh weight of *Stachybotrys chartarum* grown at different concentrations of Cu and Co.

Metal	Conc. mgl^{-1}	Fatty acids						
		C14:0	C16:0	C16:1	C18:1	C18:2	C18:3	Δmol^{-1}
Cu	0.0	0.4±0.02 (0.66%)	17.6±0.8 (29.28%)	1.4±0.2 (2.32%)	25.8±1.2 (42.92%)	14.9±0.3 (24.79%)	Tr.	0.948
	400	0.6±0.1 (0.7%)	23.8±1.4 (27.93%)	14.9±0.6 (17.48%)	28.7±3.3 (33.68%)	15.8±0.5 (18.54%)	1.4±0.02 (1.6%)	0.929
	800	3.8±0.2 (2.37%)	34.6±4.2 (21.6%)	34.9±0.8 (21.6%)	50.2±2.4 (31.4%)	33.7±1.5 (21.06%)	2.8±0.22 (1.75%)	1.001
Co	0.0	Tr.	17.9±1.1 (25.21%)	14.8±1.2 (20.84%)	27.9±1.4 (39.29%)	9.0±0.8 (12.67%)	1.4±0.12 (1.97%)	0.914
	400	0.4±0.1 (0.36%)	38±3.1 (35.05%)	9±0.3 (8.3%)	34.6±2.5 (31.91%)	23.9±2.2 (22.04%)	2.5±0.24 (2.3%)	0.912
	800	Tr.	15.4±3.4 (14.3%)	27.9±0.4 (25.9%)	41.4±4.2 (38.47%)	20.9±0.6 (19.4%)	2.0±0.21 (1.8%)	1.115

Data are expressed as μg g^{-1} fresh weight mycelium. ± SE of three investigations. Values in parenthesis are percentages.

respectively higher than in the control while, at 800mg Col l^{-1}, they also increased to approximately 1.9, 1.2 fold respectively higher than in the control. It was also observed that phospholipids biosynthesis was highly affected by the presence of Co in the growth medium where phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid not detected at 800mg Col l^{-1}. In general, the total amount of phospholipids in the plasma membrane decreased at 800mg Cu or Co l l^{-1}.

Fatty acids composition of pure plasma membrane. Most of the detected fatty acids in the plasma membrane of *S. chartarum* showed a marked increase with increasing both Cu and Co concentrations in the growth medium. Fatty acids with carbon number (16:0, 16:1, 18:1 and 18:2) were the major detected fatty acid in membranes and represented the highest percentage. At 800mg Cu l^{-1}, C18:1 represented the highest amount while C16:1 represented the highest increase to approximately 24.9 times higher than in the control. While, at 800mg Co l^{-1}, C16:1 and C18:1 showed the highest amount and increased to approximately 1.8 and 1.5 times higher than in the control. Whereas, C16:0 increased at 400mg and decreased at 800mg Co l^{-1} (Table 3).

The unsaturation index of the identified fatty acids of the plasma membrane of *S. chartarum* decreased at 400mg Cu or Co l^{-1}. This may indicate that the plasma membrane may become less fluid or more rigid at this concentration. While, at 800mg Cu or Co l^{-1} it markedly increased over

the control. This may refer that the membrane is more fluid or less rigid than the control and the membrane may becomes unable to control the entry of metal ions into the cells.

Plasma membrane fluidity. The plasma membranes were labeled with a fluorescence probe (DPH), to measure the fluorescence polarization of isolated plasma membrane of *S. chartarum*. P value (fluorescence polarization) of DPH in the plasma membrane markedly increased at 400 mg Cu or Co l^{-1}. While, it sharply decreased at 800mg Cu or Co l^{-1} (Table 4). The result indicated that the plasma membranes might be more rigid or less fluid at 400mg Cu or Co $l^{-1}$and might aid in controlling entry of metal ions to the fungal cells. While, it might be more fluid at 800mg Cu or Co l^{-1} and therefore, the membrane may be disordered and can not retard the entry of metal ions across the membrane.

Table 4. Fluorescence polarization values (P) of isolated plasma membrane of *Stachybotrys chartarum* grown at different concentrations of Cu and Co. ± SE of three investigations.

Heavy Metals	Conc. (mgl^{-1})	Fluorescence polarization (P)
Cu	0.0	0.12±0.02
	400	0.24±0.05
	800	0.08±0.01
Co	0.0	0.1±0.01
	400	0.35±0.06
	800	0.06±0.04

DISCUSSION

Copper and cobalt were toxic to *S. chartarum.* However, it tolerated both metal ions up to a concentration of 800mg l^{-1}. 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 mgl l^{-1} respectively (Azab and Hefnawy, 1999). Other fungal species such as *Thelephora terrestris* proved high tolerance to Cu at 100 and 500ppm in agar plates (Jones and Muehlchen, 1994). 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 organellar membrane integrity (Ochiai, 1987).

Total carbohydrates content in *S. chartarum* plasma membrane were increased with increasing Cu and Co concentrations in the growth medium whereas, total proteins and lipids slightly increased at 400mg l^{-1} and decreased at 800mg l^{-1}. The increasing amount of lipid and carbohydrate and proteins at 400mg l^{-1} may important in maintenance of the membrane integrity and retard the entry of toxic metals. Much work has been done on the role of protein, especially metallothioneine in metal tolerant fungi and yeasts whereas, lipids have been neglected in this regard. The role of lipids in fungi are generally not well understood. Phospholipids are important structural components of biological membranes. Some phospholipids have been implicated in the active transport of ions across membranes and are also essential for the activity of some membrane bound enzymes (Weete, 1980). There is a direct relationship between lipid composition and metal tolerance by fungi. Most phospholipids in *S. chartarum* were increased under Cu and Co stress. Phosphatidyl ethanolamine and phosphatidyl glycerol exhibited an increase at 800mg Cu or Co l^{-1}. Similar observation was also found in *Fusarium oxysporum* grown under Cu stress, total phospholipids of the whole mycelium were increased with increasing copper concentrations in the growth medium. Phosphatidyl ethanolamine and cardiolipin showed the greatest increase under copper stress (Hefnawy, 1996). It was also reported that *Microsporum gypseum* respond to calcium stress by increasing the content of phospholipids due to increased fatty acids biosynthesis (Giri *et al*., 1995). Phosphatidyl ethanolamine showed the greatest increase in plasma membrane of *Fusarium oxysporum* with increased copper in the medium, it increased at 600mg Cu l^{-1} to approximately 2 fold higher than in the relevant control (Hefnawy, 1999). It was also found that Phosphatidyl ethanolamine in the plasma membranes of *Penicillium expansum* grown under selenium stress increased at 200mg Se l^{-1} to approximately 2.37 times higher than in the control (Hefnawy, 2002).

The tested fungus may respond to the effect of copper and cobalt stress by increased synthesis of some phospholipids which are membrane components and these may be important in repairing the injured membranes and might be one of the mechanisms involved in heavy metal tolerance by fungi. It was also reported that 300mg Zn l^{-1} inhibit the growth of *Candida utilis* and at this concentration the content of protein, RNA, DNA and polysaccharides fell while, lipid content increased (Andreeva *et al*., 1983).

It has been found that environmental conditions affect the fatty acid compositions of the fungal cells. The detected fatty acids in the plasma membranes of *S. chartarum* were markedly increased with increasing Cu and Co in growth medium. Fatty acids C16:0, C16:1 C18:1 and C18:2 were the major detected fatty acids in the plasma membranes and represented the highest amounts and percentages. Quite similar observation was also found in the whole mycelium of *Fusarium oxysporum* grown under copper stress, the major fatty acids detected were oleic acid (C18:1) and palmitic acid (C16:0) (Hefnawy, 1996). It was also observed that most of the detected fatty acids increased in plasma membranes of *Fusarium oxysporum* at all tested copper concentrations. The highest amounts

were obtained at 200mg Cu l^{-1} and the major fatty acids at this concentration were C18:1 and C17:0 (Hefnawy, 1999). Quite similar observations were also found in membranes of *Penicillium expansum* grown under different concentrations of Selenium. The major detected fatty acids were C16:0, C18:1, C18:2 which showed the greatest increase in the presence of 100mg Se l l^{-1} in the growth medium (Hefnawy, 2002). In contrast, to these observations it was reported that phospholipids fatty acids composition of soil contaminated with copper was decreased consistently with increasing levels of copper (Yao *et al*., 2006).

The unsaturation index of fatty acids in plasma membranes of *S. chartarum* showed a decrease at 400mgl^{-1} Cu and Co while, it increased at 800mg. These observations may indicate that the membrane is less fluid or more rigid at 400mg and more fluid at 800mg of Cu and Co. To confirm these observations the plasma membrane fluidity was measured by labeling the membranes by DPH and the fluorescence polarization values (P) were measured. The P value was record to be increased at 400 mg and sharply decreased at 800mg Cu and Co concentration in the medium. This indicates that the membrane fluidity decreased and increased at both concentrations respectively. This result was in contrast to that found by Zel and Gogala (1989), they found that aluminum stimulated mycelial growth of *Lactarius piperatus* and increased membrane fluidity. Whereas, (Zel *et al*., 1993) reported that aluminum stress decreased membrane fluidity of *Amanita muscari*. Also, Hefnawy (1999) found that copper stress decreased membrane fluidity of a copper tolerant strain of *Fusarium oxysporum*. It was also found that the membranes of *Penicillium expansum* becomes less fluid or more rigid under selenium stress (Hefnawy, 2002). The rigidity of plasma membranes of *S. chartarum* might aid in retarding the flux of metal ions into the fungal cells and enable the fungi to tolerate Cu and Co in the growth medium to a certain limit.

In conclusion, fungal cells may respond to heavy metal stress by increasing the synthesis of some membrane phospholipids and its fatty acids acyl chain. Decreasing the unsaturation index of the membrane fatty acids indicate that the membrane becomes less fluid or more rigid, this may enable the organism to tolerate toxic metals to certain concentration and might be one of the tolerance mechanisms to heavy metals stress by filamentous fungi.

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