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Ultrastructure, Glutathione and Low Molecular Weight Proteins of *Penicillium brevicompactum* in Response to Cobalt

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Abstract

Penicillium brevicompactum highly tolerated cobalt concentrations of 50, 200, 800 and 1000 ppm both through cell wall and intracellular sequestration – immobilization of the metal on/within the cell wall, cell wall thickness, presence of electron-dense deposits inside vacuoles (thiol peptides sequestering cobalt) and in the cytoplasm (cobalt), and presence of matrixed electron-dense deposits, only at 800 and 1000 ppm, were observed. Increased vacuole formation and plasmolysis were also observed. Fraction number 9 of the cell free extract showed maximum cobalt uptake for all the investigated cobalt concentrations. In this fraction, glutathione was only induced at 500, 800 and 1000 ppm. Maximum glutathione concentration supported maximum cobalt uptake at 800 ppm. Low molecular weight protein profiles of fraction number 9 revealed that the presence of cobalt induced the appearance of new proteins that were not detected in the same fraction of the control. These low molecular weight peptides (12–5 KDa) suggest the production of Co-metallothioneins. This is the first report of cobalt-induced glutathione by *P. brevicompactum* and suggests the possible production of phytochelatins.

Key words: cell response to cobalt, cell wall, thiols, transmission electron micrographs

Introduction

Cobalt is a naturally occurring element found in rocks, soil, water, plants and animals. Cobalt is used to produce alloys used in the manufacture of aircraft engines, magnets, grinding and cutting tools, artificial hip and knee joints. Cobalt compounds are also used to color glass, ceramics and paints, and used as a drier for porcelain enamel and paints. Cobalt has both beneficial and harmful effects on health. At low levels, it is part of vitamin B12, which is essential for good health. At high levels, it may harm the lungs and heart. When cobalt salts were added to beer as foam stabilizers, it led to an epidemic of cardiomyopathy and congestive heart failure among beer drinkers. Other effects of overdosing on cobalt (>5 mg/day) include abnormal thyroid functions, polycythemia and overproduction of red blood cells (erythropoiesis), with increased production of the hormone erythropoietin (EPO) from the kidneys. The International Agency for Research on Cancer (IARC) has determined that cobalt and cobalt compounds are possibly carcinogenic to humans, however, the Federal Republic of Germany has classified cobalt as carcinogenic to experimental animals (ATSDR, 2004).

Studies conducted on the tolerance of fungi to heavy metals revealed not only a high tolerance of some fungi, but outright accumulation of heavy metals in the biomass of some strains (Volesky, 1990). Specific mechanisms are employed for specific metals in particular fungal species. It is also possible that more than one mechanism may be involved in reducing the toxicity of a particular metal (Hall, 2002).

Biodegradation is the process employed in the cleanup of complex organic pollutants. In this procedure, microbes seeded onto the contaminated area breakdown the complex hydrocarbons into simpler organic compounds. Bioaccumulation is used when the contaminants are simple compounds such as metals that cannot be further degraded. In this latter case, the microbes used usually accumulate the polluting substance by cell wall binding and/or internalization of the compound through thiol compound formation (Hall, 2002).

The cell wall consists of a variety of polysaccharides and proteins and hence offers a number of active sites capable of binding metal ions. Difference in cell wall composition among different groups of microorganisms, *viz.* algae, bacteria, cyanobacteria and fungi and the intragroup differences can thus

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cause significant differences in the type and amount of metal ion binding to them (Gupta *et al*., 2000).

Thiol compounds, including reduced glutathione (γ-glutamyl cysteinyl glycine) (GSH), phytochelatins (PCs) and metallothioneins (MTs), are essential components of heavy metal detoxification, in this respect Cd has been extensively studied (Courbot *et al.*, 2004).

GSH is a well-known thiol-containing tripeptide (307 Da) whose properties in binding and transformation suggest a role as a detoxification agent (Riccillo *et al*., 2000) and a key player in the mechanism against heavy metals in organisms that possess the GSH metabolic pathway (Figueira, 2005).

PCs are a family of small cysteine-rich peptides capable of binding heavy metal ions *via* their SH group. Their general structure is [GluCys]*n*-gly (*n* = 2 to 11). PCs are enzymatically synthesized by phytochelatin synthase from glutathione and have been found in some fungi, algae, and all plant species examined so far. This enzyme is activated by a broad range of heavy metals, including Cd, Ag, Pb, and Cu. PCs are with molecular masses which may vary, but which, according to the current literature, hardly seem to exceed 2 kDa in lower fungi (Collin-Hansen *et al.*, 2007). In the plant kingdom, the molecular weight of the native metal-containing phytochelatin complex is 1800–4000 Da, high molecular weight phytochelatins were isolated from Cd and Cu treated maize (6200–7300 Da) (Tomaszewska, 2002).

Metallothioneins are cysteine-rich, low molecular weight (3500–14 KDa) peptides that chelate metal ions by thiolate coordination. MTs have received their designation from their prominent metal and sulfur content which, varying with the metal species present, together may contribute to over 20% of their weight (Mir *et al.*, 2004). MTs are implicated in a variety of physiological processes, including maintaining homeostasis of essential metals, metal detoxification, scavenging free radicals, and regulating cell growth and proliferation (Palmiter, 1998; Vasak and Hasler, 2000).

In the current study, the high tolerance of *P. brevicompactum* towards cobalt (Co) was assessed through its ultrastructure, and detection of Co-induced GHS as well as Co-induced low molecular weight peptides in the fraction of maximum Co uptake at all the investigated concentrations.

Experimental

Materials and Methods

All the experimental work was conduced at the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt.

Fungal isolate. *Penicillium brevicompactum* was obtained from the culture collection unit of the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

Media and growth conditions. Czapek's Dox medium and broth were supplemented with different cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg Co/l). Except for the control, cobalt replaced $FeSO_4$. $CoCl_2 \times 6H_2O$ was the salt used to prepare the different cobalt concentrations. Media were autoclaved and the media with agar were poured into sterile Petri dishes (two Petri dishes for each cobalt concentration). The plates were inoculated with a 7-day old *P. brevicompactum* and incubated at 25°C to be used for transmission electron microscopy. The autoclaved broth (triplicate 250 ml-flasks for control and each concentration) were also inoculated with a 7-day old *P. brevicompactum* and incubated at 25°C to be used for further biochemical investigations.

Transmission Electron Microscopy (TEM). One mm^3 Blocks of *P. brevicompactum* were fixed by immersion in freshly prepared 1% (w/v) aqueous potassium permanganate solution for 5 min at 25°C. Specimens were then washed in distilled water (3 times each for 15 min), dehydrated through graded, 10% steps, ethanol series till absolute ethanol (Zain, 1998). Fixation and dehydration procedures were performed using the programmable LEICA EM TP tissue processor model (A-1170). Specimens were infiltrated with epoxy resin and acetone through a graded series till finally in pure resin, transferred to an oven at 60°C for 72 hrs to polymerize and then allowed to reach 25°C in desiccators for 24 hrs. Mounted blocks were trimmed with razor blades to give a trapezoid-shaped face of less than 1 mm width and height. Ultrathin sections were cut with a Leica Ultracut UCT Ultramicrotomy. Ultrathin sections were placed onto copper hexagonal mesh, 2.05 mm grids. Sections were then double stained in lead citrate followed by uranyl acetate. Stained sections were examined with a JEOL 1010 Transmission Electron Microscope at 80 kV. Measurements of cell wall thickness were performed by an image analysis software (analysis 2.1) where the mean, minimum and maximum of 50 measurements for each case were taken.

Cell free extracts. The harvested mycelia from the broth media were homogenized using tris-HCl buffer, pH 7.1. The slurry of each concentration was filtered through Whatman no 1 filter paper and then the filtrates were centrifuged at 2000 g for 10 min at 5°C. The filtrates were then concentrated to be used for gel filtration.

Gel filtration. The concentrates, 5ml each, were applied on top of a Pharmacia column (2.6 × 70 cm) packed with Sephadex G-200 and allowed to pass into the gel by running the column with Tris-HCl buffer, pH 7.1, at a flow rate of 0.02 ml min^{-1} using an LPLC Gilson pump. After discarding the void

volume, 12 fractions of 5 ml each were collected. Cobalt was determined in all fractions using atomic absorption spectroscopy.

Atomic Absorption spectroscopy. The GBC Model 932 atomic absorption spectrophotometer was used. Instrument and element (Co) conditions used were adjusted similar to those in the GBC manual. The blank and standards were prepared using tris-HCl buffer, pH 7.1, to match the samples.

Determination of protein. Proteins in fraction number 9 of control and all concentrations was determined by the method of Bradford (1976) using Bovine serum as the standard protein.

Determination of reduced glutathione (GHS). GHS detection in fraction number 9 was carried out using a GBC high performance liquid chromatography (HPLC) system equipped with a binary pump (LC 1110; GBC) C18 column (Hypersil Elite C18; ODS; 5 µm, 250×4.6 mm). The mobile phase was 50 mM $NaClO_4$ 0.1% H_3PO_4 whose flow rate was adjusted at 1ml/min. Detection was at 215 nm by UV detector. Working standard solutions were daily prepared in 50 mM $NaClO_4$ 0.1% H_3PO_4 buffer.

Low molecular weight protein determination. A BIO-RAD model Biofocus 3000 capillary zone electrophoresis (CZE) system was used to determine the low molecular weight proteins present in fraction number 9. SDS-kit was used where an uncoated capillary (24 cm × 0.5 µm) at 25°C, 15kV, 80 psi, 200 nm wavelength and a run time of 15 min were the separation instrumental conditions.

Results

P. brevicompactum was grown on Co concentrations of 0, 50, 200, 500, 800 and 1000 ppm. Its ultrastructural responses through TEM, fractionation of its cell free extracts of all concentrations, determination of Co concentration in all fractions, and detection of GSH as well as low molecular weight proteins in the fraction of maximum Co uptake were determined.

Ultrastructural response. Compared to the control, more vacuoles of bigger size were observed at a cobalt concentration of 50 ppm (Fig. 2c) and some of the vacuoles accumulated electron-dense deposits (Fig. 2b) which were absent in the control (Fig. 1). Dark electron-dense deposits were observed on the outer layer of the cell wall which became multilayered and thickened reaching a maximum of 697 nm (Fig. 2a, b, c) compared to a maximum control value of 200 nm (Table I).

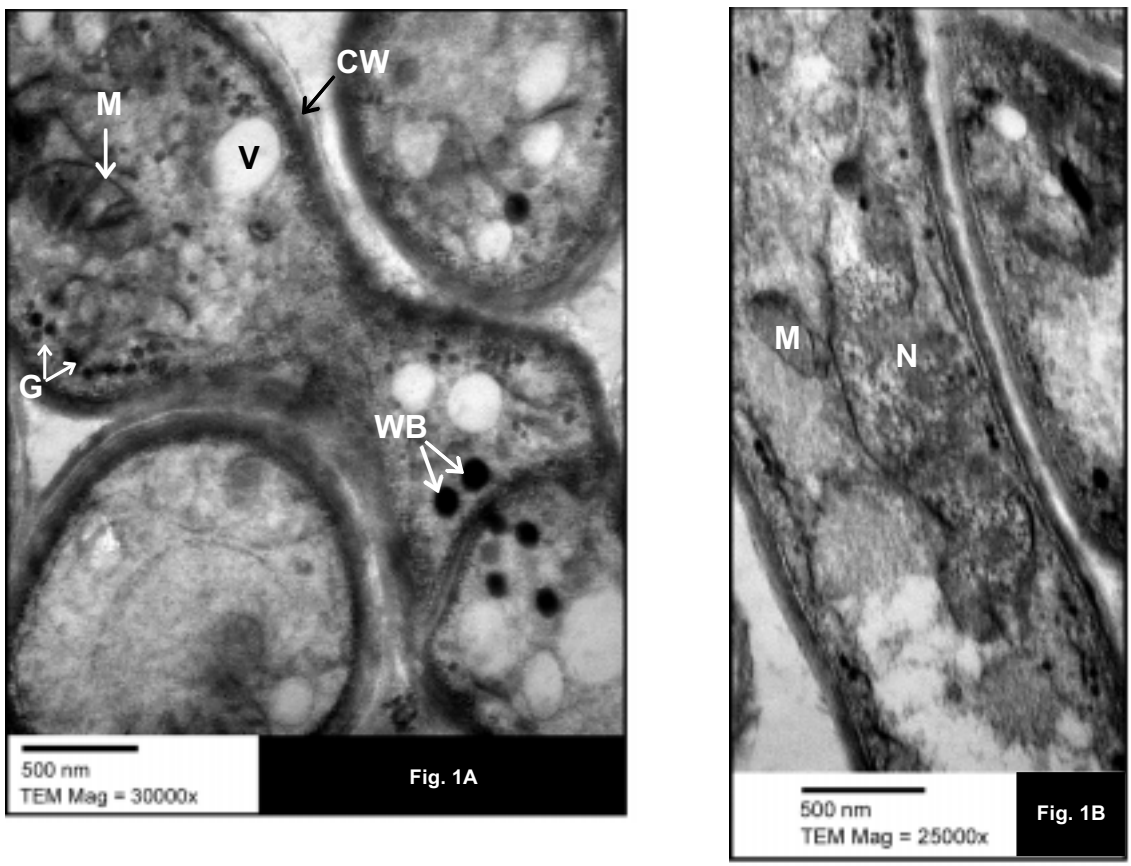
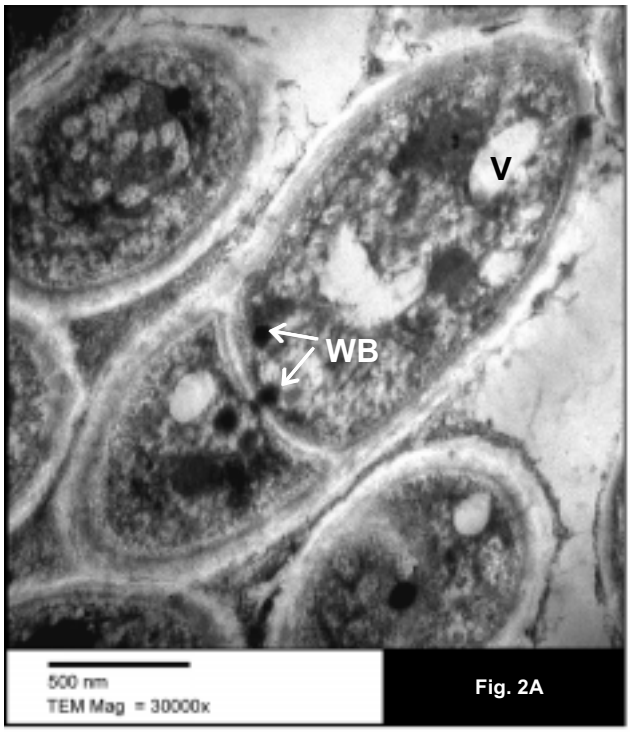
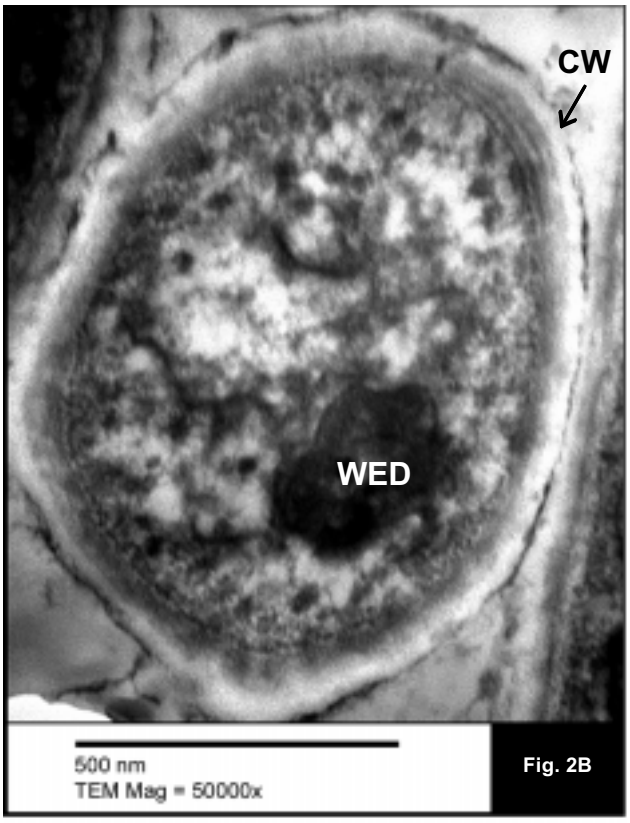


Fig. 1. Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 0 ppm (control). CW – cell wall; M – mitochondrion; N – nucleus; V – vacuole; WB – Worrion bodies; G – polysaccharide granules.





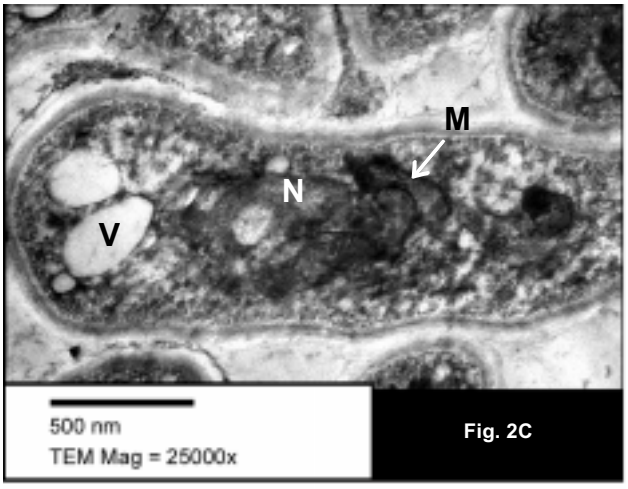


Fig. 2: Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 50 ppm. CW – cell wall (multilayered) with electron dense border; M – mitochondrion; V – vacuole; WB – Worrion bodies; VED – vacuolated electron dense bodies.

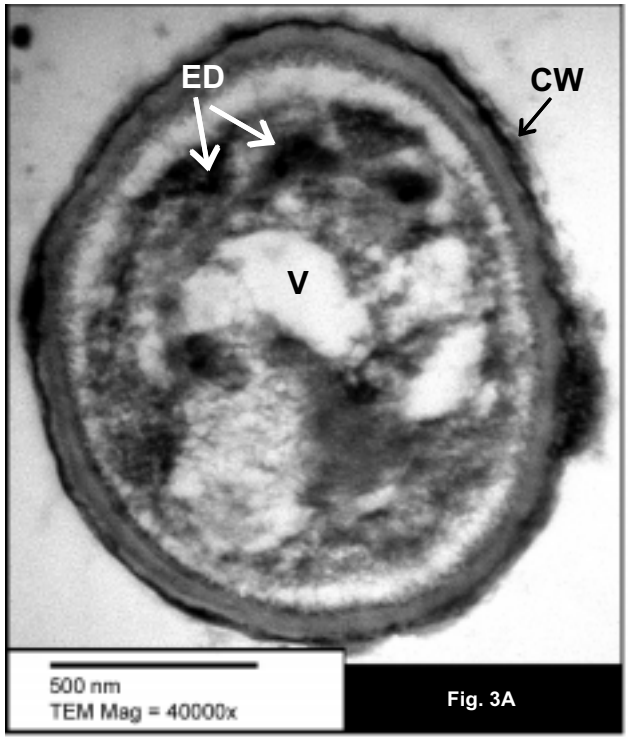
Increased number of vacuoles (Fig. 3), electron-dense deposits inside vacuoles (Fig. 3d) and in the cytoplasm (Fig. 3a), as well as increased darkening of the border and layers of the cell wall were observed at 200 ppm (Fig. 3). Cell wall thickness reached a maximum of 617 nm compared to a maximum control value of 200 nm (Table I). Sections close in appearance to control were observed however with increased vacuole production and increased cell wall darkening (Fig. 3c). Plasmolysis was also observed (Fig. 3).

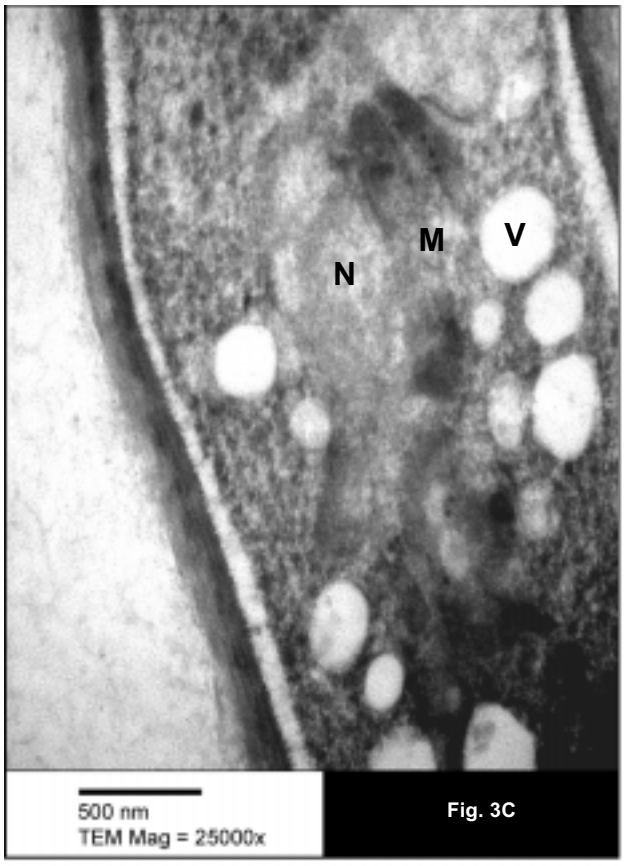
Table I
Cell wall thickness of *P. brevicompactum* at different cobalt concentrations

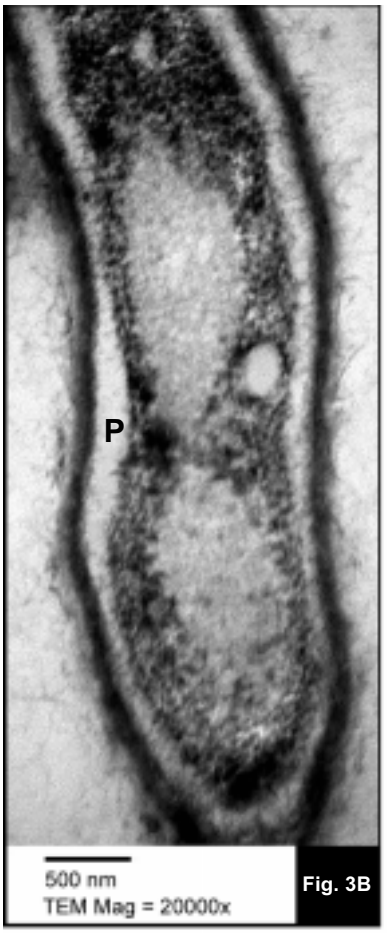
Cobalt concentration (ppm) / Cell wall thickness (nm)	0	50	200	500	800	1000
Minimum	97	155	130	121	150	172
Maximum	200	697	617	390	334	366
Mean	149	290	260	190	183	279

Vacuoles with electron-dense deposits were observed at 500 ppm (Fig. 4a) as well as cytoplasmic electron-dense deposits (Fig. 4b) together with irregular darkening and thickening of the cell wall which reached a maximum of 390 nm (Table I). Plasmolysis was also observed in some of the sections (Fig. 4c).

At 800 and 1000 ppm, circular electron-dense deposits were observed in a matrix (Fig. 5a and 6b, respectively) in addition to the vacuolated electron dense deposits and cytoplasmic electron-dense deposits observed in the other investigated concentrations (Figs. 5b, c and 6d respectively). Thickened (maximum of 334 nm at 800 ppm and 366 nm at 1000 ppm)







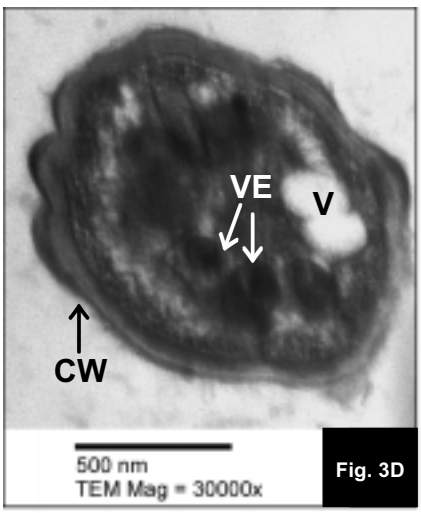
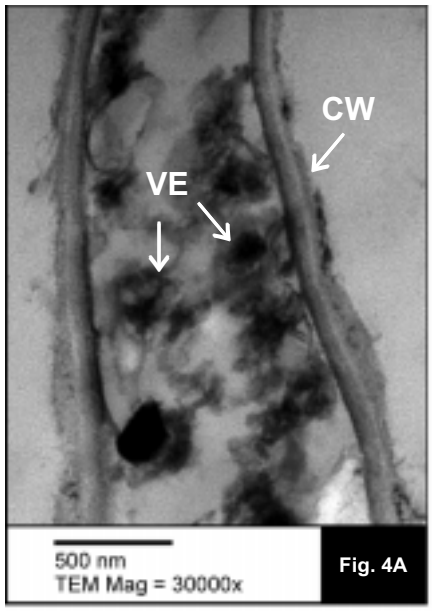


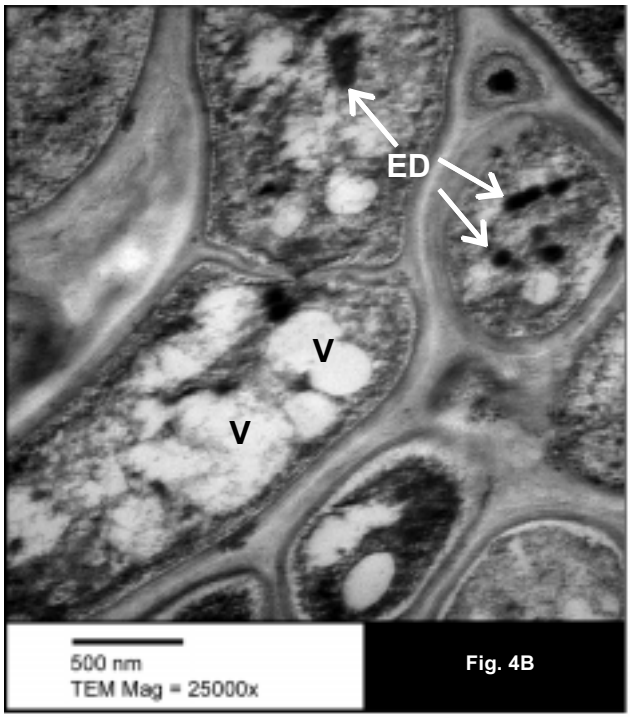
Fig. 3. Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 200 ppm. CW – cell wall (multilayered) with electron-dense border and layers; VED – vacuolated electron-dense bodies; ED – electron-dense bodies; P – plasmolysis.

(Table I) electron-dense cell wall was observed at 800 (Fig. 5) and 1000 ppm (Fig. 6). Mitochondria and nucleus were still observed at such high concentrations (Fig. 5c for 800 ppm and Fig. 6c for 1000 ppm).

At 1000 ppm, some sections were empty of any organelles and with thick, dark electron-dense wall (Fig. 6a) while others were dark and of high electron density (Fig. 6d).

Fractionation of the cell free extracts of *P. brevicompactum* grown at different cobalt concentrations. The fresh weight of *P. brevicompactum* grown at cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm





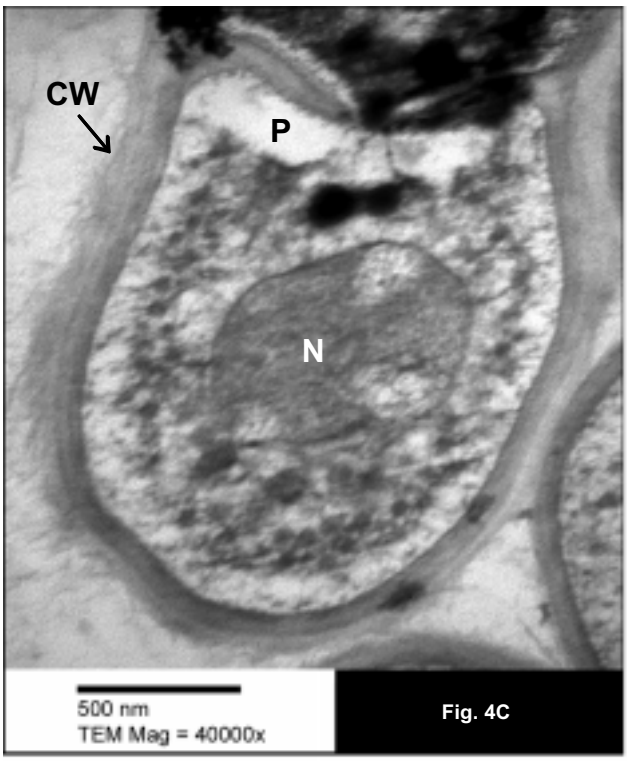
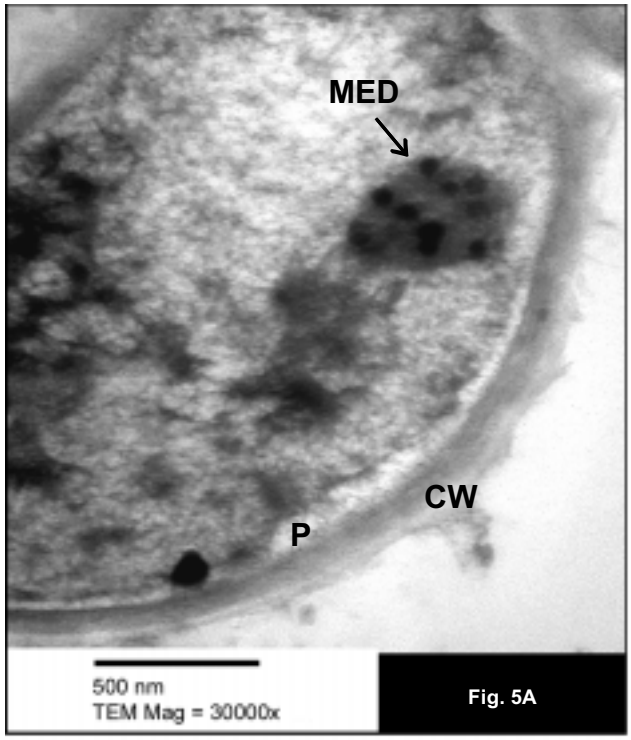


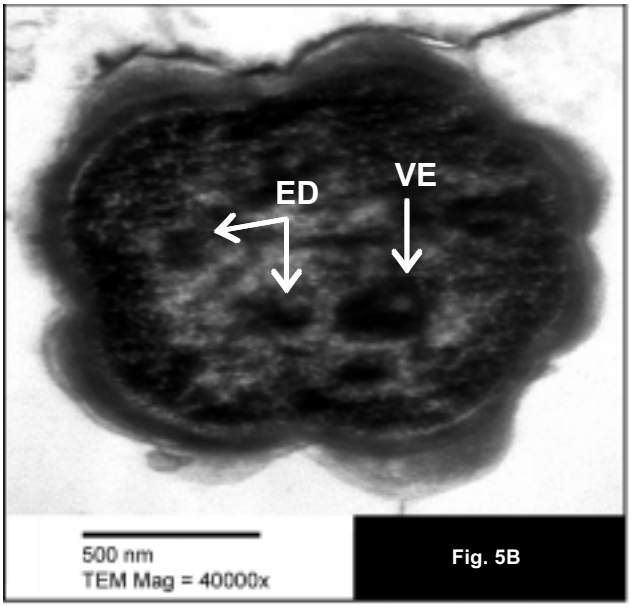
Fig. 4: Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 200 ppm. CW – cell wall (multilayered) with electron dense border; N – Nucleus; ED – electron dense wall-bounded compartment; VED – vacuolated electron-dense bodies; P – plasmolysis.

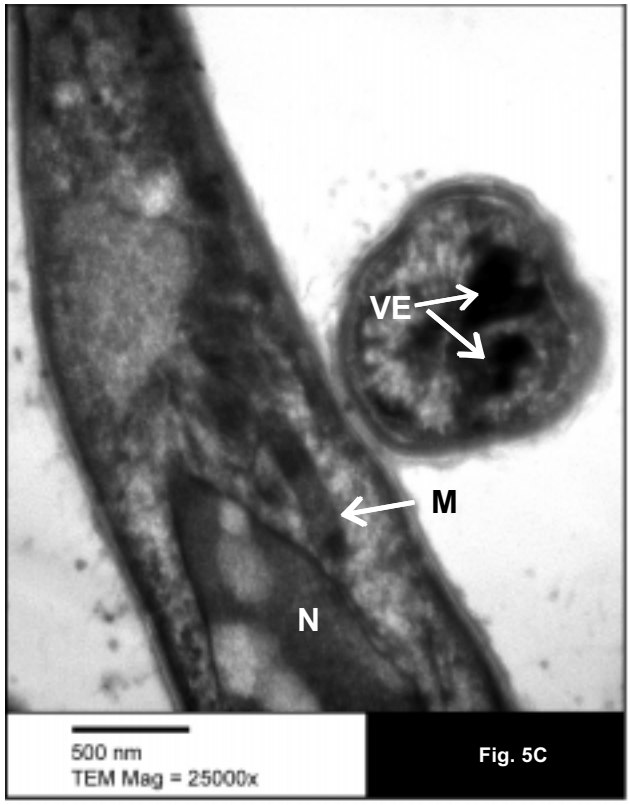
was 15, 13, 11.5, 4.3, 10.5 and 5.7 gm respectively. The cell free extract of *P. brevicompactum* grown at each cobalt concentration was fractionated. Cobalt concentration in each fraction was determined using atomic absorption (Table II). It could be clearly observed that Co is associated over a wide range of fractions. Maxi-

Table II
Cobalt concentration in fractions of the cell free extract of *P. brevicompactum* grown at the investigated cobalt concentrations

Fraction no / Co Conc.	1	2	3	4	5	6	7	8	9	10	11	12	mg Co/gm fresh weight
50 ppm	0	0	0	0	0	0	0.72	7.46	21.09	14.4	1.22	0	0.07
200 ppm	0	0.3	0.25	0.47	1.23	5.57	7.05	46.25	121.4	10.8	5.9	0	0.35
500 ppm	0.18	0.3	0.92	0.82	0.56	1.18	12.33	15	16.2	6.88	0	0	0.15
800 ppm	5.77	4.93	3.1	4.48	3.96	3.03	48.65	146.85	150	32.4	8.3	0	0.47
1000 ppm	2.96	5.4	6.17	5.8	4.87	4.76	10.25	50.8	57.2	17.5	6.96	2.71	0.36







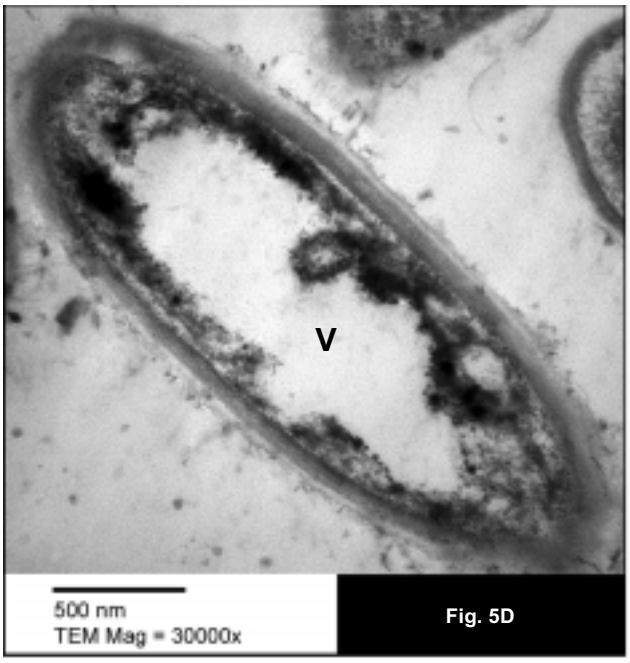


Fig. 5: Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 800 ppm. CW – cell wall (multilayered) with electron dense border and layers; M – mitochondrion; V – vacuole; ED – electron dense wall-bounded compartment; VED – vacuolated electron-dense bodies; MED – matrixed electron-dense bodies; P – plasmolysis.

mum Co uptake per gram fresh weight was at 800 ppm (0.47 mg/gm) followed by 1000 ppm (0.36 mg/gm), 200 ppm (0.35 mg/gm), 500 ppm (0.15 mg/gm) and finally 50 ppm (0.07 mg/gm) (Table II). In all the investigated Co concentrations, fraction 9 represented the fraction with maximum cobalt uptake; 21.09, 121.4, 16.2, 150 and 57.2 ppm at 50, 200, 500, 800 and 1000 ppm, respectively (Table II). Hence, fraction 9 of all the investigated concentrations was further studied using HPLC for GHS detection and capillary electrophoresis for detection of low molecular weight proteins.

Detection of reduced glutathione (GSH). GSH was undetected in fraction 9 of each of the control, 50 and 200 ppm. However, at 500, 800 and 1000 ppm,

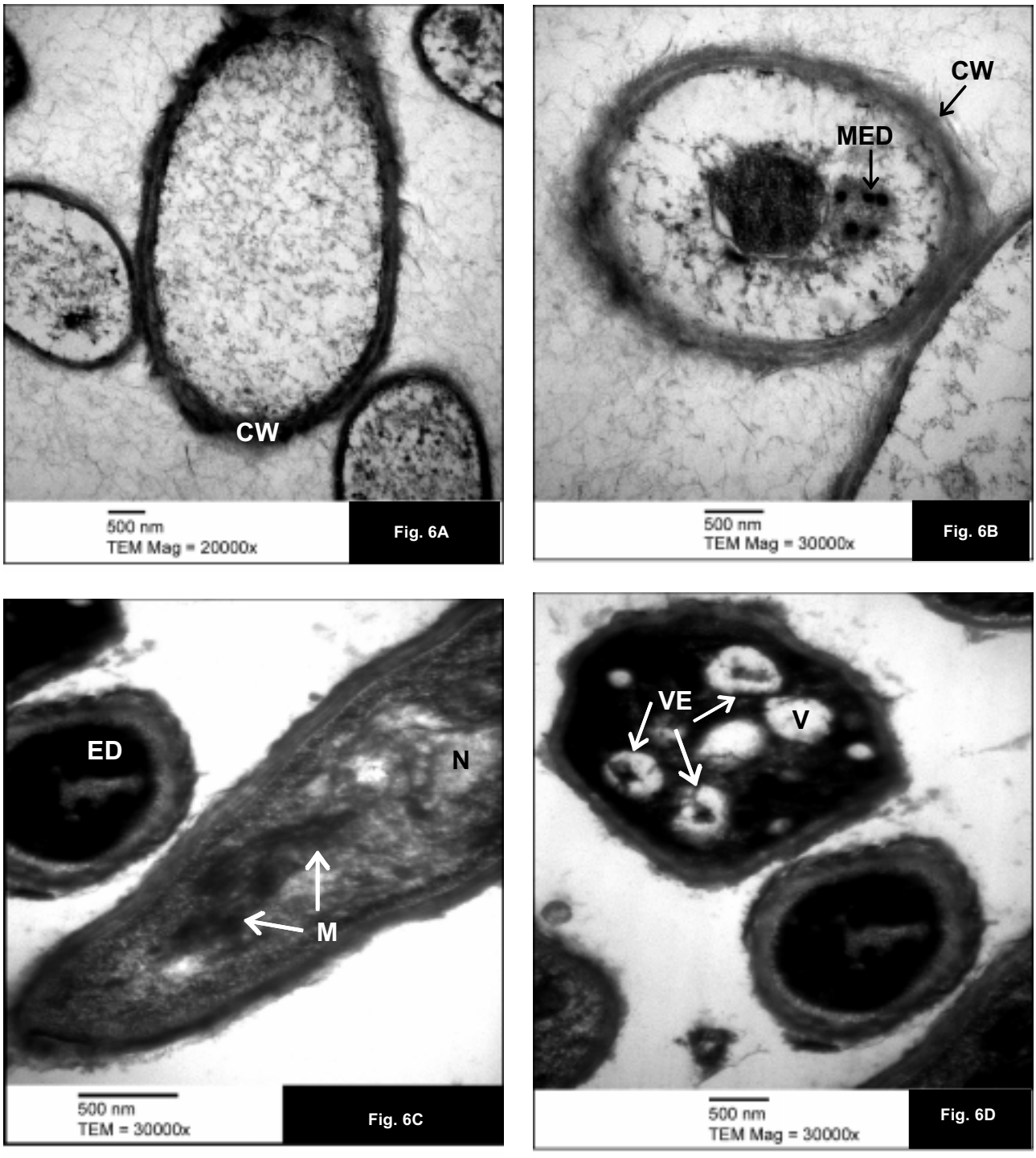


Fig. 6. Transmission electron micrographs of *P. brevicompactum* grown on Dox medium amended with a cobalt concentration of 1000 ppm. CW – cell wall (multilayered) with electron dense border and layers; M – mitochondrion; V – vacuole; ED – electron dense wall-bounded compartment; VED – vacuolated electron-dense bodies; MED – matrixed electron-dense bodies.

GSH was detected in fraction 9 at concentrations of 1.677, 5.284 and 5.258 mg/ml respectively (Table III). Concerning Co concentration in the same fraction, maximum Co conc. was at 800 ppm (0.15 mg/ml) followed by 1000 ppm (0.053 mg/ml) and then 500 ppm (0.0162 mg/ml); *i.e.*, maximum glutathione concentration supported maximum cobalt uptake (at 800 ppm) (Table III).

Detection of low molecular weight proteins. Very low protein content was detected in fraction 9 (0.015–0.02 mg/ml) of all the investigated Co concentrations. Hence, capillary electrophoresis was the method used for the detection of the present proteins. A peak corresponding to a molecular weight of 25 KDa was detected in fraction 9 of the control while was not detected at all the investigated cobalt con-

Table III
Concentration of reduced glutathione (GSH) in fraction 9 and corresponding Co concentration in the same fraction

Co Concentration (ppm) / Fraction 9	0	50	200	800	500	1000
GSH concentration (mg/ml)	0	0	0	1.677	5.284	5.258
Co Concentration (mg/ml)	0	0	0	0.0162	0.15	0.053

Table IV
Low molecular weight proteins in fraction 9 of each investigated Co concentration

Co Concentration (ppm)	Detected molecular weights (KDa)
0	25
50	12, 10, 7
200	12, 10, 7, 5
500	10, 7, 5
800	12, 10, 7, 5
1000	12, 10, 7, 5

centrations. Ten and 7 KDa were detected in fraction 9 of all the investigated Co concentrations. Twelve KDa was detected at all concentrations except at 500 ppm while 5 KDa was not detected at 50 ppm (Table IV).

Discussion

The role of terverticillate penicillia in heavy metal detoxification has been reported, where Nazareth and Marbaniang (2008) reported that resistance to heavy metals was highest by the terverticillate *Penicillium*, decreasing in the biverticillate isolates, while the monoverticillate isolates were the least. Being a terverticillate *Penicillium* (Frisvad and Filtenborg, 1983), *P. brevicompactum* was investigated for cobalt uptake, however and according to the available research, only through two researches; Tsekova *et al.* (2007) and Farrag *et al*. (2008).

The current work continues the former of Farrag *et al*. (2008) and studies ultrastructural responses, glutathione and low molecular weight protein production by *P. brevicompactum* in response to different cobalt (Co) concentrations in an attempt to elucidate its mode of tolerance towards Co. It should also be noted that the used cobalt salt is $CoCl_2 \times 6H_2O$ which is categorized as toxic and dangerous for the environment (ATSDR, 2004).

Transmission electron micrographs of *P. brevicompactum* under the investigated Co concentrations revealed that the fungus depends on cell wall and intracellular Co deposition in its tolerance to Co.

Co was deposited as electron-dense deposits on the surface and within the cell wall structure of *P. brevicompactum*. It has been reported that fungal cell wall tends to be the cellular structure coming first into contact with the ions of metals from the surrounding environment. The chemical makeup of the cell wall and its structural organization is such that metals can become deposited either on its surface and/or within its structure before they penetrate into the cellular interior (Volesky, 1990). Also it has been reported that Cu (II) grown cells of *Candida guilliermondii* strain DS31 revealed electron-dense deposits in its cell wall (Saxena and Srivastava, 1999).

Cell wall thickness of *P. brevicompactum* was also observed in response to Co. Increased cell wall thickness was the response for other metals in other fungi; an additional cell wall layer was visualized when *Gaeumannomyces graminis* var. *graminis*, a filamentous soil ascomycete, was grown in the presence of copper (Caesar-Tonthat *et al.,* 1995). Also, thickened cell wall was observed for *Acremonium pinkertoniae* at high copper concentrations (Zapotoczny *et al*., 2007).

Intracellular Co deposition by *P. brevicompactum* included aggregation into particles in the cytoplasm or in the vacuoles in the form of black, electron-dense deposits.

The presence of vacuolated electron-dense deposits indicates the ability of the fungus to produce MTs and/or PCs. Sequestration of the heavy metal inside vacuoles is important to confer tolerance. This has been insured by Ortiz *et al.* (1992) and Rea *et al*. (1998) who reported that a Cd-sensitive fission yeast was able to synthesize PCs, but was unable to accumulate the Cd-PC-sulphide complex inside vacuoles. Also, Prasad (2004) reported the formation of electron dense intracellular complexes formed by low molecular weight thiol peptides (GSH, PCs and MTs) and detoxifying the metal by sequestration in the vacuole.

Non-vacuolar, black, electron-dense deposits were also observed in *P. brevicompactum* treated with Co. Similar results were reported for other microorganisms and other heavy metals; electron-dense particles were observed when *Enterobacter* was grown in the presence of Hg (Vaituzis *et al*., 1975). Electron-dense bodies, produced in response to silver, mercury, and zinc have been observed in several fungi (Volesky, 1990). Cells of *P. aeruginosa* treated with Cd (Bhagat and Srivastava, 1994) and those of *P. stutzeri* RS34 (Gelmi *et al*., 1994) exposed to Zn also showed deposition of cytoplasmic electron-dense particles. Also, when growing a lead resistant *Penicillium* sp. (isolated from the Pacific sediment) in the presence of 4 mM $Pb(NO_3)_2$, the fungus accumulated a large amount of dark, electron-dense lead granules inside the cell (Sun and Shao, 2007). When grown with tellurium, cells of

Salinicoccus sp. strain QW6 accumulated electron-dense, black intracellular deposits (Amoozegar *et al.*, 2008). Such deposits have previously been shown to consist of elemental tellurium in several strains of bacteria and archaea (Rajwade and Paknikar, 2003; Borghese *et al.*, 2004).

Two other ultrastructural effects due to the presence of Co in the culture medium of *P. brevicompactum* were observed; plasmolysis and increased vacuole formation (probably preparing for heavy metal sequestration by low molecular weight proteins). Ultrastructural studies of *Candida guilliermondii* strain DS31 which could resist copper up to 25 mM indicated plasmolysis and increased vacuole formation (Saxena and Srivastava, 1999). Also, Copper-induced plasmolysis was reported in the case of *Pseudomonas syringae* (Cabral, 1990). Other heavy metals, *e.g.* nickel and mercury were also reported to induce plasmolysis (Vaituzis *et al.*, 1975; Sigee and AL-Rabaee, 1986).

GSH synthesis has been reported to be involved in metal tolerance and the presence of increasing GSH concentrations may be a marker for high metal stress (Figueira *et al.*, 2005).

Hence in this study and owing to the high tolerance of *P. brevicompactum* towards Co, it was important to investigate GHS concentration which was explored in the fraction with maximum cobalt uptake (fraction no 9). GHS was detected in fraction 9 at 500, 800 and 1000 ppm with maximum glutathione concentration supporting maximum cobalt uptake (at 800 ppm). It should be noted that 500 ppm was the most drastic to the fungus, however the fungus was of better performance at 800 and 1000 ppm at which GHS concentrations increased as well as Co uptake. Also, at 800 and 1000 ppm, the fungus, despite high concentrations, produced nuclei and mitochondria reflecting its ability to survive and cope with high cobalt concentrations. This might be attributed to GHS presence, where Hutchison *et al*. (2000) reported that GHS interacts with NADP/NADPH and NAD/NADH, providing the conditions to support mitochondrial oxidative phosphorylation, generation of ATP, and hence key anabolic activities.

GSH has been shown to be related to intracellular detoxification in several organisms (Li *et al.*, 1997; Zhu *et al.*, 1999; Xiang *et al.*, 2001; Figueira *et al*., 2005), however, very little research has been conducted on detection of fungal GSH levels in response to heavy metals (Jaeckel *et al*., 2005). Collectively, tolerance to other heavy metals has been related to GHS levels; a clear correlation between glutathione levels and cadmium tolerance is reported (Figueira *et al*., 2005). Also, in liquid culture, 0.1 mM cadmium increased GSH content in an aquatic hyphomycete (*Heliscus lugdunensis*) (Jaeckel *et al*., 2005).

Being the fraction of interest, low molecular weight peptides in the range from 5 to 12 KDa were detected in fraction 9 of *P. brevicompactum* at the investigated Co concentrations. These low molecular weight peptides are in the molecular weight range of metallothioneins and a former work on the same fungus using the same Co concentrations under the same cultural conditions revealed the decrease in the level of sulfur with increasing Co concentrations suggesting sequestration of Co into metallothioneins inside vacuoles (Farrag *et al*., 2008). Also, together with the previous conclusion, detection of vacuolated, black electron-dense deposits that appeared in transmission electron micrographs of the current work greatly suggests that Co-metallothioneins are produced by *P. brevicompactum*. Razak *et al*. (1990) reported the presence, in fractions no 5 and 6 using sephadex G-25, the presence of high levels of proteins of molecular weight around 5kDa by *Aspergillus fumigatus* in response to the presence of selenium (Se-metallothioneins). They also studied the response of *P. chrysogenum* towards Se and reported the presence of several metallothioneins in the 6th and up to 15th fraction.

Also, the detection of GSH (precursor of PCs) and the presence of vacuolated electron-dense deposits suggest the possibility of PC production in response to Co by the studied fungal isolate.

Using *Arabidopsis*, Xiang and Oliver (1998) showed that the treatment with Cd and Cu resulted in increased transcription of the genes for GHS synthesis, and the response was specific for those metals thought to be detoxified by PCs.

Genes encoding for PC synthases (substrate is GHS) in higher plants and yeast have been identified, and it has been shown that the *Arabidopsis* gene could confer substantial increases in metal tolerance in yeast (Clemens *et al*., 1999; Vatamaniuk *et al*., 1999). A mutant of *Schizosaccharomyces pombe* with a targeted deletion of *CADI* (gene for PC synthase) was PC-deficient and Cd-sensitive (Ha *et al*., 1999).

Zhu *et al.* (1999) overexpressed the γ-glutamylcycteine synthetase gene from *E. coli* in *Brassica juncea* resulting in increased biosynthesis of GHS and PCs and an increased tolerance to Cd.

In liquid culture, 0.1 mM cadmium increased GSH content and induced the synthesis of additional thiol peptides in an aquatic hyphomycete (*Heliscus lugdunensis*). This is the first record reporting that the induction of metallothionein and phytochelatin are accompanied by an increase in GSH level in a fungus under cadmium stress, indicating a potential function of these complexing agents for *in vivo* heavy metal detoxification (Jaeckel *et al*., 2005).

Collin-Hansen *et al*. (2007) reported the appearance of PCs, the first report demonstrating the pres-

ence of PCs in a macromycete (the mushroom *Boletus edulis*), which was coupled to the presence of GSH.

Further ensuring studies for the detection of metallothionein genes and genes for phytochelatin synthase will be conducted on *P. brevicompactum*.

Conclusively, in this study, both intracellular bioaccumulation and extracellular biosorption had contributed to the high resistance of *P. brevicompactum* to Co. These results suggest that this fungus can be used in biotreatment as a Co-trapper. This study also confirms the possibility of using energy dispersive X-ray microanalyzer (EDX) in studying fungal mode of tolerance as has been reported by Farrag *et al*. (2008) who studied the mode of tolerance of this *P. brevicompactum* isolate using the same Co concentrations and concluded that it is both cell wall and thiol peptides that confer tolerance to this fungal isolate.

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