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Intracellular Siderophore Detection in an Egyptian, Cobalt-Treated *F. solani* Isolate Using SEM-EDX with Reference to its Tolerance

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Abstract

An Egyptian, plant pathogenic *Fusarium solani* isolate was grown on cobalt concentrations of 0, 50, 200, 500, 800, and 1000 ppm. The isolate survived concentrations up to 800 ppm, however failed to grow at 1000 ppm. Morphology and elemental analysis of the isolate under the investigated Co concentrations were studied using Scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis (EDX). The isolate reserved its morphology up to a concentration of 200 ppm. Morphological distortions were dramatic at 500 and 800 ppm. EDX detected Co uptake through the hyphae, microconidia, macroconidia, and chlamydospores. Iron, calcium, and potassium were also detected. EDX results showed a linear relationship between Co% and Fe% up to a concentration of 500 ppm reflecting the possible ability of the isolate to synthesize intracellular siderophores storing iron and their release from the vacuoles. The participation of such siderophores in conferring tolerance against cobalt is discussed. At 800 ppm, the % of Fe was greatly reduced with an accompanying increase in morphological distortions and absence of microconidia. Increasing the implicated cobalt concentrations resulted in increasing the percentages of the chelated cobalt reflecting the possible implication of the cell wall as well as extracellular siderophores in the uptake of cobalt. The current results recommend the absence of cobalt in any control regime taken to combat the investigated *F. solani* isolate and highlights the accomplishment of biochemical, ultrastructural, and molecular studies on such isolate to approve the production of siderophores and the role of cell wall in cobalt uptake.

Key words: *Fusarium solani*, cobalt uptake, cobalt tolerance, siderophores

Introduction

Ions of the metals sodium, magnesium and potassium participate in the basic metabolism of cells and are therefore absolutely essential for life. They are present in high quantities in microbial cells with concentrations in the mM range (Heldal *et al*., 1985). The metals iron, cobalt, nickel, copper, manganese, zinc and molybdenum are also involved in cellular processes and are present in mM concentrations (Heldal *et al*., 1985). Most of the other metals, including lead, mercury, aluminum and silver, have absolutely no biological function and are therefore termed abiotic elements. Metals involved in cellular processes are often cofactors of enzymes. A systematic analysis of 1371 enzymes showed that 47% contain a metal ion (Andreini *et al*., 2008).

Hence, cobalt is an essential metal and is needed in trace amount by the organisms; it is used as a cofactor of vitamin B12 and other enzymes in yeast, animals, bacteria, archaea and plants. However, at higher concentrations, cobalt becomes toxic for living systems but the exact mechanism of this toxicity is still poorly understood. Metal resistant microorganisms are important as they help in understanding metal-microbe interactions (Saad, 2014).

In general, two mechanisms have been proposed for heavy metal tolerance in fungi: 1. Extracellular (chelation and cell-wall binding) sequestration. 2. Intracellular physical sequestration of metal by binding to proteins or other ligands to prevent it from damaging the metal sensitive cellular targets (Anahida *et al*., 2011). Binding to the cell wall is called biosorption. The cell surface of fungi is negatively charged owing to the presence of various anionic structures, such as glucan and chitin (Bellion *et al*., 2006; Maghsoodi *et al*., 2007). This gives fungi the ability to bind metal cations. In the intracellular mechanism, metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into vacuolar compartment (Bellion

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et al., 2006; Abdul-Tawab and Maqsood, 2007). Besides, the mechanisms of heavy metals toxicity and resistance are different and they depend on the fungal genera and species (Al-Yemeni and Hashem, 2006).

Iron is an essential nutrient for virtually all forms of life (Winkelmann, 1991). All microorganisms examined so far, with the exception of certain lactobacilli, require iron (Haas *et al*., 1999). Iron participates in a large number of cellular processes, the most important of which are oxygen transport, ATP generation, cell growth and proliferation, and detoxification. It is a coenzyme or enzyme activator of ribonucleotide reductase, a key enzyme for DNA synthesis, which catalyzes the conversion of ribonucleotides to deoxyribonucleotidides and particularly of deoxyuridine to thymidine (Thelander *et al*., 1983).

In the aerobic environment, iron exists mainly as Fe(III) and tends to form insoluble hydroxides and oxyhydroxides (Lesuisse and Labbe, 1994), making it largely unavailable to microorganisms. Iron uptake by fungi is accomplished by specific transport systems, in which an initially Fe^{3+} form is reduced to Fe^{2+} iron, through the action of specific cell surface reductases (ferroxidases). Ferrous iron is then internalized by three different mechanisms, one of such mechanisms or iron acquisition involves the production of siderophores, which are excreted through the fungal wall in the deferric form, bind iron, and then are taken up by the fungi (de Locht *et al*., 1994; Nyilasi *et al*., 2005).

Siderophores are low molecular weight (500–1000 Da), iron-chelating ligands synthesized by microorganisms (Winkelmann, 1991). Most bacteria and fungi produce siderophores (Neubauer *et al*., 2000). Most of the characterized fungal siderophores belong to hydroxamate-type whose basic structural unit is N^5-acyl-N^5-hydroxyornithine (Winkelmann, 1991; 1992). With one exception (neurosporin), all are derived from L-ornithine. Fungal hydroxamate siderophores can be divided into three structural families: fusarinines, coprogens and ferrichromes (van der Helm and Winkelmann, 1994).

Since the Fe level in the cell is a critical factor, microorganisms possess a tight regulation in enzymes and transport systems for siderophore biosynthesis, secretion, siderophore-delivered iron uptake, and iron release. Nonribosomal peptide synthetases (NRPS) pathway and independent of NRPS are the major pathways that are reported for siderophore biosynthesis (Miethke and Marahiel, 2007). It has been reported that many siderophores are peptides biosynthesised by the well-studied nonribosomal peptide synthetase (NRPS) multienzyme family (Seneviratne and Vithanage, 2015).

Fusarium solani, belongs to ascomycetes, is widely found in soil and constitutes one of the most important phytopathogens in agriculture. It infects cultivars like soybean, bean, cassava, and potato, causing root and fruit rot, as well as wilting of the plant upper parts (Olivieri *et al*., 2002; Poltronieri *et al*., 2002).

Expert *et al*. (2012) reported that the plant iron status can influence host-pathogen relationships in different ways by affecting the pathogen's virulence as well as the host's defense. Also, Haas (2012) reported that the control over access to iron is one of the central battlefields during infection as pathogens have to "steal" the iron from the host.

Like other members of the microbial world interacting with animals and humans, plant pathogenic microorganisms have evolved a diversity of systems allowing them to capture iron from various environments in response to their metabolic needs. Indeed, experimental investigations have permitted to underscore the importance of siderophores and corresponding transport machineries in iron nutrition of phytopathogenic species (Expert *et al*., 2012).

Regarding the ascomycete *Fusarium graminearum*, two NRPSs were identified, one encoded by the *NPS2* gene and the other by the *NPS6* gene; *NPS2* gene responsible for the production of an intracellular siderophore while *NPS6* gene for an extracellular one (Oide *et al*., 2006; 2007). Another study on *Fusarium oxysporum* (strain FGSC 9935) reported that this fungus produces three different ferrichrome-type siderophores, ferricrocin, ferrichrome C, and malonichrome (Lopez-Berges *et al*., 2012)

The current work aims at investigating the effect of different cobalt concentrations on an Egyptian, plant-pathogenic *F. solani* islolate using the rapid, cost-effective, successful SEM-EDX technique in an attempt to study the possible tolerance approaches exhibited by the fungal isolate to cope with cobalt.

Experimental

Materials and Methods

Fungal isolate. *F. solani* was isolated from an Egyptian broad bean cultivar suffering root rot and identified at 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 was supplemented with different cobalt concentrations of 0, 50, 200, 500, 800 and 1000 ppm (mg Co/l). Except for the control, cobalt replaced ferrous sulfate. Cobalt chloride was the salt used to prepare the different cobalt concentrations. Media were autoclaved and poured into sterile Petri dishes (two Petri dishes for each cobalt concentration). The plates were inoculated with a 7-day old *F. solani* and incubated at 25°C for seven days.

F. solani was grown on media supplemented with different cobalt concentrations (50, 200, 500, 800 and

1000 ppm). Morphological investigations were carried out using scanning electron microscopy. Energy-dispersive X-ray spectroscopy (EDX) was used to identify the elements associated with *F. solani* together with their percentage with respect to one another at the different investigated cobalt concentrations. The distribution of the elements in macroconidia, microconidia, chlamydospores and hyphae was also investigated.

Scanning electron microscopy (SEM). Blocks of the investigated fungal isolate were prepared for SEM at The Regional Center for Mycology and Biotechnology, Al-Azhar Univ. according to Zain (1998). Fixation and dehydration procedures were performed using the programmable LEICA EM TP tissue processor model (A-1170), where six to eight millimeter squares of agar with fungal growth were cut from the 14-day old cultures. The squares were then fixed by immersion in 2% (w/v) aqueous osmium tetroxide (OsO_4) at 4°C for 12 h. Fixed material was allowed to attain room temperature and then washed in distilled water (3 times, 10 min each) to remove excess of OsO_4. Fixed and washed materials were submerged and dehydrated through a graded, 10% steps, ethanol series from 10% to 90% and finally absolute ethanol. Dehydrated specimens were critical point-dried using the Critical Point Dryer EMS (Electron Microscopy Sciences) model EMS 850. The critical point-dried specimens were then attached to 0.9 mm diameter copper stubs using a carbon adhesive. Specimens were gold-coated (nearly 50 nm thickness) using an SPI ModuleTM Sputter Coater and then examined using the high-vacuum mode of a JEOL JSM-5500LV Scanning Electron Microscope. Energy-dispersive X-ray spectroscopy (EDX). Elemental analysis (the percentage of the detected elements with respect to one another) of the samples was carried out using the X-ray detector (INCAxsight, Oxford Instruments) of the scanning electron microscope (Jeol JSM-5500LV). Window Integral was the mode of analysis. The given individual percentages represent the average of ten measurements for each of the macroconidia, microconidia, hyphae and chlamydospores, and then the total average of each element for each cobalt concentration was calculated. It should be noted that SEM-EDX measures the percentage of the detected elements with respect to one another so that the sum of percentages of all the elements detected is 100.

Results

The investigated *F. solani* isolate exhibited growth on 50 and 200 ppm. Poor growth was observed at 500 and 800 ppm, while no growth was found at a cobalt concentration of 1000 ppm.

Scanning electron micrographs (Figs. 1 and 2) illustrate the ability of the investigated *F. solani* isolate to cope with high Co concentrations up to a Co conc. of 800 ppm.

Clumps or aggregates of hyphae appeared at a concentration of 50 ppm (Fig. 1d and e) and 200 ppm (Fig. 1f). Additionally, at 200 ppm, fusion among the hyphae was observed (Fig. 1h). Hyphae were greatly distorted at higher cobalt concentrations of 500 ppm and 800 ppm (Fig. 2).

At a concentration of 200 ppm, conidia were found to decrease in amount and were not easy to find. At such concentration, most of the found conidia possessed rough surfaces which, on a high magnification of 25000X (Fig. 1g), appeared like having tiny protrusions. At 500 and 800 ppm (Fig. 2), more reduction in the amount of conidia was observed. Furthermore, macroconidial shape was greatly distorted (Figs. 2b, e). Microconidia were completely absent at a conc. of 800 ppm. Additionally, chlamydospore production was greatly enhanced at Co concentrations of 500 and 800 ppm, where most of the hyphae possessed chlamydospores (Figs 2 a, c, and d).

Results of EDX on *F. solani* grown are shown in Table I. The elemental analysis was conducted on

Table I
EDX of different morphological structures of *F. solani* isolate grown at cobalt concentrations of 0, 50, 200, 500, and 800 ppm.

Cobalt concs.	Hyphae Individual average % of detected elements				Macroconidia Individual average % of detected elements				Microconidia Individual average % of detected elements				Chlamydospores Individual average % of detected elements				Total average %			
	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K	Co	Ca	Fe	K
Control	–	8 C: 92	–	–	–	6.7 C: 93.3	–	–	–	3.6 C: 96.4	–	–	No chlamydospores were detected				–	7.35 C: 93.9	–	–
50 ppm	35.5	60.2	2.4	1.9	33.55	49	7.35	10.1	28.3	54.5	3.8	13.4	32.15	46.5	3.3	18	33.73	51.9	4.35	10
200 ppm	43.8	35.5	4.9	15.9	40.65	51.35	2.15	5.9	39	42.8	2	16.4	34.1	46.2	7.4	12.3	39.52	44.35	4.82	11.37
500 ppm	58.1	25.3	6.53	10	58.82	27	4.23	9.95	53	30.1	14.6	2.3	52.4	27.42	4.23	15.95	56.44	26.57	5	12
800 ppm	92.5	3.3	0.7	3.5	96.45	1.6	0.45	1.5	No microconidia were detected				95.5	3.2	0.3	1.1	94.81	2.73	0.45	2.03

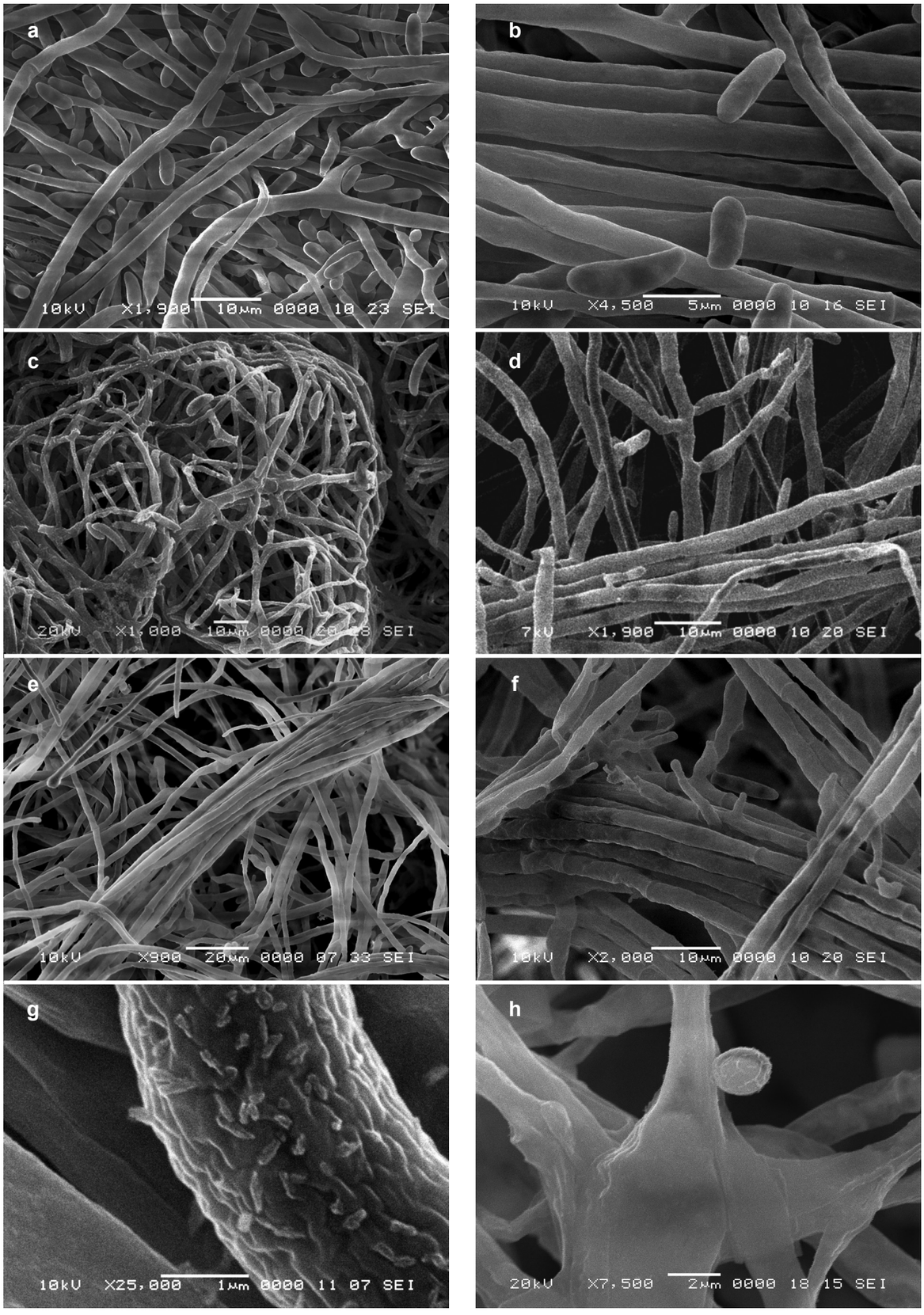

Fig. 1. Scanning electron micrographs of *F. solani*; control, a and b; 50 ppm Co, c, d, and e; 200 ppm Co, f, g, and h.

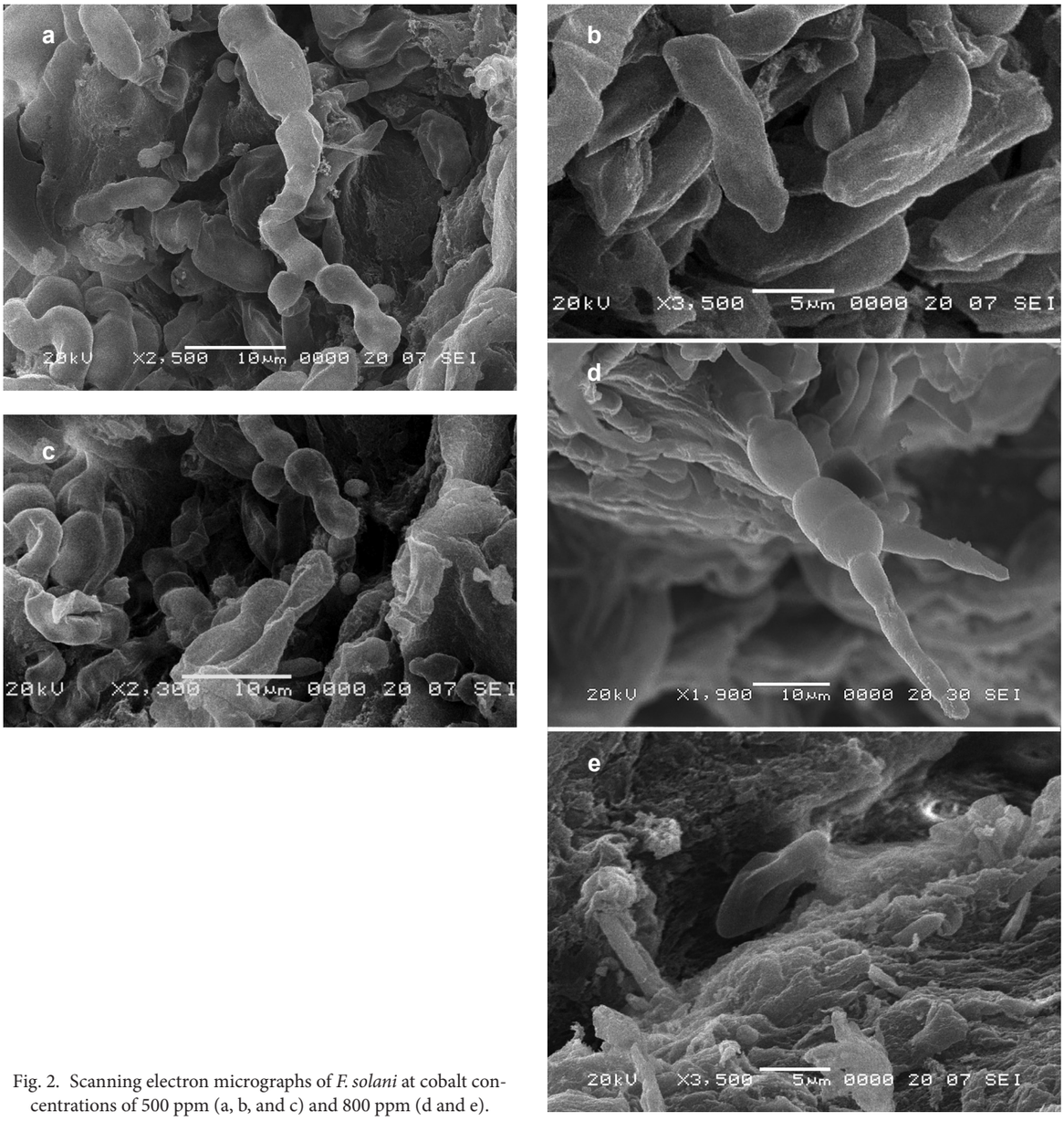

Fig. 2. Scanning electron micrographs of *F. solani* at cobalt concentrations of 500 ppm (a, b, and c) and 800 ppm (d and e).

the hyphae, macroconidia, microconidia, and chlamydospores of *F. solani* at all the investigated cobalt concentrations.

Regarding the control, in the absence of cobalt, only calcium was the detected element. The increase in the concentration of cobalt resulted in increasing cobalt uptake by the investigated *F. solani* isolate from a total average % of 33.73 at 50 ppm to 94.81 at 800 ppm. Increasing cobalt concentration also resulted in the detection of other elements which were not detected in the control, iron and potassium.

For calcium, its total average percentage jumped from 7.35, in case of the control, to 51.9 at a cobalt conc. of 50 ppm. Increasing Co conc. resulted in a gradual decrease in the total average percentage of Ca; 44.35, 26.57, and 2.73 at 200, 500, and 800 ppm respectively, however still high with respect to Fe and K. Regarding the *F. solani* morphological structure having the maximum Ca %, it was found to be different with the difference in Co Conc.; being the hyphae at 50 ppm (60.2%), the macroconidia at 200 ppm (51.35), the microconidia at 500 ppm (30.1), and the hyphae again at 800 ppm (3.3%). Chlamydospores never ranked the first as the structure possessing maximum Ca % at any of the investigated concentrations.

However for iron, increasing cobalt concentration up to a conc. of 500 ppm resulted in an increase in its total average percentage starting at a percentage of 4.35

at 50 ppm to 4.82% and then 5% at 200 and 500 ppm respectively. At a Co Conc. of 800 ppm, the pattern was reversed and the total average percentage of Fe dropped to a value of 0.45. Additionally, it is worth noting that different parts of *F. solani* responded differently to the increase in Co conc in the view of Fe detection; where at a conc. of 50 ppm, the maximum iron average % was detected in macroconidia, at 200 ppm in chlamydospores, at 500 ppm in microconidia, and at 800 ppm in hyphae.

Potassium followed the same pattern as iron showing a gradual increase in its values with increasing cobalt concentration up to 500 ppm, after which, at 800 ppm, the total average % of K dropped from 12% (at 500 ppm) to 2.03%. Chlamydospores represent the site where potassium percentage is usually elevated, at a Co conc of 50 and 500 ppm. However at 200 and 800 ppm, K % was the highest in microconidia and hyphae respectively.

Discussion

Fusarium, as a plant pathogen, have higher requirements for Fe or higher utilization efficiency compared with higher plants. Therefore, Fe differs from the other micronutrients such as Mn, Cu and B, for which microbes have lower requirements (Dordas, 2008). Furthermore, Sow *et al*. (2009) reported that particularly for fungi, iron is a major virulence factor.

F. solani is a widely distributed soil-borne fungus causing wilt and rot diseases on a wide variety of crops (Bogale *et al*., 2009). According to the available research, the effect of cobalt on *Fusarium* spp. has been investigated with little reference to the species *solani*. Hashem and Bahkali (1994) showed that a strain of *F. solani* could grow in the presence of cobalt up to a concentration of 300 ppm, however the scavenging mechanisms of *F. solani* towards cobalt was not studied. Hong *et al*. (2009) studied the effect of *F. solani* isolates on Cu, Zn, and Pb, *i.e*., cobalt wasn't involved in the study.

Hence according to the available research, the current study could be regarded the first dealing with the effect of cobalt on plant-pathogenic *F. solani* with reference to the possible tolerance mechanisms implicated using SEM-EDX.

Scanning electron micrographs revealed that different structures of the investigated isolate responded morphologically to the different cobalt concentrations implicated through forming hyphal clumps or aggregates (at 50 and 200 ppm), hyphal fusion (at 200 ppm), hyphal distortion (at 500 and 800 ppm), reduced macroconidial amounts (at 200, 500 and 800 ppm), absence of microconidia (at 800 ppm), and excessive formation of hyphal chlamydospores (at 500 and 800 ppm). Bautista-Baños *et al*. (2012) studied morphological and cellular alterations in *A. alternata*, *F. oxysporum* and *R. stolonifer* due to chitosan application using SEM and reported the value of EM technology as a tool that enables visualizing internal and external changes occurring in fungi treated with the investigated natural compounds.

Energy dispersive X-ray microanalysis (EDX) of the current study revealed that iron was not detected in the control, however the introduction of cobalt concentrations resulted in considerable iron levels detected by EDX in the form of considerable percentages. Such detectable levels could never be only due to iron sequestration from the distilled water used in preparing the growth medium from which ferrous sulphate was removed to eliminate any source for heavy metal other than cobalt. Hence, the only possible explanation for Fe detection is the ability of the investigated plant pathogenic *F. solani* isolate to synthesize intracellular siderophores capable of storing iron inside vacuoles. Being subjected to high toxic cobalt concentrations, the *F. solani* isolate responded by releasing the vacuolar Fe-siderophore complexes in an attempt to survive the stress exerted by such cobalt concentrations.

This conclusion could be supported by not detecting any iron percentages in the control sample for being stored inside the vacuoles away from the sensitivity of the electron beam which detects elements only in the cell wall, cell membrane and cytoplasm but cannot penetrate deep into the cellular organelles (Farrag *et al*., 2008; Farrag, 2009).

As revealed by SEM micrographs, morphological distortions in hyphae and macroconidia regarding shape and number were observed at 500 and 800 ppm with the last concentration resulting in more adverse morphological effects and accompanied with the least individual and total iron percentages as well as the highest cobalt percentages.

Hence, without refraining the role of cobalt, a relationship between such distortions and the release of vacuolar intracellular siderophores in response to cobalt might be assumed, supported by Eisendle *et al*. (2006) who reported that deficiency in intracellular siderophores causes a reduction of asexual conidiospore production in *Aspergillus nidulans*, which can be partly cured by supplementation with high concentrations of iron. They also concluded that the intracellular siderophore is a central component of the fungal physiology, as it is involved in iron storage, oxidative-stress resistance, and germination. Additionally, it has been demonstrated that intracellular siderophores in *F. graminearum* have roles in certain types of fungal development such as asexual and sexual sporulation (Eisendle *et al*., 2003; Oide *et al*., 2007; 2015).

Schrettl *et al*. (2007) and Wallner *et al*. (2009) reported that intracellular siderophores are believed to function in iron storage.

Recently, Oide *et al*. (2015) reported the presence of the intracellular siderophore ferricrocin in the culture filtrate of *F. graminearum* (meaning it could also be regarded as an extracellular siderophore) and stated that designation of ferricrocin as strictly intracellular appears to be an oversimplification. They also reported its doubtful role in iron uptake as an extracellular siderophore. They additionally added that due to its property as an iron storage molecule, ferricrocin iron release may be strictly regulated, and therefore iron bound to ferricrocin may not be a good source of nutritional iron.

However, several studies indicated that cobalt might compete with iron for specific binding sites in certain proteins, thereby impairing their functions (Goldberg *et al*., 1988; Bunn *et al*., 1998). Also, Stadler and Schweyen (2002) reported that cobalt might displace iron in yeast iron-sensing factors.

In the current study and in the light of the above mentioned references, it could be assumed that the presence of cobalt facilitated the release of iron from the vacuolar-free, intracellular siderophore so that the released iron can participate in overcoming cobalt stress and at the same time cobalt is chelated by forming a Co-siderophore complex.

Another possible assumption, is the ability of the *F. solani* isolate to synthesize other types of extracellular siderophores, forming Co-siderophore complexes, in response to cobalt presence. This could be supported by the fact that intracellular siderophores are only found in siderophore-producing fungi (Haas, 2012).

EDX results could support the first assumption regarding iron release where a pattern of increase in the total average % of Fe with increasing cobalt concentrations was observed up to a concentration of 500 ppm. However, at 800 ppm, the total average % of Fe was decreased to a very low % of 0.45. At 1000 ppm, the fungus failed to grow reflecting the possible inactivation of the enzymes controlling the release process of iron whose activity was greatly reduced with the high cobalt conc. of 800 ppm and completely inhibited at 1000 ppm.

The above results could be supported by Philpott (2006) who reported the presence of enzymes responsible for catalyzing the reduction or degradation of intracellular siderophores. Additionally, the strong inhibition of enzyme activity exerted by high heavy metal concentrations has been well documented by many researchers (Oliviera and Pampulha, 2006; Wang *et al*., 2008).

Furthermore, cobalt competition might have impaired iron-dependent enzymes such as aconitase and catalase A, thereby decreasing conidial size and conidial resistance to oxidative stress (Schrettl *et al*., 2007; Wallner *et al*., 2009).

In support for cobalt uptake by siderophores, it has been reported that not only with Fe, siderophores are able to form complexes with other metals as well (Yeterian *et al*., 2010). Additionally, it has also been reported that toxic metals induce the production of some siderophores suggesting that these chelators may play a role in microbial heavy metal tolerance (Schalk *et al*., 2011). Braud *et al*. (2010) reported that growth assays showed that *Pseudomonas aeruginosa* strains capable of producing pyoverdine and pyochelin, siderophores, appeared to be more resistant to metal toxicity than a siderophore non-producing strain.

Additionally, *F. solani* production of siderophores for sequestering heavy metals was reported by Hong *et al*. (2009) who observed that all the *F. solani* isolates tested showed positive CAS reactions (chrome azurol S agar plate siderophore assay) and hence reported that it is possible that the *F. solani* siderophores may have solubilized and sequestered Cu and Zn from wastes contaminated singly or in combination with these heavy metals.

Cell wall can be assumed to participate in elevating the observed percentage of the chelated cobalt due to the observed elevations in the % of Ca and K, where studies have shown that Mg, Ca, K and hydrogen ions were released from fungal biomass as a result of biosorption (Akthar *et al*., 1996; Kapoor and Viraraghavan, 1997). Muraleedharan *et al*. (1994) reported that the majority of the metal taken up through biosorption was exchanged with calcium present on the cell wall. Additionally, Tsekova *et al*. (2006) studied heavy metal biosorption in *Penicillium cyclopium* and reported that the biosorption of copper and cobalt on raw biomass was accompanied with the release of Mg, Ca and K ions into the reaction mixture.

It is worth noting that SEM-EDX has proved successful in explaining the tolerance of *Penicillium brevicompactum* against cobalt where Farrag *et al*. (2008) reported that SEM-EDX might reflect the possible presence of two mechanisms conferring tolerance to *P. brevicompactum* against cobalt; cell wall and thiol peptides. Then, Farrag (2009) confirmed such conclusions through biochemical and ultrastructural studies proving the efficiency of SEM-EDX as a rapid, cost-effective, and successful technique to investigate and explain tolerance of fungi to heavy metals. SEM-EDX (used in Farrag *et al*., 2008) reached the same results that were reached using the simultaneous work of high performance liquid chromatography (HPLC), gel electrophoresis, capillary electrophoresis, and transmission electron microscope (TEM) (used in Farrag, 2009).

It should also be noted that SEM-EDX guides and paves the way to reach the correct chosen tolerance mechanism(s) of the fungus. For instance, the detection of sulphur by SEM-EDX in *P. brevicompactum* in response to cobalt highlights the possible ability of the fungus to imply metallothioneins in its tolerance

(Farrag *et al*., 2008; Farrag, 2009). However, the detection of Fe in the current study underscores the possible implication of siderophores in the tolerance of the investigated *F. solani* isolate against cobalt.

For the investigated *F. solani* isolate, due to the presence of a relationship between cobalt and increased iron demands, even at the least investigated cobalt concentration, as a mean to overcome the stress and toxicity of cobalt, it is not recommended to use cobalt or cobalt-amended fungicides for its combating as cobalt might induces the *in vivo* uptake of iron in such isolate and hence its survival and virulence. Also, this increased iron uptake boosts the competition on iron and affects the iron levels available for the plant.

Further biochemical, ultrastructural and molecular studies will be conducted on the investigated *F. solani* isolate to ensure the possible role of siderophores and cell wall in tolerance to cobalt.

Conclusively, the current study reflects the possible ability of the investigated plant pathogenic *F. solani* isolate to synthesize intracellular siderophores for iron storage and highlights the importance of such iron in conferring tolerance against cobalt. The role of extracellular siderophores as well as cell wall in cobalt tolerance is also possible.

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