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Transformation of copper oxychloride fungicide into copper oxalate by tolerant fungi and the effect of nitrogen source on tolerance

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Abstract

Aspergillus niger and *Penicillium chrysogenum* were able to grow on Czapek Dox medium amended with elevated concentrations [up to 500 ppm active ingredient (ai)] of the fungicide copper oxychloride. Solubilization of the fungicide in solid medium was evident by the appearance of a clear (halo) zone underneath and around the growing colonies. The halo formed with *A. niger*, grown on fungicide-containing nitrate nitrogen medium, was found subsequently to enclose concentric rings of newly crystalline precipitate. These crystals were extracted, examined by scanning electron microscopy and IR, and identified as copper oxalate. The supplemented nitrogen source to the medium greatly affected both fungicide solubilization and fungal tolerance. Ratios of fungicide solubilization rate (R_S) in relation to the colony growth rate (R_G) were significantly higher on ammonium than on nitrate nitrogen medium for both fungal strains. Growth ratios (the colony extension rate in the presence of a given concentration of the fungicide in relation to the control colony growth rate) of *A. niger* were markedly lower on ammonium than on nitrate nitrogen medium. The cellular copper contents, taken up from the fungicide, and the medium titratable acidity were higher in ammonium than in nitrate medium for both fungi. These results suggested fungal possession of variable tolerance mechanisms to this fungicide by complexation and/or precipitation of copper in the medium. Additionally, this work emphasizes the activity of fungi in transformation of insoluble inorganic metal-containing fungicides into insoluble organic metal compounds, which has a potentiality in metal cycling in biogeochemical and environmental context.

Introduction

Despite the production of a wide variety of synthetic organic fungicides, copper fungicides still predominated the field of fungicidal plant disease control. The copper fungicides have been used for the control of many vegetable, fruit and flowering plant diseases. Copper oxychloride is probably the most widely employed copper fungicide. It is a low soluble copper in different formulation as wettable powder, colloidal liquid or ready to use dust. Extensive studies indicated its effecacy against a wide variety of plant pathogenic fungi such as *Rhizoctonia solani*, *R. bataticola*, *Botrytis cinerea*, *Fusarium semitectum*, *F. culmorum*, *F. moniliforme*, *F solani*, *F. oxysporum*, *Stemphylium radicinum*, *Hirschmanniella oryzae*, *Sclerotinia sclerotiorum*, *Colletotrichum gloeosporioides* (Gokulapalan et al. 1988; Kucmierz et al. 1989; Sugha et al. 1989; Bhaskar & Ahmad 1991; Thakare & Patil 1995). It also controls the pathogenic and non pathogenic fungi associated with seeds including *Helminthosporium* sp. *Rhizopus nigricans*, *Chaetomium* sp., *Aspergillus niger*, *A. flavus* and *Stachybotrys atra* (Narayanappa & Sohi 1985). It may be applied alone, in combination with other fungicides, seed treatment, foliar application, soil drenching pre- or post-emergence.

The toxicity of copper compounds is due to their ability to precipitate proteins, which causes the coagulation of the cytoplasm. Copper reacts with the sulphhydryl groups of acids and other compounds (Hugges & Poole 1989). Mehta et al. (1990) con-

cluded that the inhibitory effects of different fungicides including copper oxychloride were due to their overall adverse effect on the growth of some fungal strains. However, Karpagavalli (1997) reported that copper oxychloride, out of different investigated fungicides, was the least inhibitory to the radial growth of *Trichoderma harzianum* and *T. viride*. El-Mehalawy et al. (1999) stated that copper oxychloride administration led to an inhibition of growth, and a reduction in auxin, gibberellin and cytokinin levels in *Aspergillus flavus*, *A. terreus* and *Penicillium palitans*, however, the resistant *Aspergillus niger* showed little response. El-Mehalawy (1999) also reported that neither lipids nor pattern of amino acids played a role in the process of adaptation of *Aspergillus terreus*, *A. nidulans*, *Penicillium palitans*, and *Fusarium sporotrichoides* to higher concentrations of this fungicide.

Mechanisms of fungicide tolerance by fungi appear to be of increasing interest due to its potentiality in fungicidal application and controlling plant diseases. Copper fungicide formulations are poorly soluble in water and its fungicidal activity depends mainly on its solubilization and availability of copper ions. Free-living fungi are ubiquitous in soil, and agricultural application of metal-containing fungicides forms a potential hazard to the environment. Therefore the present study was undertaken to investigate the activity of some non-target soil-borne tolerant fungi in mobilization of the fungicide copper oxychloride and the mechanism(s) possibly implicated in fungal tolerance. Additionally, this work also aimed at studying the influence of the most agronomically important nitrogen, such as nitrate and ammonium, in fungicide tolerance which may help in application.

Materials and methods

Fungicide, organisms, medium and culture conditions

The fungicide copper oxychloride ($CuCl_2.3Cu(OH)_2$ is insoluble powder, marketed under the tradename "coprovit" (50% active ingredient), and produced by Prochemiocos, Ugota, Columbia. A stock suspension [50,000 ppm ai] of this fungicide was prepared in sterile distilled water under aseptic conditions to be used in the experimentation. Fungicide tolerant fungal strains were isolated from a reclaimed and recent cultivated area nearby Sadat city district, Menoufia governerate, Egypt, using soil plate methods (Warcup 1950). The highly tolerant strains were identified as *Aspergillus niger* and *Penicillium chrysogenum* according to Gilman (1957), Barnett (1962), and Raper & Fennell (1977), and confirmed by "The Regional center for Mycology and Biotechnology" (RCMB), El-Azhar University, Egypt. They were routinely maintained on Czapek-Dox medium of the following composition ($g.l^{-1}$ distilled water): sucrose, 30; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeCl_3.6H_2O$, 0.001. NH_4Cl at concentration of 1.27 $g.l^{-1}$ was used as a nitrogen source instead of $NaNO_3$ to prepare ammonium nitrogen medium. pH for both media were initially adjusted to 6.0 by the addition of HCl and NaOH. 15.0 $g.l^{-1}$ agar (Lab M no. 2) was added to prepare solid medium. 10 ml aliquots of agar Dox medium (6 replicates) were autoclaved, and after colling to $\sim$55 °C and just before pouring, an aliquot volume from the stock fungicide suspension was added to obtain the desired concentrations. Control (fungicide-free) treatment was also prepared by addition of sterile distilled water instead of the fungicide. The media were mixed well to insure homogenous distribution of the fungicide particulates before pouring into 90 mm diameter Petri dishes. 80 mm diameter discs of autoclaved sterile dialysis membrane (BDH, Poole, UK) were placed under aseptic conditions onto the surface of three replicate out of the solidified agar plates in each treatment. 9 mm diameter discs of mycelium cut from the margin of 24 h grown mycelia on agar Dox medium were used as an inoculum in the centre of the plates. Relationships between colony extension and fungicide solubilization activity were determined as described by Gharieb et al. (1999). The colony diameter and any clear (halo) zones present after peeling the membranes were measured over 7d incubation at 26 °C. The same treatments were prepared using liquid medium in 250 ml Erlenmeyer conical flasks, each containing 50 ml sterile media. The flasks were inoculated with 0.2 ml aliquot of spore suspensions, and then they were incubated for 7 days at 26 °C.

Electron microscopy

Agar was taken from the crystallized zone that newly formed under and around the growing colonies of *A. niger*. The crystals were extracted from the agar by gentle maceration with warm water in a crystallizing dish followed by settling and further washing of the settled crystals several times with distilled water. Crystals and samples of the fungicide were mounted on aluminum stubs and dried in a vacuum desiccator

at room temperature for at least 24 h as previously described by Gharieb & Gadd (1999). The dried crystals were sputter-coated with palladium-gold alloy using a JFC-1100E coating unit. Samples were finally examined using a Jeol JSM 3500 scanning electron microscope with an accelerating voltage of 25 kv.

Infra red (IR) spectroscopy

Infrared spectra of pure (BDH) copper oxalate, oxalic acid and the newly formed crystals underneath the growing colonies on medium containing the fungicide after purification and drying were measured with KBr discs using a Perkin Elmer 1430 spectrophotometer.

Cellular content of copper and other analysis

After the incubation period on liquid medium, the fungal biomass was separated using nylon mesh (100 μm aperture size). The mycelial dry weight was determined by collection of the mycelial mat, washing three times with dH_2O and dried at 80 °C for 24 h using aluminum foil cups. Definite weight of the dried mycelial was digested in 0.5 ml 6 M HNO_3 by heating at 90° for 1 h. After cooling, the digest was diluted with an appropriate volume of dH_2O. Copper content of diluted mycelia extracts and culture filtrates were determined using Perkin Elmer, Norwalk, Connecticut atomic absorption spectrophotometer (AAS) with reference to appropriate standard solutions. Medium pH was determined with a combined glass electrode (Cole-Parmer), and titratable acidity was determined by titrating a 20-ml sample of 7 days old medium filtrate to pH 7.0 with 20 mM NaOH.

Results

Solubilization of the fungicide and the fungal growth response

Preliminary experiments indicated that both *A. niger* and *P. chrysogenum* tolereted up to 1000 ppm fungicide copper oxychloride. There was a significant clearing of the agar containing the fungicide occurred around the growing colonies that manifesting solubilization of the fungicide. Colony growth rate (R_G) and the rate of extension of the clear zones of fungicide solubilization (R_S) were calculated, using least squares regression, over the linear portion of the growth and solubilization graph. The fungicide solubilization ratio (R_S/R_G) was expressed as the rate of extension of the clear zone of solubilization (R_S) in relation to the extension rate of that colony (R_G). Additionally, ratios of the colony extension rate in the presence of a given concentration of the fungicide (R_G) to that in the control colony (R_C) were also presented as growth ratios (R_G/R_C). Ratio of 1.0 indicates that the rate of extension of the clear zone of solubilization on a given fungicide concentration is the same as the colony extension rate on that concentration, and the colony extension rate in the presence of the fungicide is the same as the control colony. Figure 1 shows that solubilization ratios produced by both fungal strains on nitrate were significantly lower than on ammonium nitrogen medium. This means that the mycelial growth in relation to fungicide solubilization became higher on nitrate than on ammonium medium. The fungicide solubilization ratio produced by growth of *A. niger* on both nitrogen medium decreased with increasing fungicide concentration, and colony extension ratio of this fungus was higher on nitrate medium (Figure 1a). There was a growth stimulation of *A. niger* by low concentrations of this fungicide amended to nitrate nitrogen medium. Up to approximate 50% stimulation of colony growth was detected at 250 ppm fungicide as the growth ratio increased to 1.6 at this concentration (Figure 1a). Growth of *P. chrysogenum* on ammonium medium showed a dramatic increase in solubilization activity to be three times higher than the colony growth by increasing the fungicide concentration to 250 ppm (Figure 1b). Despite very low concentration of the fungicide slightly stimulated growth, there was no influence of the nitrogen source on the fungicidal action against this fungus.

Formation of copper oxalate crystals

During growth of *A. niger* on nitrate medium containing the fungicide copper oxychloride, new crystals in concentric rings inside a marginal clear zone was evident around the growing colonies. Scanning electron microscopy confirmed the production of morphologically different crystals to the amorphous particulates of the fungicide copper oxychloride (Figure 2). These crystals were blue and arranged into spherical structures with stratified walls and holes in the center. Each sphere was approximately 16 μm across. IR spectroscopic analysis revealed the formation of similar peaks for both pure copper oxalate crystals and such newly formed ones (Figure 3). The spectra show new bands at *ca* 1320 and 574 cm^{-1}, assignable to ν (C–O) and (Cu–O) respectively. Pure oxalic acid crystals

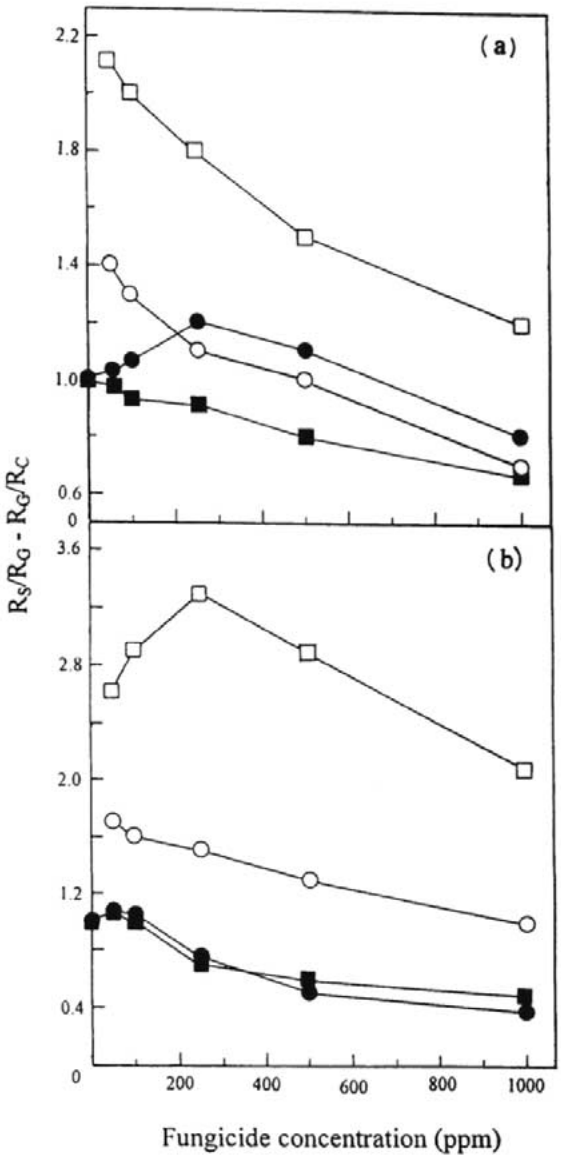

Figure 1. Ratios of fungicide solubilization rate in relation to the fungal growth rate (R_S/R_G) (○, □), and ratios of fungal growth rate on the fungicide-containing medium in relation to the control growth rate (R_G/R_C) (●, ■) of (a) *Aspergillus niger*, and (b) *Penicillium chrysogenum*. The fungi were incubated for 7 d at 26 °C on nitrate nitrogen (○, ●) or ammonium nitrogen (□, ■) Czapek Dox medium containing different concentrations of the fungicide copper oxychloride.

also displayed a broad band in the range 3650–3100 cm^{-1} assigned to μ(OH), while the band at 1680 cm^{-1} assigned to μ(C=O) (Figure 3c). These results suggests the chemical nature of these new crystals being possibly copper oxalate.

Influence of the fungicide on medium pH and titratable acidity

The responses of *A. niger* and *P. chrysogenum* mycelial dry weight, and the final pH and titratable acidity of the medium to the presence of different concentrations of the fungicide under different nitrogen condition are shown in Figure 4. Despite the growth of these fungi over the incubation period resulted in decrease the medium pH, increasing the concentration of the fungicide alleviated such an effect i.e. marked increase in the final cultures pH was resulted from increasing the supplemented fungicide concentration. While the medium pH values after growth of *P. chrysogenum* on fungicide-free nitrate and ammonium medium were 3.4 and 2.0, the addition of 500 ppm fungicide increased these values to 6.0 and 3.8 respectively (Figure 4b). Additionally, growth of both fungal strains on ammonium nitrogen medium resulted in significant lower pH values than that obtained on nitrate medium. This variation was clearly higher in *P. chrysogenum*. Figure 4 also shows that at different concentrations of the fungicide, the titratable acidity is obviously higher in ammonium than in nitrate growth medium of both fungi. Patterns of the titratable acidity obtained after growth of *P. chrysogenum* were significantly decreased with increasing the fungicide concentration and it reverses the medium pH pattern (Figure 4b). However, growth of *A. niger* on both nitrogen sources displayed a peak pattern of medium titratable acidity over the fungicide concentrations being maximum at 100 ppm fungicide (Figure 4a). This indicates lack of a concise relationship between the medium pH and the titratable acidity by growth of this fungus.

Dry weight and copper contents of mycelia grown on the fungicide

The dry weight of the fungal mycelia and the cellular copper contents after incubation on medium containing the fungicide are indicated in Figure 5. It is obvious that increasing the fungicide concentrations significantly decreased the fungal growth. Moreover, by using ammonium nitrogen medium there was a higher growth inhibition than that on nitrate. Accordingly, the nitrogen source supplemented to the medium

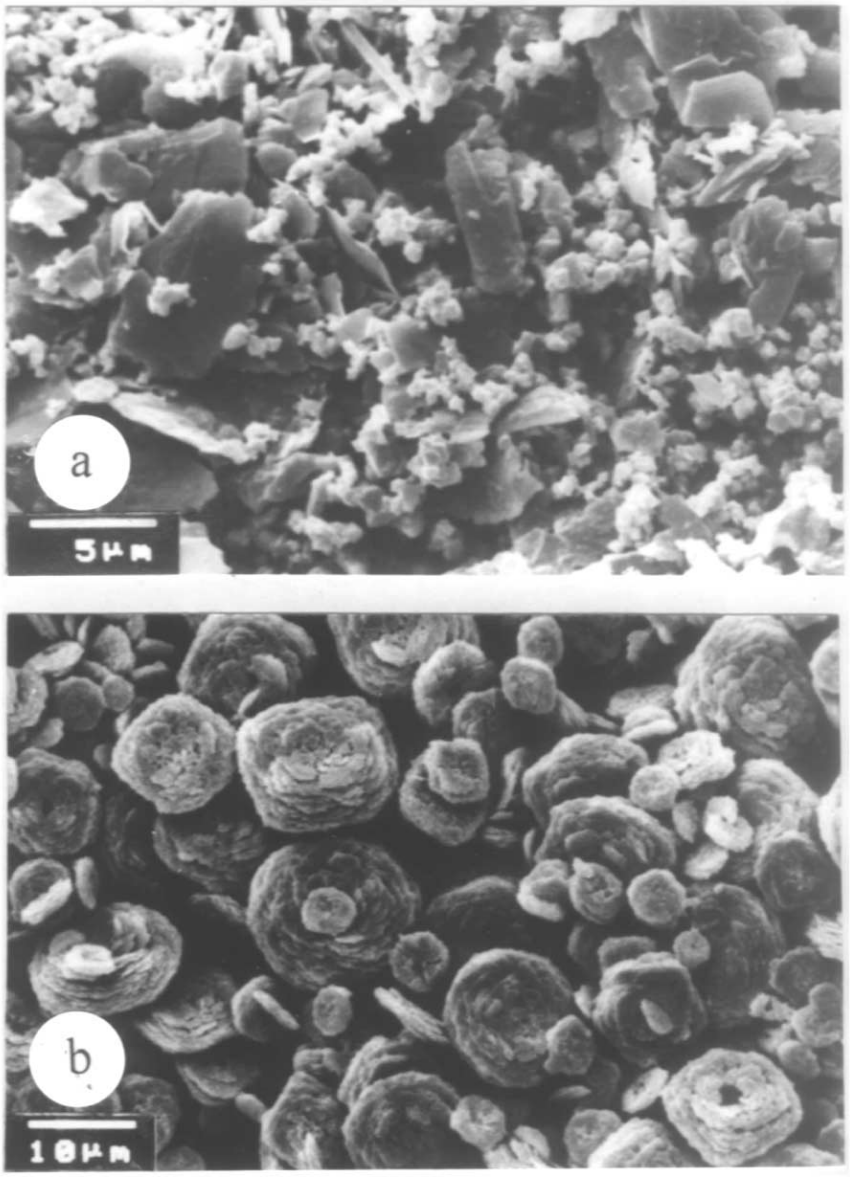

Figure 2. Scanning electron micrograph (SEM) of (a) particulates of the fungicide copper oxychloride, and (b) the newly formed crystals around and underneath the growing colonies of *Aspergillus niger* after 7d incubation at 27 °C on agar nitrate Czapek Dox medium containing 500 ppm fungicide.

appeared to greatly affect the tolerance of these fungi to the fungicide copper oxychloride. The cellular copper content of the fungi grown on elevated concentrations of the fungicide is also illustrated in Figure 5. There was a significant increasing in the cellular copper content by increasing the fungicide concentration for both fungi which were substantially higher in the mycelia grown on ammonium nitrogen medium. Additionally, the mycelia of *P. chrysogenum* grown on this medium displayed higher cellular copper content than the corresponding treatment of *A. niger*.

Discussion

Since the copper-containing fungicides are mostly formulated in insoluble form, its fungicidal activity depends mainly on its solubilization which lead to enhanced mobility of Cu^{2+}. Therefore, the resulted free

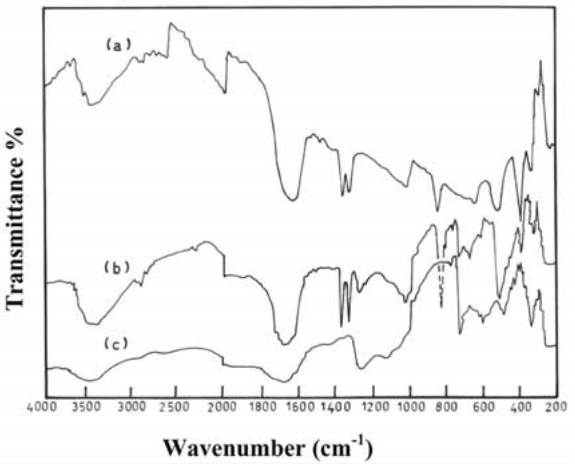

Figure 3. Infra Red (IR) spectra of (a) highly pure crystals of copper oxlate, (b) purified crystals formed underneath and around the growing colonies of *Aspergillus niger* after 7d incubation at 27 °C on agar Czapek Dox medium containing 500 ppm fungicide copper oxychloride, and (c) pure oxalic acid crystals.

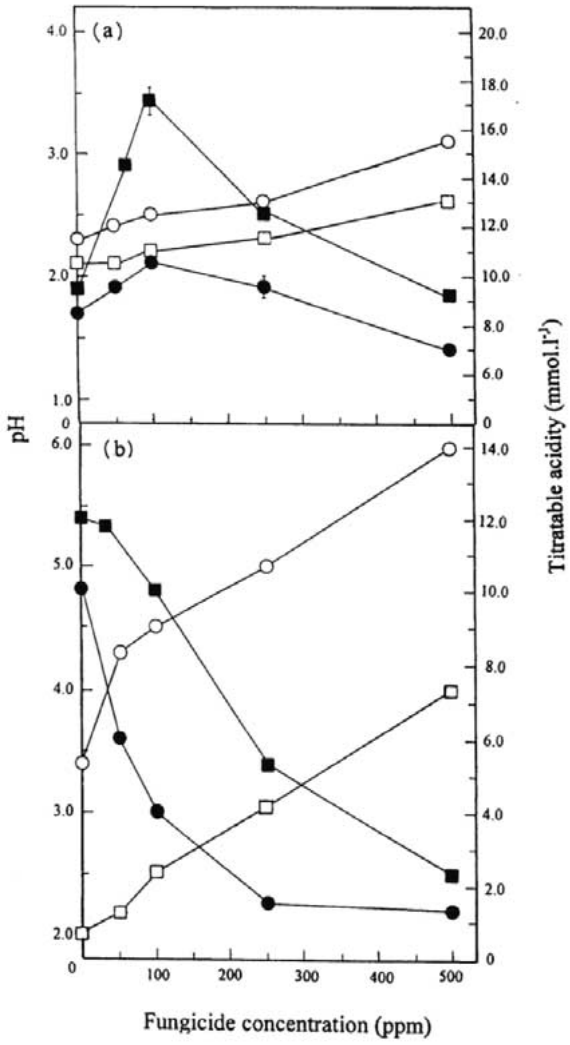

Figure 4. Medium pH (○, □) and titratable acidity (●, ■) after incubation of (a) *Aspergillus niger* and (b) *Penicillium chrysogenum* for 7d at 27 °C on nitrate (○, ●) or ammonium (□, ■) Czapek Dox medium containing different concentrations of the fungicide copper oxychloride. Bars indicate standard error of the mean (three replicates) and when not shown were smaller than the symbol dimensions.

copper ions would be presumably available for cellular uptake and subsequently exert cellular toxicity. Despite numerous mechanisms were postulated, microbial solubilization of insoluble metal compounds most probably resulting from protonation of the anion of the metal compound (Hughes & Poole 1989). Solubilization of copper fungicides is mainly brought about by fungal exudates such as amino acids and keto acids (Hassall 1990). Preliminary experiment showed that the addition of HCl solutions in wells in agar plates containing this fungicide resulted in clear zones of solubilization (data not shown). Protons can be pumped into the external medium by the proton translocating ATP_{ase} of the plasma membrane (Slayman et al. 1990; Morley et al. 1996) and/or organic acid production such as citric and oxalic acids which are major products of several fungi including *A. niger* (Kubicek & Rohr 1986). In a recent study, Gharieb (2002) reported the ability of *A. niger* to adsorb and subsequently solubilize high concentrations of the fungicide copper oxychloride *via* Ca^{+}/H^{+} exchange capacity of the fungal cells. The results obtained in this study revealed that *A. niger* and *P. chrysogenum* were able to grow on high concentrations of this fungicide though displaying solubilization activity. This clearly indicates possession of copper tolerance mechanisms by these fungal strains. Copper tolerance by fungi has been extensively investigated, and the ability to prevent cellular entry or reducing cellular accumulation of copper has been reported as the main mechanism for tolerance (Gadd & White 1989). Growth of the tested fungi on medium-containing elevated concentrations of the fungicide is characterized by increasing the cellular copper content despite displaying lower fungicide solubilization activity. This result might be due to changes in fungicide concentration as well as medium pH achieved by fungicide amendment. Gadd & Griffiths (1980) reported that a reduction in pH of the culture medium may lead to a reduction in the tox-

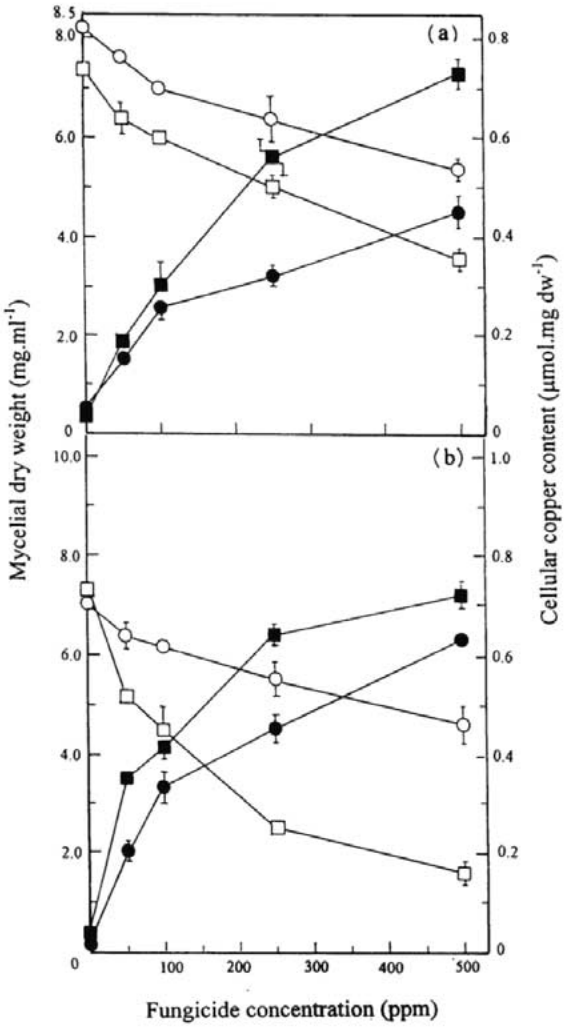

Figure 5. Mycelial dry weight (○, □) and the cellular copper contents (●, ■) after incubation of (a) *Aspergillus niger* and (b) *Penicillium chrysogenum* for 7d at 27 °C on nitrate (○, ●) or ammonium (□, ■) Czapek Dox medium containing different concentrations of the fungicide copper oxychloride. Bars indicate standard error of the mean (three replicates) and when not shown were smaller than the symbol dimensions.

icity of copper to fungi due to decrease in the amount of copper taken up by the cell.

In addition to providing protons, organic acid anion is usually capable of forming complex with the metal cation, thus affecting its mobility and toxicity (Sayer & Gadd 1997; Gharieb & Gadd 1999). Franz et al. (1991) reported that the adsorption of insoluble metal oxides onto the mycelial pellets of *Penicillium simplicissimum* was required for citric acid production and solubilization. Additionally, oxidation and solubilization of metal sulphides and releasing of free metals by *A. niger* and *Trichoderma harzianum* was linked to the ability of the fungal hyphae to adsorb these compounds (Wainwright & Grayston 1989). Gharieb (2002) suggested that adsorption of the fungicide copper oxychloride onto the fungal cells induces excretion of acidic complexing agents, which are implicated in the fungicide solubilization and tolerance. On the other hand, since solubilization of this fungicide is positively correlated with the medium pH therefore any factor affecting the medium acidity will affect the solubilization process. The influence of the amended nitrogen on the metabolites produced by fungi could emphasize and explain the present results. A stimulation of proton extrusion by ammonium ions has been documented in many instances. Roos and Lackner (1984) reported that acidification observed in cultures of *Penicillium cyclopium* was mostly due to the extrusion of protons into the medium, and in the absence of ammonium protons were extruded together with citrate. Dixon-Hardy et al. (1998) found that the rate of solubilization of $Zn_3(PO_4)_2$ and $Co_3(PO_4)_2$ by *A. niger* decreased with decreasing nitrogen (as ammonium) concentration, and increased with decreasing nitrogen (as nitrate) concentration. Therefore, it could be postulated that in liquid nitrate nitrogen medium production of complexing agents by these fungal strains resulting the formation of soluble copper complexes which render copper being less toxic in nitrate medium.

After initial solubilization of the fungicide a precipitation of newly formed crystals under *A. niger* colonies grown on solid medium has been demonstrated. Crystals of this nature have been observed under colonies of *A. niger* growing on agar amended with other insoluble metal compounds [ZnO, $Zn_3(PO_4)_2$ and $Co_3(PO)_2$], and have previously been identified as insoluble metal oxalates (Sayer & Gadd 1997). In the present study scanning electron microscopy and IR analysis also evident the possible formation of copper oxalate crystals instead of the added fungicide particulates to the growth solid medium. Oxalic acid is a common metabolite excreted in large quantities by species belonging to all classes of fungi and its role in copper tolerance was previously reported (Dutton & Evans 1996). This and other low molecular weight organic acids, e.g. citric acid are characterized by their ability to leach, complex and precipitate metals from insoluble minerals (Dutton & Evans 1996; Morley et al. 1996). Calcium oxalate precipitation by several fungal species has been documented and proposed as a mechanism, which prevents calcium

toxicity (Lapeyrie et al. 1987; Gharieb & Gadd 1999). Precipitation of copper as an oxalate has also been reported in several free-living and symbiotic fungi. Sutter et al. (1983, 1984) reported that the wood-rotting fungi *Poria placenta* and *Poria vaillantii* could immobilize copper in wood treated with copper sulphate as a preservative by the formation of insoluble and hence nontoxic copper oxalate. Murphy & Levy (1983) reported the formation of numerous copper oxalate crystals associated with the developing colonies of *A. niger*, *Penicillium spinulosum*, *Verticillium psalliotae* and *Poria placenta* grown in agar media containing copper sulphate. Formation of insoluble copper oxalate crystals provides an effective means of toxic copper immobilization, with a possible further evidence of implication for fungal tolerance to the fungicide copper oxychloride in solid environment.

Stimulation of oxalic acid production in the presence of nitrate and inhibition by ammonium was previously reported for the plant pathogenic *Sclerotium rolfsii* (Kritzman et al. 1977) and certain mycorrhizal fungi (Lapeyrie et al. 1987). Gharieb (2000) also reported that elevated concentrations of nitrate increased oxalic acid production by *A. niger* to 21 μmol/ml whereas with the same concentration of ammonium nitrogen, the highest amount of the produced oxalic acid was 3.5 μmol/ml. In certain lower plants, Meeuse & Campbell (1959) also suggested an inhibition by nitrate of oxalic acid oxidase, which breaks down oxalic acid to carbon dioxide and hydrogen peroxide. The present investigation showed that the fungicide copper oxychloride is solubilized in both nitrogen media and *A. niger* exhibited higher cellular copper content and toxicity in ammonium than in nitrate medium. These results could be also attributed to extrusion of free protons which release copper ions available for cellular uptake and exertion of copper toxicity in ammonium nitrogen medium. However, in nitrate nitrogen medium this fungal strain would excrete oxalate and citrate, relatively different according to the growth condition, which complex copper ions and subsequently preventing its entry into the fungal cells.

Conclusion

These findings emphasize the significant effect of the nitrogen source on fungal tolerance to the fungicide copper oxychloride which has a potentiality in agricultural application. Production of soluble and insoluble less toxic copper-containing compounds from the fungicide by these organisms also indicates possession of flexible tolerance mechanisms depending on the fungal species and growth conditions. Additionally, this work evident the capability of free-living soil fungi to transform the inorganic metal-containing compound (copper oxychloride) into insoluble organic compound (copper oxalate) which has a biogeochemical and environmental importance for understanding elemental cycling in nature.

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References

Barnett HL (1962) Illustrated Genera of Imperfect Fungi. Burgess Publishing Co.

Bhaskar RB & Ahmad ST (1991) Root rot disease of barseem and its control. Indian Phytopath. 43: 589–590

Dixon-Hardy JE, Karamushka VI, Gruzina TJ, Nikovska GN, Sayer JA & Gadd GM (1998) Influence of the carbon, nitrogen and phosphorus source on the solubilization of insoluble metal compounds by *Aspergillus niger.* Mycol. Res. 102: 1050–1054

Dutton VM & Evans CS (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42: 881–895

El-Mehalawy AA (1999) Role of amino acids and lipids in adaptation of fungi to higher concentrations of fungicides. Desert Institute Bull, Egypt 47: 119–133

El-Mehalawy AA, Abd Allah MM & Telwani KA (1999) Influence of copper oxychloride on growth-regulating substances of certain soil fungi. Desert Institute Bull, Egypt 47: 475–486

Franz A, Burgstaller W & Schinner F (1991) Leaching with *Penicillium simplicissimum*: Influence of metals and buffers on proton extrusion and citric acid production. Appl. Environ. Microbiol. 57: 769–774

Gadd GM &White C (1989) Heavy metal and radionuclide accumulation and toxicity in fungi and yeasts. In: Poole RK & Gadd GM (Eds) Metal-Microbe Interactions (pp 19–38). IRL Press, Oxford

Gadd GM & Griffiths AJ (1980) Influence of pH on toxicity and uptake of copper in *Aureobasidium pullulans*. Transact. Br. Mycol. Soc. 75: 91–96

Gharieb MM & Gadd GM (1999) Influence of nitrogen source on the solubilization of natural gypsum ($CaSO_4.2H_2O$) and the formation of calcium oxalate by different oxalic and citric acid producing fungal strains. Mycol. Res. 103: 473–481

Gharieb MM, Sayer JA & Gadd GM (1999) Solubilization of natural gypsum ($CaSO_4.2H_2O$) and the formation of calcium oxalate by *Aspergillus niger* and *Serpula himantioides*. Mycol. Res. 102: 825–830

Gharieb MM (2000) Nutritional effects on oxalic acid production and the solubilization of natural gypsum by *Aspergillus niger*. Mycol. Res. 104: 550–556

Gharieb MM (2002) Biosorption and solubilization of copper oxychloride fungicide by *Aspergillus niger* and the influence of calcium. Biodegradation 13: 191–199

Gilman JC (1957) A Manual of Soil Fungi. The Iowa State University Press

Gokulapalan C, Nair M, Mohandas N & Koshy G (1988) Integrated control of *Rhizoctonia solani* and *Hirschmanniella oryzae* under field conditions. Integrated Pest Control: Progress and Prospectives. In Proceedings of the National Symposium (pp 381–383), held at Trivandrum, India

Hassall KA (1990) The Biochemistry and Uses of Pesticides: Structure, Metabolism, Mode of Action and Uses in Crop Protection. Macmillan Press Ltd, London

Hughes MN & Poole RK (1989) Metals, microorganisms and biotechnology. In: Metals and Micro-organisms (pp 303–358). Chapman and Hall, London, New York

Karpagavalli S (1997) Effect of different fungicides on the growth of *Trichoderma*. Indian J. Plant Prot. 25: 82–83

Kritzman G, Chet I & Henis Y (1977) The role of oxalic acid in the pathogenic behavior of *Sclerotium rolfsii*. Sacc. Exper. Mycol. 1: 280-285

Kubicek CP & Rohr M (1986) Citric acid fermentation. Crit. Rev. Biotech. 3: 331–373

Kucmierz J, Bartynska M & Mazur S (1989) Studies of fungal diseases on carrot grown for seed. Roczniki-Akademii-Rolniczej-w-Poznaniu,-ogrodnictwo 194: 171–178

Lapeyrie F, Chilvers GA & Bhem CA (1987) Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* (Batsch. ex Fr.) Fr. New Phytol. 106: 139–146

Meeuse BJ & Campbell JM (1959) An inhibitor of oxalic acid oxidase in beet extracts. Plant Physiol. 34: 583–586

Mehta A, Chopra S & Mehta P (1990) Fungicides: inhibitory agents of cell wall degrading enzymes. Indian Phytopath. 43: 117–121

Morley GF, Sayer JA, Wilkinson SC, Gharieb MM & Gadd GM (1996) Sequestration, mobilization and transformation of metals and metalloids. In: Frankland JC, Magan N & Gadd GM (Eds) Fungi and Environmental Change (pp 235–256). Cambridge University Press, Cambridge

Murphy RJ & Levy JF (1983) Production of copper oxalate by some copper tolerant fungi. Transact. Br. Mycol. Soc. 81: 165–168

Narayanappa M & Sohi HS (1985) Seed mycoflora of marigold and its control. Indian J. Mycol. Plant Pathol. 15: 283–286

Raper KB, Fennel D (1977) The Genus *Aspergillus*. Williams and Wilkens Co., Baltimore.

Roos W & Luckner I (1984) Relationship between proton extrusion and fluxes of ammonium ions and organic acids in Penicillium cyclopium. J. Gen. Microb. 130: 1007–1014

Sayer JA & Gadd GM (1997) Solubiliozation and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger*. Mycol. Res. 101: 653–661

Slayman CL, Kaminski P & Stetson D (1990) Structure and function of fungal plasma membrane ATPase. In: Kuhn PJ, Trinci AP, Jung MJ, Goosey MW & Copping LG (Eds) Biochemistry of Cell Walls and Membranes in Fungi (pp 299–316). Springer-Verlag KG, Berlin

Sugha SK, Thakur RS & Singh BM (1989) Comparative sensitivity of *Rhizoctonia solani* isolates to selected systemic fungicides. Indian J. Plant Pathol. 7: 167–172

Sutter HP, Jones GE & Walchli O (1983) The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.) Fr. Materials and Organisms 18: 241–262

Sutter HP, Jones GE & Walchli O (1984) Occurrence of crystalline hyphal sheaths in *Poria placenta* (Fr.) Cke. J. Wood Sci. 10: 19–23

Thakare CS & Patil PY (1995) Studies on leaf blight of *Chrysanthemum* caused by *Colletotrichum gloeosporioides*. J. Maharashtra Agricultural Universities 20: 49–52

Wainwright M & Grayston SJ (1989) Accumulation and oxidation of metal sulphides by fungi. In Poole RK & Gadd GM (Eds) Metal Microbe Interactions (pp 119–130). IRL Press, Oxford

Warcup JH (1950) The soil plate method for isolation of fungi from soil. Nature (London) 166: 117