Responses of Arbuscular Mycorrhizal Fungal Spores Isolated from Heavy Metal-polluted and Unpolluted Soil to Zn, Cd, Pb and Their Interactions in vitro

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Abstract: Arbuscular mycorrhizal fungal spores of Glomus spp., isolated from heavy metal-polluted and corresponding unpolluted soil obtained from Abou-Rawash farm, Giza (Egypt), were investigated in vitro to test their sensitivity to Zn, Cd and Pb, measured as an inhibition of the germination percentage and hyphal growth from germinated spores. The spores germination and hyphal growth for both isolates showed a variable responses to different concentrations of Zn, Cd and Pb. Only, Zn at low concentrations increased germination percentage and hyphal growth for both isolates spores compared to control (without metal) and this effect was more pronounced with the spores isolated from polluted soil. Germination of the spores isolated from polluted soil were more tolerant to Zn, Cd and Pb than the spores from unpolluted, with EC50 values of 76, 34 and 14 μmol L⁻¹ for the former isolate and 30, 16 and 6 μmol L⁻¹ for the later to Zn, Cd and Pb respectively. Interaction between metals singly or in combination of 2 or 3 metals (at EC50 values) showed variation in sensitivity to Zn, Cd and Pb. The presence of Zn in the media significantly ameliorated toxicity of Cd and /or Pb in most circumstances as indicated by increasing hyphal growth of germinated spores. This effect was more pronounced with the spores isolated from polluted soil and in combination of 3 metal tested. It was concluded that, AM fungal spores from polluted and unpolluted soil differ in their heavy metals susceptibility to single or multiple metal exposure.

Keywords: Arbuscular mycorrhizal fungi, heavy metals toxicity, heavy metals interaction, Zn, Cd, Pb toxicity

Introduction
In the natural environment, contamination by single pollutants rarely occurs. Man’s energy and chemical consumption, metal smelting, mining and manufacturing process more often result in environments contaminated with a mixture of potentially toxic metals. The main sources of the heavy metals contamination in the agricultural soil are fertilizer impurities and the use of refuge-derived compost and sewage disposal, municipal and industrial waste. Soil contamination with heavy metals has become a world problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain (Salt et al., 1995). The toxicity of heavy metals in soil depends on their bioavailability, defined as their ability to be transferred from the soil to a living organism (Berthelin et al., 1995), metal bioavailability is a function not only of their total concentration but also of physio-chemical (as pH and organic matter) and biological (as biosorption and bioaccumulation) factors. All organisms, including microorganisms, can achieve resistance to heavy metals by avoidance, when the organism is able to restrict metal uptake, or by tolerance, when the organism survives in the presence of high internal concentration (Turnau et al., 1996).

Arbuscular mycorrhizal (AM) fungi are abundant components of the soil biota in most terrestrial ecosystems. The influence of AM fungi in plant nutrition is thought to be greater for elements such as phosphorus and heavy metals (Lambert et al., 1979). Heavy metals have been reported to inhibit spore germination and hyphal extension in vitro (McGee, 1987) and to reduce or completely eliminate AM colonization of plant roots in pot experiment (Chao and Wang, 1991). However, AM fungi occur in metal polluted soil, Glickman and Tinker (1983) demonstrated that the strain of Glomus mosseae isolated from heavy metals contaminated soil was much more tolerant to Zn and Cd than that from uncontaminated soil. These results are confirmed by Weissenborn et al. (1993), who demonstrated that soils polluted by applications of heavy metals-contaminated sewage sludge and by atmospheric deposition from a smelter contained AM fungi more tolerant to Cd than reference strains isolated from noncontaminated soils. Griffioen et al. (1994) found tolerant AM fungal strains associated with Agrostis capillaries only at Zn/Cd contaminated sites, whereas no tolerant strains were detected at Cu contaminated sites. AM fungal spores isolated from zinc- polluted or cadmium-polluted soil proved more tolerant to cadmium than a laboratory
reference strain *Glomus mosseae* regarding spore germination ability in sand amended with Cd solution (Weissenhorn et al., 1994). The aim of this study was to investigate the response of two different local isolates spores of *Glomus* spp., one isolate obtained from polluted soil site and other from corresponding unpolluted site, to different doses of heavy metals, zinc, cadmium, and lead with their interaction on germination and hyphal growth in vitro.

**Materials and Methods**

Two isolate spores of arbuscular mycorrhizal (AM) fungus, *Glomus* spp. were used in this investigation. They were obtained from the rhizosphere soil of wild plant *Panicum turgidum* Forsk grown in Abou-Rawash farm which is located in Giza, Egypt, as previously described by Shalaby et al. (1999). One isolate spores was obtained from heavy metal polluted soil (Spoll) irrigated with heavy metals-polluted sewage effluent for 10 years application, other isolate spores obtained from corresponding unpolluted soil (Sunpoll) irrigated with well water. AM fungi resting spores were isolated from rhizospheric soil in each site by wet sieving (63 sieve) according to Gerdenmann and Nicolson (1963). Only predominate, yellowish brown spores with the characteristic of *Glomus* spp. as described by Trappe (1982) were isolated under a binocular microscope and maintained in sterile distilled water at 4-6 °C after surface sterilization in 3.5% calcium hypochlorite solution for 2 min and rinsing with sterilized distilled water several time and used within month. The soil samples were analysed according to Black et al.(1965) to determine physico-chemical composition and heavy metal content (Table 1).

**Experiment 1**

**Effect of heavy metals treatments on the spores germination and hyphal growth:** The spore germination and hyphal growth for each isolate were tested, on minimal (M) medium with 1% agar as described by Becard and Fortin (1988), for their sensitivity to zinc as (Zn SO₄. 7H₂O), cadmium as (Cd SO₄. 8H₂O) and lead as (Pb(NO₃)₂), which was added to the medium before autoclaving at 120 °C for 20 min and pH was adjusted to 6.5 using KOH. The final concentrations of each metal tested in minimal (M) agar medium were: 0, 5, 10, 20, 40, 80, 160 μmol L⁻¹. For each treatment five replicates plates were prepared in petri-dish with diameter (16 cm). Each plate was inoculated with five spores which were placed at the vertices of an imaginary pentagon 6 cm far from edge. The Petri-dishes, were sealed with parafilm to prevent drying out and contamination, incubated at 30 °C in the dark. After incubation for 3 weeks, the plates were flooded with acid glycerol trypan blue (glycerol 500 ml; H₂O, 45 ml; 1% HCl, 50 ml; trypan blue, 0.5 g) for one hour, then examined under microscope (40x). Following two parameters were investigated:

1. The spores germination percentage,
2. The hyphal length from germinated spore, estimated by a line-intersect method (Tennant, 1975). The effective concentration at which 50% of the spores that germinated without metal addition failed to germinate (EC₅₀) was estimated graphically (Fig. 1).

**Experiment 2**

**Effects of interactions between Zn, Cd and Pb on hyphal growth:** The effect of heavy metals (at EC₅₀ values) were investigated singly or in combination of 2 or 3 metals, on hyphal growth of isolates spores, using the methods of each isolate outlined above in experiment 1. The results were then expressed as a percentage of each individual metal treatment and displayed as histograms.

**Data analysis:** Data were presented as mean with standard errors (n=5). The mean effective concentrations at which 50% of the spores that germinated without metal addition failed to germinate (EC₅₀) was estimated graphically.

**Results:** The spores of *Glomus* spp., isolated from polluted soil (Spoll) and corresponding unpolluted soil
Fig. 1: Effect of Zn(a, d), Cd(b, e) and Pb(c, f) on spore germination and hyphal growth of two Clomus spp one isolated from unpolluted soil (oo) and the other from polluted soil (••). Mean of 5 replicates plate (each with 5 phases), bars represent the standard error of mean.

(Sunpoll) showed a variable responses for Zn, Cd or Pb treatments on spores germination percentage and hyphal growth from germinated spores (Fig. 1). In control treatment (without heavy metal) and at low concentration of Zn and Cd (5 μmol L⁻¹) the spore germination percentage and hyphal growth from germinated spore were higher for the spores isolated from unpolluted soil (Sunpoll) than spores isolated from polluted soil (Spoll). In contrast, with increasing heavy metal concentrations, percentage of the spore germination and hyphal growth of the spores isolated from (Spoll) were significantly higher than the spores isolated from (Sunpoll) in most treatments of three heavy metals tested (Fig. 1). Also, Zn at 5 μmol L⁻¹ non-significantly increased germination percentage and hyphal growth for the spores isolated from unpolluted soil, while spores isolated from polluted soil showed highly significant increasing in germination percentage and hyphal growth with Zn treatments up to 20 μmol L⁻¹ compared to control treatment, with less pronounced effect on hyphal growth (Fig. 1 a, d). In the
Each individual heavy metal treatment reduced germination percentage for both isolates spores by 50% of control treatment, as expected from the results of EC_{50} values graphically estimated (Fig. 1). The EC_{50} values for both isolates varied significantly for each heavy metal tested, 76, 30 μmol L^{-1} Zn more tolerant; 34, 16 μmol L^{-1} Cd less sensitive and 14, 6 mol Pb, more sensitive, for the spores isolated from polluted and corresponding unpolluted soil respectively. These results were confirmed with hyphal growth per germinated spore (Fig. 1 d-f).

The results of heavy metals interaction indicated a wide variation in response of the two isolate spores to heavy metals singly or in combination. In the meantime, the spores isolated from polluted soil (Spoll) was more tolerant to toxicity in all combination of metals treatments than the spores isolated from unpolluted soil (S unpoll) (Fig. 2 a-c). Cd and Pb interacted with Zn singly (Zn + Cd), (Zn + Pb) or in combination (Zn + Cd + Pb) to reduced Zn toxicity by increasing hyphal growth for both isolates spores compared to action of Zn alone (Fig. 2 a). This effect was more pronounced with 3 heavy metals combination (Zn + Cd + Pb) which increase hyphal growth by 35 and 30% for the spores isolated from polluted and unpolluted soil respectively. Also, Zn ameliorated toxicity of Cd and/or Pb by increasing hyphal growth. Zn with Cd treatments increased relative hyphal growth by 20 and 35% compared to Cd treatment alone, while Zn with Pb increase hyphal growth by 50 and 65% compared to Pb treatment alone for the spore isolated from unpolluted and polluted soil respectively. In the meantime, Zn highly significantly ameliorated Cd and Pb toxicity in combination of 3 metals treatments (Zn + Cd + Pb) and hyphal growth increased by 45 and 60% compared to Cd treatment and by 60 and 85% compared to Pb treatment alone for spore isolated from unpolluted and polluted soil respectively. In contrast combination of Cd and Pb, (Cd + Pb) treatments, increased toxicity of Cd or Pb by decreasing hyphal growth by 25 and 40% compared to Cd treatment and by 20 and 30% compared to Pb treatment alone for the spores isolated from polluted and corresponding unpolluted soil (Fig. 2 a, b).

**Discussion**

The spores, of AM fungus Glomus spp., isolated from polluted soil were more tolerant to heavy metals, Zn, Cd and Pb, compared to the spores isolated from corresponding unpolluted soil with over double magnitude EC_{50} values. In the meantime, germination percentage and hyphal growth of both isolates spores appeared more tolerant to Zn treatments than Cd while Pb was more toxic (Fig. 1). The isolation of metal-tolerant mycorrhizal fungi from polluted soil sites has been
documented (Leyval and Weissenhorn, 1996). AM fungi, 
*G. mosseae* and *G. macrocarpum*, isolated from heavy 
metal contaminated soil showed higher percentage 
of spore germination in the presence of increasing doses of 
added Pb than a *G. mosseae* reference strain (Diaz et al., 
1996). The results showed that, the spores originally isolated 
from polluted soil differ in their metal susceptibility than 
the spores isolated from corresponding unpolluted. This 
variation may be due to ecophysiological adaptation of 
the spores isolated from polluted soil for heavy metals. 
The mechanism of adaptation to elevated heavy metal 
concentration are not known. However, regarding their 
relatively long generation time and the large number of 
 nuclei in one spore, the observed rapid appearance of 
metal tolerance may be based on phenotypic plasticity 
rather than on selection of the tolerant genotypes 
(Weissenhorn et al., 1994). They suggested that, the 
spores of *G. mosseae* and *G. etunicatum* isolated from Cd 
polluted soil lost their tolerance to Cd and Zn compared 
to reference *G. mosseae* after one reproduction cycle (6 
months) on metal-free substrate. 
A number of studies had compared the response of AM 
fungus isolated from contaminated and uncontaminated 
soil sites in an attempt to relate metal sensitivity to the 
contamination of the soil origin. The results of 
investigations were contradictory. Weissenhorn et al. 
(1994) showed that, the spores of *G. mosseae* spp. isolated 
from polluted soil (long application of Zn polluted sewage 
sludge) culture exhibited a specific tolerance to Zn than 
unspecific tolerance isolate from other polluted soil 
treated with Cd, for one year) culture to both Cd and 
Zn, despite the much higher Zn availability in sewage 
sludge polluted soil. Diaz et al. (1996) found that, 
sensitivity of two *G. mosseae* spp. isolates from different 
polluted soil sites was shown to vary significantly. In 
contrast, Weissenhorn et al. (1993) noted that, similar 
sensitivity (EC$_{50}$) values for Cd on *Glomus* spp. spores 
isolated from soil polluted with sewage sludge and from 
corresponding unpolluted soil. Willenborg et al. (1990) 
showed that, the susceptibility of ectomycorrhizal fungi to 
environmental stress factors, including heavy metals, 
varied from species to species but also from strain to 
strain within species. Also, other worker found wide 
variation in sensitivity of ectomycorrhizal fungi to Zn, 
with EC$_{50}$ values ranging from 0.8-1.5300 m mol m$^{-2}$ in agar 
medium and 1.35-3.060 m mol m$^{-2}$ in liquid medium (Denny 
and Wilkins, 1987; Tam, 1995). 
Zn, but not Cd and Pb, particularly at low concentrations 
increased hyphal growth for germinated spores in both 
isolates and the effect was less pronounced than spore 
germination percentage (Fig. 1 a-d). This may be 
contributed to high affinity for Zn as essential element for 
fungal nutrition than for non essential Cd and Pb, where 
Zn is one constituent of Minimal (M) medium used for 
culturing AM fungi spores (Becard and Fortin, 1988). 
Weissenhorn et al. (1994) reported that, Zn tolerance was 
more pronounced at the level of spore germination than at 
the level of hyphal growth from germinated spores for 
AM fungi isolated from heavy metal-polluted and 
unpolluted soil. Smylla and Mroczkowska-Badner (1991) 
also observed higher Cd sensitivity of spore germination 
compared to hyphal growth from pre-germinated spores of 
*Streptomyces* they suggested that certain germination 
phases are particularly effected by competition between 
Cd and essential divalent cations or by inhibition of the 
enzyme activity. As known from higher plant, activities of 
stress related enzymes as oxidative enzymes, should be 
included as biomarkers in phytotoxicity of Cd 
contaminated soil (Lagariffoul et al., 1998). In the meantime, 
plant tolerated Cd toxicity by exhibited antioxidative 
defense mechanism by elevated antioxidative enzymes 
activities (Schickler and Caspi, 1999). Similar mechanisms 
may be assumed for AM fungi. 
Interaction between heavy metals influence their relative 
toxicity to AM fungi. Inhibition of hyphal growth from 
germinated spores caused by multiple metals interaction 
cannot be predicted from their individual toxicity and 
varies between two isolates. The most interactions of 
heavy metals reduced toxicity for both isolates compared 
to the toxicity of individual metals. Zn ameliorated toxicity 
of Cd and/or Pb for both isolates and was more pronounced 
with spores isolated from polluted soil (Fig. 2 a-c). This would suggest that soil contaminated by 
a mixture of metals might not be as to AM fungi as 
predicted from toxicity assessment of individual metals. 
Amelioration of Cd toxicity with Zn interaction was 
recorded in ectomycorrhizal fungus *Suillus variegatus* 
where appeared to be highly sensitive to Cd in absence of 
Zn, with Cd EC$_{50}$ value 0.8 m mol m$^{-2}$, yet in the presence 
of 500 m mol m$^{-2}$ Zn, the Cd EC$_{50}$ increased significantly 
to 6.5 m mol m$^{-2}$ (Hartley et al., 1997). Furthermore, Colpaert 
and Van Asche (1992) recorded amelioration by Zn to Cd 
toxicity on the ectomycorrhizal fungus *Suillus bovinum* 
where 765 m mol m$^{-2}$ Zn reduced the toxicity of 8.9 to 89 
m mol m$^{-2}$ Cd. This amelioration by Zn was also recorded 
in the basidiomycete *A. aegerita* where increasing the 
concentration in agar media suppressed Cd translocation 
into the mycelium (Brunnert and Zachrasi, 1985). Metals 
tolerance interaction have also been documented in 
plants, while Zn was shown to interacted with Cd and 
Pb, both metals were competitively inhibited (Symeonidis 
There are a number of possible explanation for the
interaction between Zn with Cd and/or Pb. The plasma membrane is the main selective barrier to influx / efflux of cations and anions and is the principal site of active transport (Meharg and Macnair, 1992). Ions being transported from external to the cytoplasm bind to transport sites in the plasma membrane. It is therefore, possible that competition between ions of the same valancy or size may occur, assuming that the number of binding sites is small in relation to the concentration of competing ions (Marschner 1995). It is possible, therefore, that the interaction of Zn with Cd and/or Pb would vary dependent on the concentration of metals involved. At relative high Zn concentrations amelioration of Cd and/or Pb toxicity for both isolates was significantly high in most treatments (Fig. 2), where the metals interaction takes place at EC50 value for each metal (EC50 values used were 76, 36 μmol L−1 Zn, 34, 16 μmol L−1 Cd and 14, 6 μmol L−1 Pb for spores isolated from polluted and unpolluted soil respectively). Also, as Zn is an essential element for fungal nutrition, divergent metal transporters might have a higher affinity for Zn than for the non-essential Cd and Pb. A second hypothesis could be that a physiological response in mycorrhizal fungi is induced by the presence of Zn, but not Cd and Pb. This response might result in decreased sensitivity to Zn and increased Cd and/or Pb sensitivity to mycorrhizal fungus. A range of mechanisms have been proposed by which mycorrhizal fungi protect themselves and their hosts against metal toxicity fall into four main categories: reduced influx; extracellular binding; complication with polyphosphate granules; and intracellular chelation (Hartley et al., 1997). These results indicated that, in most cases, AM fungal spores isolated from heavy metals polluted soil could be adapted to tolerate toxicity of heavy metals investigated as singly or in combination than the spore isolated from unpolluted soil.

Whilst single metal studies are essential for investigating the relationships between AM fungi and potentially toxic metals, it is likely that this investigation would have little practical relevance for predicting the effect of multiple metal exposure on AM fungi in contaminated soil. Potentially toxic metals are very rarely present singly in the environment but occur in mixture. Further work is required to examine the interactive effects of metals on AM fungi in symbiosis with host plants.

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