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Persistence of heavy metal tolerance of the arbuscular mycorrhizal fungus *Glomus intraradices* under different cultivation regimes

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Abstract

The difference in development of arbuscular mycorrhizal (AM) symbiosis of maize with various *Glomus intraradices* strains isolated from Mn-contaminated (BEG140), Pb-contaminated (PH5) or non-polluted (BEG75) soils was studied in polluted substrates from waste disposal sites to test persistence of heavy metal (HM) tolerance. Four inoculation treatments were established in HM-contaminated soils and in sand as an inert control: (i) non-inoculated plants, (ii) plants inoculated with the reference isolate BEG75, (iii) plants inoculated with the lineage of the indigenous isolate (PH5 or BEG140) maintained for a long term in the original contaminated soils, (iv) plants inoculated with the lineage of the indigenous isolate maintained in the inert substrate. In both HM-contaminated soils, lineages of both indigenous isolates maintained in original soils developed much better than the reference isolate, while all isolates developed similarly in sand. Moreover, the lineage of the BEG140 isolate maintained in inert substrate for almost 5 years showed a loss of HM tolerance when returned to the original polluted soil. In contrast, no such differences were observed between both lineages of the isolate from Pb-contaminated soil. These results suggest that continuous cultivation of indigenous isolates from contaminated soil under HM stress should be recommended to maintain their HM tolerance.

Keywords: Mycorrhizal symbiosis, arbuscular mycorrhiza, HM tolerance, lead, manganese, extraradical mycelium, maize

1. Introduction

Arbuscular mycorrhizal (AM) fungi have been shown to affect plant growth and fitness under heavy metal (HM) stress, although great variability among AM isolates has been reported (e.g,. Leyval et al., 1997). The benefits of being mycorrhizal include plant growth promotion related to improved nutrient supply and modifications in uptake and distribution of HMs between roots and shoots (Schüepp et al., 1987; Weissenhorn et al., 1995; Meharg and Cairney, 2000). Some indigenous AM isolates from HM-contaminated soils showed adaptation to high HM concentrations, sustaining higher HM concentrations than reference AM isolates from non-contaminated soils (e.g., Gildon and Tinker, 1981; Weissenhorn et al., 1993; del Val et al., 1999; Tullio et al., 2003). Certain isolates from contaminated soils not only tolerated higher HM concentrations, but also more effectively stimulated plant growth, increased host HM tolerance or decreased HM uptake into plant tissues (Hildebrandt et al., 1999; Vivas et al., 2005, 2006). Interestingly, the duration of HM stress required for the occurrence of higher HM tolerance in AM fungi does not necessarily need to be in terms of many years because 1- or 2-year periods of HM treatment only were reported as sufficient for a noticeable induction of HM tolerance (Weissenhorn et al., 1994; Tullio et al., 2003). Different mechanisms of AM-induced HM tolerance in plants have been proposed, including HM accumulation in hyphal walls, cytoplasm and vacuoles (Dehn and Schüepp, 1989; Turnau et al., 1993; Joner et al., 2000). Differences in HM accumulation in fungal tissues among isolates have

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also been reported (Joner et al., 2000; Gonzales-Chavez et al., 2002).

AM fungi are considered to be of importance especially in contaminated waste substrates with poor nutrient status, low water holding capacity and susceptibility to wind and water erosion. Such sites are targeted for revegetation programmes where utilization of indigenous AM fungi has been proposed (Perotto and Martino, 2001; Turnau and Haselwandter, 2002; Gaur and Adholeya, 2004). From a practical perspective, a study of the stability of HM tolerance in indigenous AM isolates from contaminated soils is of pivotal significance for determining proper maintenance and preparation of suitable inocula. To our knowledge, very little attention has been paid to this topic, except for the study by Weissenhorn et al. (1994) who identified loss of HM tolerance in AM culture after 1-year maintenance without HM stress.

In our previous sand-based hydroponics experiment (Malcová et al., 2003a), the persistence of HM tolerance was not shown as a significant decrease in Mn tolerance of the isolate *G. intraradices* BEG140 from a Mn-contaminated soil was observed after its 2-year cultivation in inert substrate. In comparison with cultivation systems where HMs are added artificially to inert substrate or liquid/agar media, the use of original HM-contaminated soils is more complex and realistic, because AM fungi in contaminated soils are exposed not only to higher HM concentrations, but often face other edaphic stresses, such as nutrient deficiency, low content of organic matter, extreme pH values and others. Moreover, the whole microbial community in the given contaminated substrate can significantly affect plant interactions with HMs (Vivas et al., 2003a,b, 2005), AM fungi being only one, although substantial, component. For the reasons given, the present follow-up study was conducted in original contaminated soils. Specifically, we studied the differences in HM tolerance (i) between a reference isolate from non-polluted soil and indigenous AM isolates from HM-contaminated soils and (ii) between lineages of indigenous isolates cultured long term with or without HM stress. Moreover, element analysis of ERM of AM isolates/lineages was conducted using energy dispersion spectroscopy (EDS) to test the hypothesis that fungal strains with different HM tolerance differ in HM accumulation in mycelium.

Table 1. Chemical characteristics of HM-contaminated soils used for the study.

Soil	pH_{KCl}	C	N	S	*Na	*Mg	*Ca	**P	*K	Cl^-	SO_4^-
		(%)			(mg kg^{-1})						
Mn-polluted	6.1	11.6	0.5	0.5	32	27	1,288	33	78	<0.1	760
Pb-polluted	5.2	1.5	0.2	0.3	59	109	1,875	27	65	<0.1	<500

Soil	Mn			Cu (mg kg^{-1})			Zn		
	Total[1]	Extr.I[2]	Extr.II[3]	Total	Extr.I	Extr.II	Total	Extr.I	Extr.II
Mn-polluted	69,000	1,057	460	28	4	2	87	20	2
Pb-polluted	688	124	4	610	180	39	796	359	140

Soil	Pb			Fe (mg kg^{-1})			Cd		
	Total	Extr.I	Extr.II	Total	Extr.I	Extr.II	Total	Extr.I	Extr.II
Mn-polluted	30	4.4	0.04	58,400	398	49	0.6	0.07	0.02
Pb-polluted	30,000	9,954	890	25,900	308	49	5.7	2.5	1.1

*1 M ammonium acetate-extractable. **0.5 M sodium bicarbonate-extractable (Olsen-P).
[1]Total = aqua regia-extractable metal concentrations. [2]Extr.I = 1 M EDTA + 0.1 M ammonium acetate-extractable metal concentrations (Lakanen and Erviö, 1971; Borggaard, 1976). [3]Extr.II = 0.005 M DTPA + 0.1 M triethanolamine + 0.01 M $CaCl_2$-extractable metal concentrations (Lindsay and Norvell, 1978).

2. Material and Methods

AM isolates

Two fungal isolates of *Glomus intraradices* Schenck and Smith obtained from two HM-contaminated sites of anthropogenic origin in the Czech Republic were chosen for the study: (i) isolate PH5 from a Pb-contaminated waste disposal site in the proximity of the Příbram lead smelter and (ii) isolate BEG140 from a Mn-contaminated sedimentation pond at Chvaletice. Both isolates were obtained via trap cultures and subsequent multispore cultures were initiated from about twenty morphologically identical spores. After successful sporulation, two lineages of each isolate were established either in the original HM-contaminated soil (referred to as Mn or Pb) or in metal-free substrate (clinoptinolite/sand 1:1, referred to as inert). *G. intraradices* PH5 and BEG140 were cultured in two lineages from November and August 1998, respectively, in pots on maize and sub-cultured approximately every six months. Isolate *G. intraradices* BEG75 served as the reference isolate from non-contaminated soil.

Experimental design

Four different inoculation treatments with five replicates were established for both HM-contaminated soils (Pb-polluted and Mn-polluted) from which AM fungal isolates originated: (i) non-inoculated plants, (ii) plants inoculated with the lineage of indigenous isolates grown in original contaminated soils, (iii) plants inoculated with the lineage of indigenous isolates grown in inert substrate and (iv) plants inoculated with a reference isolate from non-polluted soil. At the time of experiment establishment, the lineages of *G. intraradices* PH5 and BEG140 had been maintained separately for 55 and 58 months, respectively. Chemical characteristics of both contaminated soils are given in Table 1. Each inoculation treatment was established also in sand to separate the differences in AM development induced by substrate from the differences in development of particular AM isolates *per se*. Maize seeds (*Zea mays* L., cv. TATO-260) were surface-sterilised, pre-germinated in Petri dishes and transplanted to 375-ml pots (one per pot) filled with γ-irradiated substrates (5 Mrad). Maize was chosen as a host plant to enable comparison of results with experiments conducted previously with the same AM isolates under simulated HM stress (Malcová et al., 2003a,b). All mycorrhizal treatments received 10-ml suspension of the inoculum that was prepared by wet sieving (Gerdemann and Nicolson, 1963) and contained colonised root segments, extraradical mycelium and spores. Non-inoculated plants were treated with the same amounts of inoculum autoclaved at 121°C for 25 min. In an effort to equalize possible differences in microbial communities between inoculation treatments, non-inoculated plants were also irrigated with a filtrate from a mixture of all relevant non-sterile inocula (1:10 suspension of soil inocula filtered through a filter paper (Whatman No. 1, UK) to remove AM fungal propagules). In the attempt not to affect original HM availability in contaminated soils, the plants were not supplied with nutrient solution during cultivation.

Harvest and plant analyses

The plants were grown for 16 weeks (May to September) in a temperature-controlled greenhouse with supplementary 12-hour light supplied by metal halide lamps (400 W). After harvest, shoot and root dry biomass of maize plants was assessed after drying at 80°C. Shoots and roots were ground and decomposed by a dry ashing procedure (Mader et al., 1998). Plant material (1 g) was decomposed in 50-ml quartz-glass beakers at 500°C for 16 h on a hot plate and in muffle furnace with stepwise increase of the ashing temperature. The ash was dissolved in 20 ml of 1.5% solution of HNO_3. The digest was used for the determination of the concentrations of phosphorus, manganese (in Mn-polluted soil) or lead (in Pb-polluted soil) in the roots and shoots. Mn and Pb concentrations were determined by atomic absorption spectrometry (AAS) with flameless atomisation (Varian SpectrAA-300). To determine P concentrations in dry biomass, 9 ml of 1.5% solution of HNO_3 was added to 1 ml of the digest. P analysis was conducted using the Scalar (San^{Plus} System) segmented continuous flow analysis with photometric detector (420 nm).

Mycorrhizal parameters

To evaluate AM colonisation in plant roots, root samples were stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989) and then assessed under microscope at 100× magnification using the method of Trouvelot et al. (1986). The following parameters of mycorrhizal development were evaluated on fifty 1-cm root fragments per plant: frequency of mycorrhizal colonisation in the root system (F%), intensity of the mycorrhizal colonisation in the root system (M%), intensity of the mycorrhizal colonisation in the colonised root fragments (m%), arbuscule and vesicle abundance in the root system (A% and V%, respectively), arbuscule and vesicle abundance in mycorrhizal parts of root fragments (a% and v%, respectively). The substrate from each pot was mixed and small samples were taken to assess the length of ERM using the aqueous extraction and modified membrane filtration technique (Jakobsen et al., 1992). The sample of the substrate was blended at high speed using a household mechanical blender with 300 ml of distilled water for 30 s. Then, 1 ml of the supernatant from this blended suspension was removed (always from the same position in blender jar), pipetted onto a membrane filter (24 mm diameter, 0.40

Table 2. Shoot and root biomass of maize plants and development of mycorrhizal symbiosis (ERM length and activity of NADH-diaphorase) as influenced by cultivation substrate (Pb-polluted soil/sand) and AM inoculation (PH5 = *Glomus intraradices* PH5, inert = lineage maintained long term in metal-free substrate, Pb = lineage maintained long term in original Pb-polluted soil, BEG75 = reference isolate *G. intraradices* BEG75). Data are means (±SE) of 5 replicates. Means marked with the same letters are not significantly different within one substrate at P<0.05 according to Duncan's Multiple Range test.

Substrate	AM inoculation	Dry weight of shoots (g)	Dry weight of roots (g)	ERM length (m g^{-1})	NADH-d. activity (%)
Pb-polluted	Non-inoculated	1.59 ± 0.16 a	0.72 ± 0.04 b	–	–
	PH5-inert	1.97 ± 0.13 a	1.29 ± 0.15 a	8.6 ± 1.6 a	30 ± 6 a
	PH5-Pb	1.86 ± 0.18 a	1.18 ± 0.13 a	8.7 ± 1.4 a	22 ± 5 ab
	BEG75	1.78 ± 0.32 a	1.05 ± 0.23 ab	4.1 ± 0.9 b	7 ± 4 b
Sand	Non-inoculated	1.11 ± 0.16 a	0.66 ± 0.06 a	–	–
	PH5-inert	1.01 ± 0.11 ab	0.77 ± 0.05 a	8.6 ± 1.5 a	75 ± 5 a
	PH5-Pb	0.83 ± 0.10 ab	0.63 ± 0.08 a	11.2 ± 1.3 a	75 ± 6 a
	BEG75	0.73 ± 0.06 b	0.75 ± 0.11 a	8.0 ± 1.5 a	68 ± 6 a
Analyses of variance[1]					
Substrate (A)		***	***	ns	***
Inoculation (B)		ns	ns	*	ns
A × B		***	***	ns	***

[1]Significant effect at P<0.05*, P<0.001***, ns = non-significant effect.

Table 3. Shoot and root biomass of maize plants and development of mycorrhizal symbiosis (ERM length and activity of NADH-diaphorase) as influenced by cultivation substrate (Mn-polluted soil/sand) and AM inoculation (BEG140 = *Glomus intraradices* BEG140, inert = lineage maintained long term in metal-free substrate, Mn = lineage maintained long term in original Mn-contaminated soil, BEG75 = reference isolate *G. intraradices* BEG75). Data are means (±SE) of 5 replicates. Means marked with the same letters are not significantly different at P<0.05 within one substrate according to Duncan's Multiple Range test.

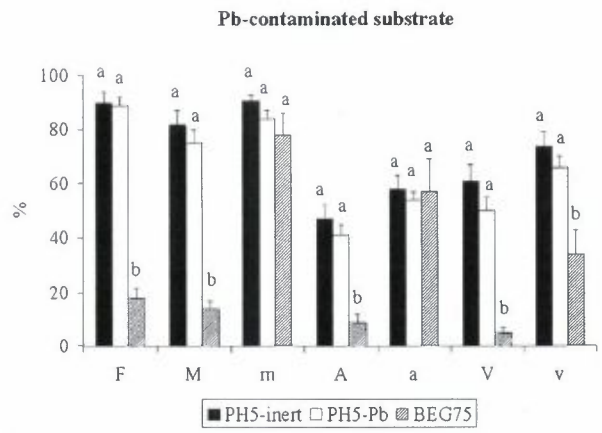
Substrate	AM inoculation	Dry weight of shoots (g)	Dry weight of roots (g)	ERM length (m g^{-1})	NADH-d. activity (%)
Mn-polluted	Non-inoculated	2.95 ± 0.21 a	1.11 ± 0.08 a	–	–
	BEG140-inert	3.40 ± 0.26 a	1.15 ± 0.15 a	2.4 ± 0.4 b	10 ± 3 b
	BEG140-Mn	2.85 ± 0.14 a	1.34 ± 0.06 a	8.1 ± 1.4 a	39 ± 8 a
	BEG75	3.19 ± 0.10 a	1.05 ± 0.07 a	3.0 ± 0.3 b	10 ± 4 b
Sand	Non-inoculated	1.01 ± 0.10 a	0.86 ± 0.08 ab	–	–
	BEG140-inert	0.92 ± 0.05 ab	1.11 ± 0.13 a	9.5 ± 1.3 a	64 ± 3 b
	BEG140-Mn	1.12 ± 0.09 a	1.09 ± 0.12 a	7.0 ± 1.0 ab	79 ± 2 a
	BEG75	0.73 ± 0.10 b	0.64 ± 0.06 b	5.7 ± 0.8 b	51 ± 3 c
Analyses of variance[1]					
Substrate (A)		***	**	***	***
Inoculation (B)		ns	**	**	***
A × B		*	ns	**	ns

[1]Significant effect at * P<0.05, ** P<0.01, *** P<0.001, ns = non-significant effect.

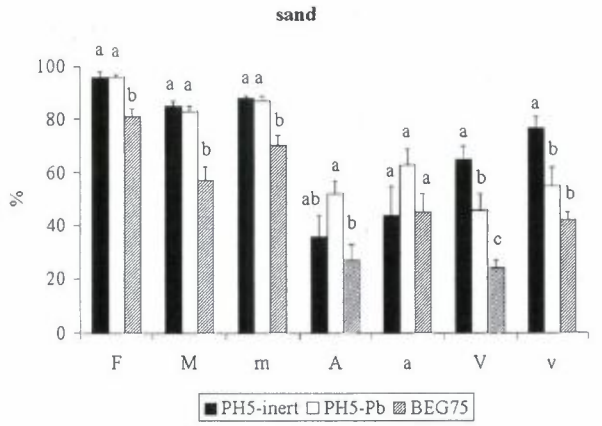
μm pore size; Pragopor 6, Pragochema, Czech Republic) and vacuum filtered. The ERM retained on the filter was stained with 0.05% trypan blue in lactoglycerol. The total length of ERM was assessed using the grid-line intersect method under a microscope at 100× magnification and expressed in meters of hyphae per 1 g of air-dried substrate. The background length of fungal mycelium observed in non-inoculated treatments was subtracted from the values found for the inoculated treatments in the same substrate. Small aliquots of mycelium extracted from a homogenised substrate by wet sieving were used for vital staining of NADH-diaphorase activity in ERM (Sylvia, 1988; Hamel et al., 1990). ERM clusters were incubated in the tubes with NADH-staining solution at room temperature in the dark for 14 h. Then, the proportion of viable ERM containing red precipitate (NADH-diaphorase activity; Fig. 3) to non-stained hyphae was assessed using a grid-line intersect method under a microscope at 400× magnification (500 intersections per sample).

ERM analyses

To find out if the isolates studied differed in HM accumulation in their ERM, SEM/EDS analysis was

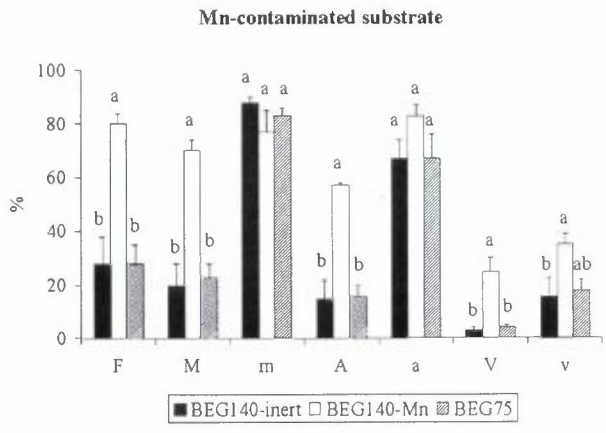


(a)

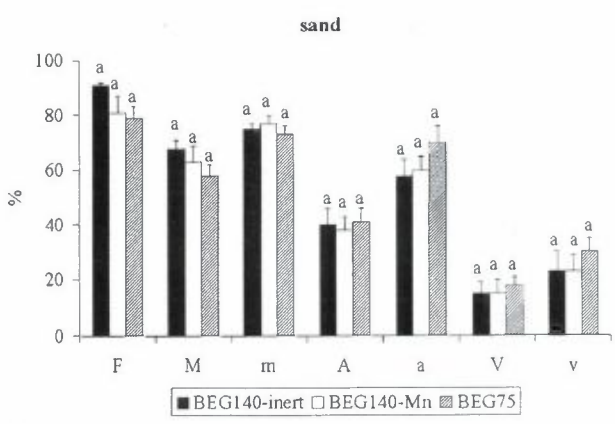


(b)

Figure 1. Differences in mycorrhizal colonisation among three different *Glomus intraradices* isolates (PH5 = *G. intraradices* PH5, inert = lineage maintained long term in metal-free substrate, Pb = lineage maintained long term in original Pb-contaminated soil, BEG75 = reference isolate *G. intraradices* BEG75) grown either in Pb-contaminated soil (a) or in sand (b). Parameters of root colonisation according to Trouvelot et al. (1986): F = frequency of mycorrhiza in the root system, M = intensity of mycorrhizal colonisation in the root system, m = intensity of mycorrhizal colonisation in mycorrhizal parts of root fragments, A = arbuscule abundance in the root system, a = arbuscule abundance in mycorrhizal parts of root fragments, V = vesicle abundance in the root system, v = vesicle abundance in mycorrhizal parts of root fragments. Data are means (±SE) of 5 replicates. Columns marked with the same letters are not significantly different at $P<0.05$ according to Duncan's Multiple Range test.



(a)



(b)

Figure 2. Differences in mycorrhizal colonisation among three different *Glomus intraradices* isolates (BEG140 = *G. intraradices* BEG140, inert = lineage maintained long term in metal-free substrate, Mn = lineage maintained long-term in the original Mn-contaminated soil, BEG75 = reference isolate *G. intraradices* BEG75) grown either in Mn-contaminated soil (a) or in sand (b). Abbreviations and symbols as in Fig. 1. Data are means (±SE) of 5 replicates. Columns marked with the same letters are not significantly different at $P<0.05$ according to Duncan's Multiple Range test.

conducted on mycelium samples obtained from three randomly selected pots per inoculation treatment. Mycelium samples were collected by wet sieving and purified using fine tweezers. The samples were embedded in isopentan cooled in liquid nitrogen to –145°C and lyophilised with a tissue dryer at –30°C and vacuum 2×10^{-2} mBarr, mounted on carbon stubs, covered with the 15 nm layer of carbon and analyzed with SEM JEOL JSM 5410 equipped with a SiLi detector (NORAN Instruments Inc., USA). The analytical conditions were established on a basis of Monte Carlo simulations for chitin matrix ($C_8H_{13}NO_5$) (Win X-Ray by Demers et al., version 1.2.1.13). The estimated depth of the electron beam penetration did not exceed 6 μm and 10 μm at 15 keV and 20 keV, respectively. The maximum depth of X-ray emission, however, was 4 μm for Cu K_α, 7 μm – Zn K_α, 5 μm – Pb L_α and 9 μm – Pb M_α. Thus, the microscope was operated at 15 or 20 kV depending on the element analyzed (Cu at 15 kV and Zn or Pb at 20 kV), at a magnification of 3500–7500× in point mode, probe current set on 300 pA and 100 s of analytical time. Computer analysis of spectra was carried out with the Voyager 3.6 program (NORAN Instrument Inc., USA). Net count data and element mg kg^{-1} dry mass data were determined on the basis of reference standard

calculation. The standards were obtained by preparing a dilution series of each element in double distilled water with 20% gelatine type A from porcine skin (SIGMA, G-1890), followed by stirring and heating the solutions in water bath at the temperature below 60°C till the solution became clear (without air bubbles). The drops were frozen into pellets in isopentane cooled in liquid nitrogen, cut with cryotome into 16 and 5 μm thick sections. The sections were placed on a carbon holder, lyophilised, carbon coated and analyzed in conditions identical to sample analysis. The obtained net counts were plotted against concentration in mg kg^{-1} dry mass (Tylko et al., 2004). The obtained calibration curve was used to calculate concentrations of Na, Mg, P, S, Cl, K, Ca, Mn, Cu, Zn and Pb in mycelium (Table 5).

Statistical analysis

The effects of experimental treatments on the measured plant and fungal parameters were analyzed using one- or two-way analysis of variance (ANOVA). Proportional values (mycorrhizal colonisation and NADH-diaphorase activity) were arcsine transformed prior to ANOVA. Comparisons between means were carried out using Duncan's Multiple Range test at a significance level of $P<0.05$. EDS measurements were evaluated using a non-parametric U-Mann Whitney test.

3. Results

Plant growth

AM inoculation did not influence maize growth in Mn-polluted soil. In Pb-polluted soil, AM fungi did not affect shoot biomass, but plants inoculated with either lineage of PH5 isolate had higher root weights as compared with non-inoculated plants. Significant differences in shoot and root weights were noted between plants cultivated on both polluted soils and sand, with plants grown in sand showing lower dry weights. In both sand treatments, plants inoculated with the reference isolate displayed lower shoot dry weights than plants in other inoculation treatments (Tables 2 and 3).

AM development

Similar frequency of AM colonisation (F%) was observed in the roots of plants inoculated with PH5-inert vs. PH5-Pb lineages when cultivated in Pb-polluted soil and sand (over 90 % of root length colonised). Lower frequency of colonisation was observed in plants inoculated with the reference strain BEG75, with 81% in sand and only 18% in Pb-polluted soil (Fig. 1). A similar pattern was observed for other mycorrhizal parameters – M, A and V. In the Mn-polluted soil, there were significant differences in frequency of colonisation in different inoculation treatments: significantly higher values were noted for the BEG140-Mn lineage as compared with BEG140-inert lineage and BEG75 isolate. In sand cultures, no differences in any colonisation parameters were observed between the strains/lineages (Fig. 2).

In spite of the differences between inoculation treatments and substrates in the frequency of mycorrhiza in the root system (F%), no significant differences were observed in the intensity of mycorrhizal colonisation (m%) and arbuscule abundance (a%) in mycorrhizal parts of root fragments (Figs. 1 and 2). In contrast, lower vesicle abundance, which was found in both contaminated soils for the reference BEG75 isolate and in the Mn-contaminated soil also for BEG140-inert lineage, was observed in the whole root system (V%) as well as in mycorrhizal parts of root fragments (v%). Poorer development of the reference BEG75 isolate and BEG140-inert lineage in contaminated soils was evident also from their lower ERM lengths and percentage of NADH-diaphorase-active hyphae (Tables 2 and 3). The average percentage of hyphae showing the NADH-diphorase enzymatic activity was more than three times lower in HM-contaminated soils than in sand.

Metals and phosphorus concentrations in plant biomass

Inoculation did not influence the concentrations of Pb in maize shoots, whereas Pb concentrations in the roots were significantly higher in the plants inoculated with PH5-Pb lineage as compared with non-inoculated plants (Table 4a). Inoculated plants also displayed significantly higher P concentrations in the roots, but not in shoots. Interestingly, the reference isolate was able to maintain a comparably high P concentration in the Pb-contaminated soil, in spite of much lower root colonisation. In the Mn-polluted soil, significantly higher Mn concentrations were found in the shoots of BEG140-Mn inoculated maize plants when compared with non-inoculated ones (Table 4b). Plants inoculated with BEG140-Mn also exhibited significantly higher P concentrations in the shoots than plants in other inoculation treatments. Non-inoculated plants showed significantly lower concentrations of root P than the plants inoculated with *G. intraradices* BEG140-Mn or BEG75 isolate.

ERM elemental concentrations

According to EDS measurements (Table 5, Fig. 4), most element concentrations were below 500 mg kg^{-1}, except for Mn. Large within-sample variation was observed and point analysis done for the same hyphae, but in different locations (even in close vicinity), often differed strongly. In both polluted soils, no statistically significant differences

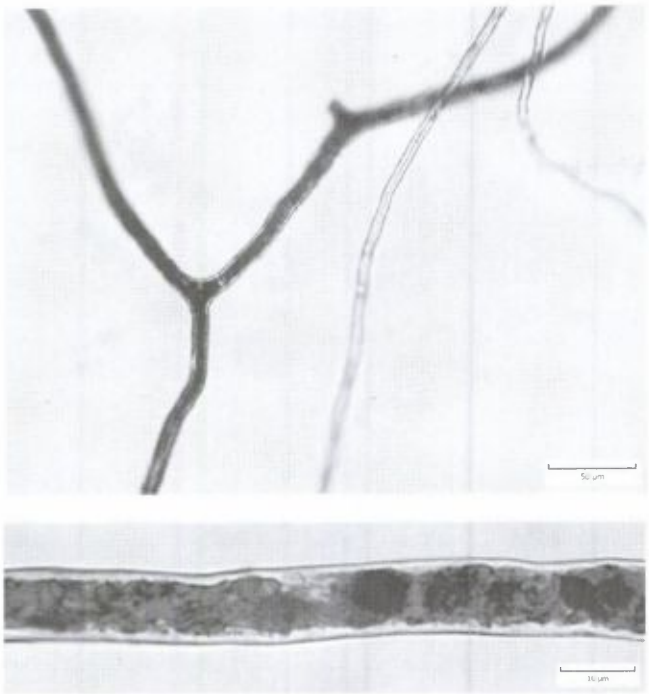


Figure 3. Extraradical hyphae of *G. intraradices* stained for NADH-diaphorase activity (viable hyphae filled with precipitate, dead hyphae empty).

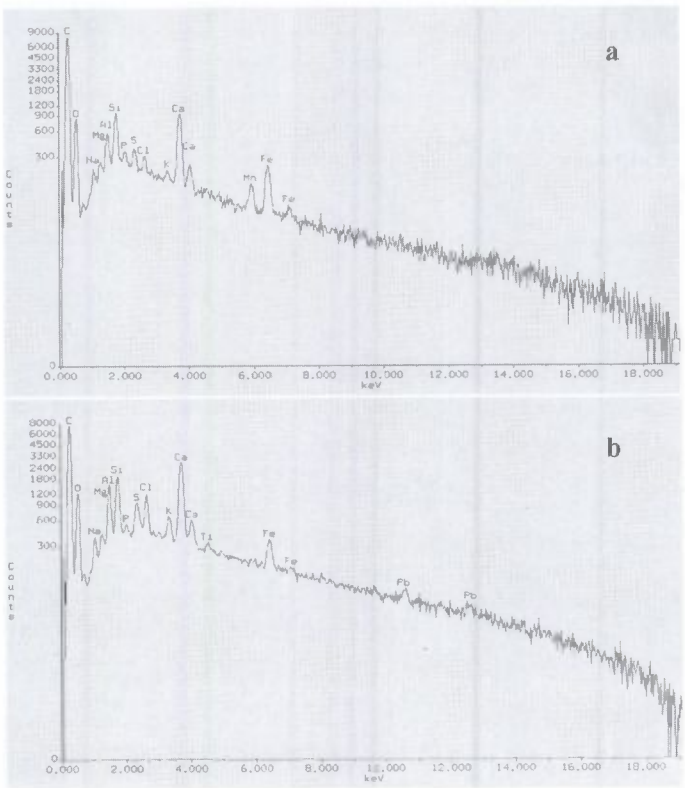


Figure 4. X-ray spectra of extraradical mycelium cultivated in Mn-contaminated (a) and Pb-contaminated soil (b) obtained after EDS analysis in scanning electron microscope at 20 keV of accelerating voltage and 100 s of exposure time.

were observed between the lineages, both in the case of BEG140 and PH5 isolates (except for a higher Mn content in mycelium of PH5-inert). In Pb-polluted soil, few statistically significant differences were found between the isolates BEG75 and PH5 (in P, Cl, Ca and Cu concentrations; Table 5). In comparison with plant material in the present experiment (evaluated with AAS), the contents of Pb and P in mycelium were lower, while mycelium usually contained several times more Mn than the plant material cultivated on Mn-rich soil.

4. Discussion

The present study conducted in HM-polluted soils confirmed the results from our previous experiments with simulated HM stress, i.e., that i) isolates from polluted soils tolerate higher HM concentrations than reference ones and that ii) HM-tolerance of indigenous AM fungi can be lost when maintained long term without HM stress. Concerning differences among AM isolates native or non-native to polluted soils, reference isolate BEG75 developed significantly less vigorously when grown in contaminated soils whereas indigenous isolates that had been maintained in original soils were able to form root colonisation and keep high ERM activity under HM contamination. The poorer development of BEG75 could not be considered as an isolate-specific feature, as colonisation levels of the reference BEG75 isolate were much higher in sand (about 80%) than in contaminated soils (18% and 28% in the Pb-polluted and Mn-polluted soil, respectively) while no pronounced differences between indigenous and reference isolates were observed in sand. It is difficult to judge which soil factor had a decisive suppressive effect on AM development in polluted soils. However, as comparable negative effects on AM colonisation as well as similar differences between the same AM isolates were previously observed due to artificial Pb or Mn application (Malcová et al., 2003a,b), it can be assumed that depression of AM colonisation in polluted soils is, at least partly, caused by HMs. The weak colonisation of BEG75 in HM-contaminated soils cannot be explained only by their lower pH (BEG75 originates from uncontaminated soil with pH of 8.2; http://www.kent.ac.uk/bio/beg/englishhomepage.htm), because high colonisation levels were observed for this isolate also in sand with pH of 6.7.

Not only the reference isolate BEG75, but also the lineage of indigenous BEG140 isolate from Mn-contaminated soil maintained in inert substrate developed weakly in the Mn-polluted soil when compared with the lineage of the same isolate maintained long term in the original soil. The level of root colonisation observed for BEG140-inert lineage was as low as for the reference isolate BEG75 and was more than three times lower than the colonisation by Mn lineage.

Table 4. The effect of different AM inoculation (PH5 = *Glomus intraradices* PH5, BEG140 = *G. intraradices* BEG140, IS = lineage maintained long term in inert substrate, Mn/Pb = lineages maintained long term in original contaminated soils, BEG75 = *G. intraradices* BEG75) on phosphorus and lead (a) or manganese (b) concentrations in the shoot and root biomass of maize plants grown in polluted soils. Data are means (±SE) of 5 replicates. Means marked with the same letters are not significantly different at the level $P<0.05$ within one substrate according to Duncan's Multiple Range test.

AM inoculation	Shoots	Roots	Shoots	Roots
a)	Pb concentration (mg kg^{-1})		P concentration (mg kg^{-1})	
Non-inoculated	52 ± 3 a	484 ± 31 b	307 ± 16 a	438 ± 17 b
PH5-inert	76 ± 11 a	617 ± 35 ab	415 ± 28 a	657 ± 36 ab
PH5-Pb	56 ± 5 a	718 ± 95 a	401 ± 30 a	778 ± 61 a
BEG75	58 ± 9 a	653 ± 80 ab	341 ± 53 a	801 ± 158 a
b)	Mn concentration (mg kg^{-1})		P concentration (mg kg^{-1})	
Non-inoculated	193 ± 23 b	548 ± 62 a	699 ± 58 b	1,250 ± 43 c
BEG140-inert	277 ± 42 ab	918 ± 219 a	871 ± 81 b	1,301 ± 23 bc
BEG140-Mn	308 ± 25 a	800 ± 165 a	1,539 ± 256 a	1,588 ± 58 a
BEG75	250 ± 27 ab	466 ± 44 a	962 ± 70 b	1,468 ± 123 ab

Table 5. Element concentrations in ERM of different AM isolates grown in the Pb- or Mn-polluted soil (BEG140 = *Glomus intraradices* BEG140, PH5= *G. intraradices* PH5, BEG75 = reference isolate *G. intraradices* BEG75, inert = lineage maintained long term in inert substrate, Pb/Mn = lineages maintained long-term in the original contaminated soils). Means (n=3) marked by different letters are significantly different at $P<0.05$ within one substrate.

Soil / AM isolate	Na	Mg	P	Cl	K	S	Ca	Mn	Cu	Zn	Pb
						(mg kg^{-1})					
Pb-polluted											
PH5-inert	61 a	101 a	84 ab	144 ab	73 a	48 a	136 a	1,740 a	41 a	22 a	76 a
PH5-Pb	74 a	104 a	117 a	195 a	109 a	65 a	105 a	885 b	36 ab	29 a	120 a
BEG75	43 a	58 b	56 b	108 b	50 a	34 a	58 b	892 b	22 b	10 a	81 a
Mn-polluted											
BEG140-inert	118 a	143 a	137 b	178 a	160 a	64 a	71 a	24,688 a	9 a	8 a	17 b
BEG140-Mn	43 a	85 a	103 b	59 a	66 a	53 a	69 a	18,862 a	5 a	6 a	18 ab
BEG75	74 a	90 a	163 a	81 a	87 a	65 a	54 a	28,257 a	15 a	13 a	22 a

In contrast, colonisation of maize roots by both lineages was at similarly high levels in sand. This difference between BEG140 lineages in tolerance to HM contamination is in accordance with the results observed under simulated Mn stress in quartz sand after 2-year cultivation of the isolate BEG140 in separate lineages (Malcová et al., 2003a). In contrast to the BEG140 isolate, no such differences between both lineages were observed for the PH5 isolate in Pb-contaminated soil. Contrasting results on persistence of HM-tolerance in PH5 and BEG140 isolates might indicate that diverse mechanisms conferring metal resistance are employed in fungal tolerance to Mn and Pb, with the more costly mechanism disappearing more rapidly under absence of selection pressure. Information on the persistence of HM tolerance in AM fungi is very scarce in the literature; Weissenhorn et al. (1994) mentioned a loss of higher HM tolerance after 6-month cultivation of Cd- and Zn-tolerant AM culture without HM stress. Recently, Kelly et al. (2005) reported considerable differences in Al tolerance of two strains of *Glomus clarum* isolated from acidic soils, with the tolerance decreasing with increased time under neutral culture conditions. The authors point out that assessments of response of AM fungi to specific edaphic stress requiring the function of specific tolerance genes may not be reliable if isolates are studied after long-term cultivation without any selection pressure.

Although total root colonisation was much lower for the reference isolate BEG75 and BEG140-inert lineage in polluted soils, no significant differences among AM isolates were found in the intensity of the mycorrhizal colonisation in the colonised root fragments (m%) and arbuscule abundance in mycorrhizal parts of root fragments (a%). Such patterns suggest that infection units develop similarly in tolerant and non-tolerant AM isolates once penetrating hyphae enter the roots. A low level of root colonisation of non-tolerant isolates observed in contaminated soils is then probably a result of a reduction in the number of spores germinating because germination

has been proved as more sensitive to HMs than hyphal growth (e.g. Weissenhorn et al., 1993). The only difference observed in the pattern of colonisation within mycorrhizal fragments between non-tolerant and tolerant AM isolates was a lower abundance of vesicles in non-tolerant isolates, which indicates a likely decreased reserve storage, which could be a potential disadvantage for long-term survival of the fungus. Alternatively, lower presence of vesicles could be caused simply by a slowing of the development of the symbiosis under adverse conditions as vesicles occur usually at later stages of symbiosis development.

Concerning plant and fungal tolerance to HM stress, a generally greater responsiveness of AM fungi observed in the present experiment is in concordance with the results obtained on the same host plant for Zn (Gildon and Tinker, 1983), Cd (Weissenhorn and Leyval, 1995) and higher doses of a mixture of micronutrients (Fe, Mn, Zn, Cu, B, Mo; Liu et al., 2000). Andrade et al. (2004) described a similar difference between response of AM fungi and soybean plants for Pb. They did not observe any effect of excessive Pb on soybean growth, although mycorrhizal colonisation and sporulation were greatly influenced. However, it is not possible to conclude that AM fungi are generally less HM tolerant than their hosts as opposed to Janoušková et al. (2005) who reported that two fungal symbionts (*G. intraradices* isolates PH5 and BEG75) showed higher Cd tolerance than tobacco plants when exposed up to 60 mg Cd kg^{-1} soil.

With regards to AM effect on HM uptake, AM colonisation did not act as a barrier against metal translocation from plant roots to the shoots as root-to-shoot ratios of HM concentrations were not significantly influenced by inoculation, despite higher Pb concentrations observed in the roots of mycorrhizal plants. A trend to higher Pb concentrations in the roots of inoculated plants might have been caused by Pb retention by fungal intra- and extraradical mycelium because high metal sorption capacity of ERM hyphae for HMs has been demonstrated (Joner et al., 2000). However, the hypothesis on Pb retention in ERM is not supported by higher Pb concentrations in plant material than ERM. On the other hand, very high Mn concentrations were found in ERM as compared to plant tissues. However, the presence of high levels of metals does not necessarily mean that they are toxic. What is important is the form and availability of the given element to the cell. A special ability to sequester metals is usually seen in or on the surface of cell walls of external tissues and the similar or even higher ability is observed in the fungal mycelium where special complexes are being formed to immobilize diverse elements (Turnau and Kottke, 2005). Nevertheless, direct comparison of data obtained with the classical AAS method used here to estimate metal content in plant material with the microanalytical EDS analysis carried out on mycelium should be avoided. This is because in the first case we obtain the mean value for the whole-plant sample, which is composed of tissues differing in ability to accumulate metals. The present results clearly show that the comparison between plant and fungus should be done using the same method. In our study, EDS measurements were conducted only within mycelia as the main aim was to compare HM accumulation in ERM of isolates of different origin or history of cultivation. Nevertheless, EDS did not prove differences in HM accumulation between lineages maintained on different substrates, which may be partly caused by the large variation of results between points located closely on the hyphae.

In conclusion, our results indicate that AM fungi originating from contaminated soils could lose their HM tolerance when sub-cultured for a long term in inert media without contamination. The tested isolates of *G. intraradices* from Mn- or Pb-contaminated soils showed higher HM resistance than the reference isolate of the same species, however this tolerance was stable only in the case of the isolate from Pb- but not from Mn-contaminated soil. Therefore, a desired HM tolerance can only be achieved when indigenous AM isolates would be maintained in inert media with simulated HM stress or optimally in original HM-contaminated soils.

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