APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2004, p. 6643–6649 Vol. 70, No. 11
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.11.6643–6649.2004

Heavy-Metal Stress and Developmental Patterns of Arbuscular Mycorrhizal Fungi

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Received 18 November 2003/Accepted 29 June 2004

The rate of global deposition of Cd, Pb, and Zn has decreased over the past few decades, but heavy metals already in the soil may be mobilized by local and global changes in soil conditions and exert toxic effects on soil microorganisms. We examined in vitro effects of Cd, Pb, and Zn on critical life stages in metal-sensitive ecotypes of arbuscular mycorrhizal (AM) fungi, including spore germination, presymbiotic hyphal extension, presymbiotic sporulation, symbiotic extraradical mycelium expansion, and symbiotic sporulation. Despite long-term culturing under the same low-metal conditions, two species, *Glomus etunicatum* and *Glomus intraradices*, had different levels of sensitivity to metal stress. *G. etunicatum* was more sensitive to all three metals than was *G. intraradices*. A unique response of increased presymbiotic hyphal extension occurred in *G. intraradices* exposed to Cd and Pb. Presymbiotic hyphae of *G. intraradices* formed presymbiotic spores, whose initiation was more affected by heavy metals than was presymbiotic hyphal extension. In *G. intraradices* grown in compartmentalized habitats with only a portion of the extraradical mycelium exposed to metal stress, inhibitory effects of elevated metal concentrations on symbiotic mycelial expansion and symbiotic sporulation were limited to the metal-enriched compartment. Symbiotic sporulation was more sensitive to metal exposure than symbiotic mycelium expansion. Patterns exhibited by *G. intraradices* spore germination, presymbiotic hyphal extension, symbiotic extraradical mycelium expansion, and sporulation under elevated metal concentrations suggest that AM fungi may be able to survive in heavy metal-contaminated environments by using a metal avoidance strategy.

Mining and smelting of metalliferous ores combined with combustion of fossil fuels have dramatically increased the global deposition of Cd, Pb, and Zn over the past two centuries (10). This trend has been somewhat mitigated in the past few decades by the use of unleaded gasoline (10), but industrial inputs and the agronomic application of fertilizers, pesticides, and metal-contaminated sewage continue to contribute to metal accumulation in the soil (20). The immediate toxicity of soil metals to soil organisms is moderated by metal immobilization by soil colloidal components. However, metal ions may be mobilized by changes in physical and chemical conditions of the soil environment, including pH decrease, change in redox potential, and enhanced decomposition of organic matter, posing a considerable challenge to heavy metal-sensitive soil biota (19, 21).

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are one of the most prominent soil microorganisms (37). They expand the interface between plants and the soil environment and contribute to plant uptake of macronutrients P (29) and N (1) as well as micronutrients Cu (29) and Zn (8). AM fungi are also involved in plant interactions with soil toxic metals, either by alleviating metal toxicity to the host or by accentuating it (for reviews, see references 9, 28, 32, and 34). The specific role of arbuscular mycorrhizae in the host exposure to metal stress and in the progression of the host stress response depends on a variety of factors, including the plant species (12) and ecotype (17); the fungal species (11) and ecotype (30); the metal (18) and its availability (15); soil edaphic conditions, including soil fertility (26); and plant growth conditions, such as light intensity (44) or root density (24). Despite the significant role that AM fungi play in plant interactions with soil toxic metals and the ubiquity of AM fungi in the soil environment, only recently has progress been made towards understanding the cellular mechanisms utilized by AM fungi to metabolize heavy metals and alleviate their cytotoxicity. That research implicates metallothionein-like polypeptides in Cd and Cu detoxification in AM fungal cells, since these polypeptides bind and sequester the toxic metals (27).

The obligately biotrophic life history of AM fungi can be divided into (i) the presymbiotic phase of spore germination and presymbiotic hyphal extension, and (ii) the symbiotic phase of hyphal proliferation inside host roots and mycelium expansion outside host roots (symbiotic extraradical mycelium expansion), which is accompanied by spore formation. The effect of heavy metal exposure on presymbiotic functioning of AM fungi can be quantified easily and can potentially be used to predict the functioning of AM symbiosis under metal stress (39), so considerable attention has been devoted to the impact of elevated metal concentrations on spore germination. Spore germination is highly sensitive to metal stress in AM fungi derived from low-metal habitats, while ecotypes from metal-enriched environments have higher levels of metal tolerance (22). A large body of conflicting data is available on toxic metal effects on the symbiotic functioning of arbuscular mycorrhizae, but little is known about the direct (not mediated by the host) effects of heavy metals on the expansion of AM fungal extraradical mycelium and sporulation. The high sorption capacity of AM fungal extraradical mycelium for Cd, Zn (23), and Cu (16) indicates that the extraradical mycelium may significantly affect plant interactions with the metal ions in the soil environment.

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The objectives of the present study were (i) to assess the effects of increased availability of Cd, Pb, and Zn on the presymbiotic and the symbiotic phase of the life cycle in AM fungi collected from low-metal environments; (ii) to determine if AM fungi from low-metal environments possess intrinsic abilities to tolerate (i.e., to be insensitive to) elevated metal concentrations and/or to avoid exposure to metal stress by increasing hyphal expansion range or hyphal proliferation in low-metal pockets of the soil environment; and (iii) to predict whether these fungi are likely to survive and grow at sites with high levels of heavy metals. Our results support the hypotheses that AM fungi are differentially sensitive to heavy metals and that the metal avoidance growth patterns exhibited by some AM fungi may help them survive at heavy metal-polluted sites.

MATERIALS AND METHODS

Fungal material. Monoxenic cultures of *Glomus etunicatum* Becker & Gerdemann, from St. Paul, Minn., were developed as described by Pawlowska et al. (35). *Glomus intraradices* Schenck & Smith, the Pont-Rouge isolate (DAOM 181602), was provided by J. A. Fortin (38). Fungal cultures were maintained in association with excised Ri T-DNA-transformed carrot roots (DC1 clone developed by G. Bécard) grown in a modified minimal White's medium, M (6). The M medium was solidified with 0.3% gellan gum (Phytagel; Sigma Chemical Co., St. Louis, Mo.) and buffered with 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] at a pH adjusted to 6.0 prior to autoclaving. The cultures were inverted and incubated in the dark at 30 ± 2°C.

The two-compartment culture system. Two-compartment 100- by-15-mm petri plates were prepared in a manner similar to that described by St.-Arnaud et al. (38). One compartment, designated the root compartment (RC), contained unamended M medium (10 mM MES [pH 6.0] and 0.3% gellan gum), whereas the other compartment, designated the hyphal compartment (HC), contained the M medium amended with $Cd(SO_4)_2$, $(C_2H_3O_2)_2Pb$, or $Zn(SO_4)_2$ at the following metal concentrations: 0.001, 0.01, 0.1, or 1.0 mM for Cd; 0.01, 0.1, or 1.0 mM for Pb; or 0.1, 1.0, or 10 mM for Zn. The control HC treatment contained 0.01 mM Zn but no Cd or Pb. Solutions of Cd, Pb, and Zn salts were filter sterilized and added to the M medium after autoclaving. Metal amendments did not affect the pH of the M medium, which was buffered at pH 6.0 with 10 mM MES. The medium layer was about 10 mm thick in both compartments. Two 5-cm-long fragments of Ri T-DNA-transformed carrot roots were placed in the RC. After 3 weeks, the RCs were inoculated with *G. etunicatum* or *G. intraradices*. The *G. etunicatum* inoculum consisted of a 1-cm^3 plug of culture medium containing axenic spores and colonized root segments, whereas the *G. intraradices* inoculum consisted of five spore clusters comprised of approximately 10 spores each. Three replicates of *G. etunicatum* cultures and eight replicates of *G. intraradices* cultures were prepared for each metal treatment. Plates were incubated for 8 weeks in the dark at 30 ± 2°C and monitored to remove roots straying from the RC into the HC.

Effects of heavy metal stress on extraradical mycelium and sporulation. The density of the extraradical mycelium was used to estimate the symbiotic extraradical mycelium expansion, and spore density was used to estimate symbiotic sporulation. Both hyphal and spore densities were assessed in the metal-containing HC and in the RC, by using a dissecting microscope at ×10 magnification (38). The density of the extraradical mycelium was expressed as the number of hyphae per square centimeter and was estimated by counting the number of hyphae crossing an imaginary plane delimited by a line on a reticule eyepiece across the depth of the medium (1 cm) in three equidistant predetermined positions along an imaginary line parallel to the plate divider, at a 2-cm distance from it. To assess the number of resting spores per cubic centimeter (spore density), spores were counted at the same positions as hyphae across the entire depth of the medium within an imaginary square delimited by a square on a reticule eyepiece. Nested analysis of variance (ANOVA) followed by the Tukey procedure for mean separation was performed separately for the RC and HC to assess the effects of metal amendments on the hyphal and spore densities. To evaluate associations between hyphal and spore densities, Pearson's product-moment correlation coefficients were calculated separately for each level of metal treatment. Hyphal densities in RCs and metal concentrations in HCs were evaluated as predictors for hyphal densities in the HCs by using multiple regression analysis.

A pilot experiment was performed to test possible effects exerted on the AM fungi by elevated concentrations of sulfate and acetate ions that accompany heavy metal ions in the medium. Sulfate ions were incorporated into the M medium as 10 mM $MgSO_4$. The source of acetate ions was 1 mM magnesium acetate. Three replicates of *G. intraradices* cultures were set up for each treatment. We did not detect any effects of elevated sulfate or acetate ions on extraradical mycelium expansion or sporulation.

Effects of heavy metal stress on spore germination and presymbiotic hyphal extension. To test the effects of various metal concentrations on spore germination and presymbiotic hyphal extension, the M media were amended with heavy metals. *G. etunicatum* and *G. intraradices* spores were extracted from the culturing media by solubilization of the media (14). Chunks of medium, approximately 1 cm^3, were suspended in 10 times their volume of 10 mM sodium citrate buffer (pH 6.0) in 50-ml tubes and shaken for 30 min at room temperature. After the spores were recovered from the liquefied medium, they were washed for 30 min in sterile deionized water and plated on the metal-amended M media. *G. intraradices* spores are formed in clusters, so great care was taken to separate individual spores for plating. Each treatment of 50 spores was replicated three times for *G. etunicatum* and six times for *G. intraradices*. Plates were incubated for 8 weeks in the dark at 30 ± 2°C. Spore germination was assessed with a dissecting microscope, and metal effects were estimated by using ANOVA followed by the Tukey procedure on the data that were arcsine transformed prior to the analysis. Presymbiotic hyphal extension was estimated by measuring the length of the longest hypha extending from a germinated spore with a dissecting microscope and an ocular micrometer. Presymbiotic spores formed by hyphae extending from germinated spores were counted. ANOVA, followed by the Tukey procedure, was performed on the mean presymbiotic hyphal extension and the mean number of presymbiotic spores per parent spore. The presymbiotic hyphal extension data were transformed by using square root transformation for *G. etunicatum* and $\log(1 + n)$ transformation for *G. intraradices* prior to the analysis. The association between the mean presymbiotic hyphal extension and the mean number of presymbiotic spores per parent spore was tested by computing the Pearson's product-moment correlation coefficient.

We also tested whether spores that failed to germinate on media containing one of the metals could germinate if the spores were transferred to a metal-free medium. Ungerminated spores were transferred to a control medium, and their germination was examined after 8 weeks. Three replicates of at least 25 spores were created for each set of spores inhibited by a specific metal at a given concentration.

G. intraradices spores formed in source media enriched with 0.1 mM Cd, 0.1 mM Pb, and 0.1 mM Zn were tested for their ability to germinate in a target medium amended with the same metal as in the source medium. Metal salts were incorporated into the target media at a 1.0 mM concentration of Cd or Pb or a 10 mM concentration of Zn, as described above. Six replicates of 50 spores were prepared for each spore source, including the control medium. Two-way ANOVA was performed separately for each metal treatment on the arcsine-transformed proportions of germinated spores with the source medium and target medium as independent variables.

Comparison of metal sensitivity of AM fungi at various life stages. Simple linear regression was used to make predictions about metal concentrations that decrease spore germination in *G. etunicatum* as well as spore germination, presymbiotic sporulation, symbiotic extraradical mycelial expansion, and symbiotic sporulation in *G. intraradices*. Based on the empirical data, we estimated metal concentrations that would cause a 50% decrease of a particular growth parameter in comparison to the control treatment.

RESULTS

Symbiotic extraradical mycelium expansion and symbiotic sporulation. To assess direct fungal responses to the metal stress, we used a compartmentalized culture system which restricted mycorrhizal host roots to the RC and permitted the expansion of AM fungal extraradical mycelium into the metal-enriched HC. In *G. intraradices*, the extraradical mycelium spread into the HC compartment. In contrast, the hyphae of *G. etunicatum* did not grow into the HC despite profuse extraradical mycelium development and sporulation in the RC.

Extraradical mycelium expansion and sporulation by *G. intraradices* in the RC were strongly correlated to each other, and they were not affected by the incorporation of heavy metal amendments in the HC (Fig. 1). Increasing metal concentra-

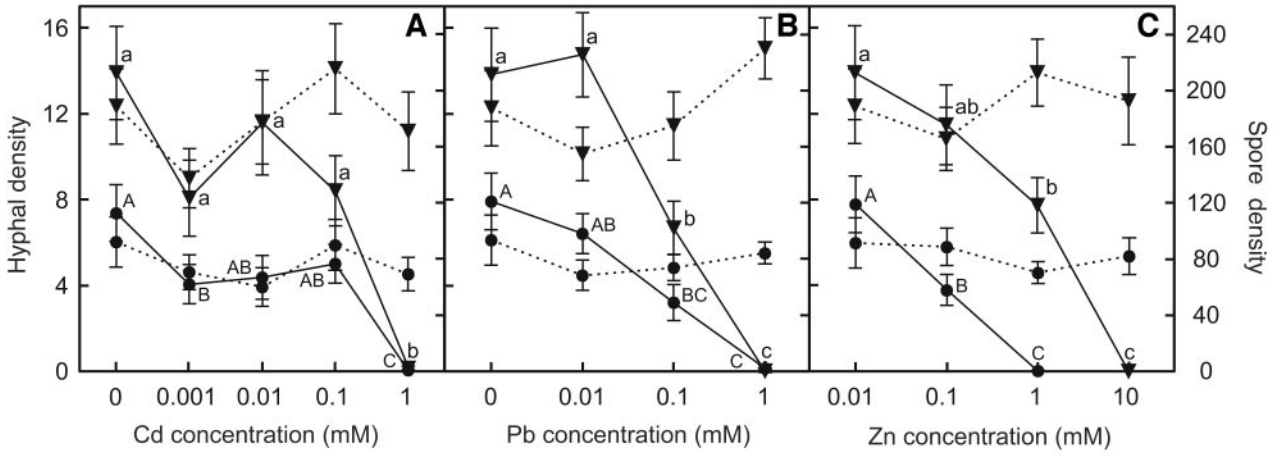

FIG. 1. Effects of Cd (A), Pb (B), and Zn (C) on hyphal density (triangles) and spore density (circles) of *G. intraradices* in the RCs (dashed lines) and the HCs (solid lines) of the bicompartmental plates. Values are means ± standard errors of the means (SEM) ($n = 24$). There were no significant effects of any of the metals on hyphal and spore densities in the RC. Hyphal and spore density values indicated by different letters (uppercase letters for the RC; lowercase letters for the HC) are different (ANOVA followed by the Tukey procedure for mean separation at $\alpha = 0.05$).

tions in the HC resulted in decreasing hyphal and spore densities in the HC (Fig. 1). Hyphal and spore densities in the HC were correlated with each other in most treatments. The only exception was the 1.0 mM Zn concentration, which inhibited sporulation in the HC but had only a moderate effect on hyphal density in the HC (Fig. 1C). In 1.0 mM Cd, 1.0 mM Pb, and 10 mM Zn, the absence of spore formation was accompanied by a far more severe inhibition of hyphal expansion.

The mycelium in the HC was an extension of the mycelium in the RC, so we expected that some of the variation in hyphal density in the HC compartment could be explained by the variation in hyphal density in the RC. Two variables, hyphal density in the RC (HD-RC) and metal concentration in the HC (metal-HC, where the metal is specified in the equations below), explained 39, 38, and 45% of the variation in hyphal density in the HC amended with the increasing concentrations of Cd, Pb, and Zn, respectively (coefficient of multiple determination $R^2_{\text{HD-RC,Cd-HC}} = 0.3941$, $F_{[2,117]} = 38.050$, $P = 0.0001$; $R^2_{\text{HD-RC,Pb-HC}} = 0.3816$, $F_{[2,93]} = 28.693$, $P = 0.0001$; $R^2_{\text{HD-RC,Zn-HC}} = 0.4499$, $F_{[2,87]} = 35.572$, $P = 0.0001$). Cd, Pb, and Zn effects alone accounted for 45, 79, and 71% of the explained variation in hyphal density in the HC, respectively (partial coefficient of determination $r^2_{\text{Cd}} = 0.1773$, $P = 0.0001$; $r^2_{\text{Pb}} = 0.3031$, $P = 0.0001$; $r^2_{\text{Zn}} = 0.3174$, $P = 0.0001$).

Spore germination. Spores of monoxenically grown *G. etunicatum* and *G. intraradices* differed noticeably in their sensitivities to the Cd, Pb, and Zn exposures (Fig. 2; Tables 1 and 2). For example, *G. etunicatum* spores did not germinate at a 1.0 mM concentration of Cd or a 10 mM concentration of Zn, but spores of *G. intraradices* germinated under both of these conditions, at rates of 70 and 60%, respectively. The two species also differed dramatically in germination rates of spores transferred to the control medium following incubation on media supplemented with high levels of a metal (Table 1). *G. intraradices* spores did not seem to suffer any latent effects of the prior exposure to the inhibitor, but only a small

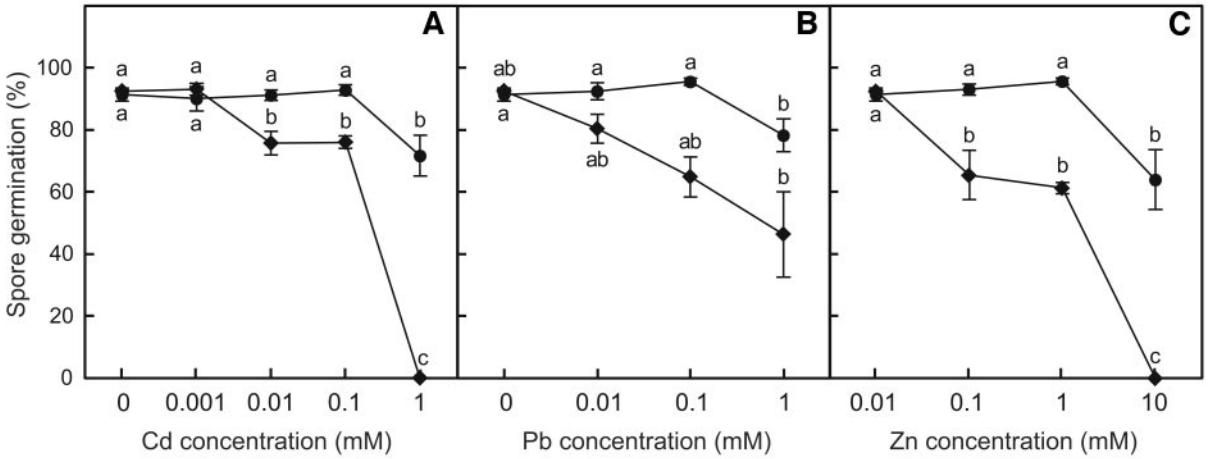

FIG. 2. Effects of Cd (A), Pb (B), and Zn (C) on spore germination for *G. etunicatum* (diamonds) and *G. intraradices* (circles). Values are means ± SEM ($n = 3$ for *G. etunicatum*; $n = 6$ for *G. intraradices*). Values indicated by different letters within a species are different (ANOVA followed by the Tukey procedure at $\alpha = 0.05$).

TABLE 1. Germination rates of spores of *G. etunicatum* and *G. intraradices* that had failed to germinate at elevated metal concentrations and were subsequently transferred to the control medium

Metal (concentration [mM])[a]	Spore germination rates (%) after transplanting indicated organism to the control medium[b]	
	G. etunicatum	*G. intraradices*
Cd (1.0)	2.4 ± 2.4	99 ± 1.2
Pb (1.0)	0	97 ± 1.3
Zn (1.0)	6.5 ± 3.2	NA
Zn (10)	0	87 ± 4.3

[a] Initial conditions inhibiting spore germination.
[b] Values are means ± SEM (n = 3). NA, not available; *G. intraradices* spores were not inhibited by a 1.0 mM Zn concentration.

proportion of the *G. etunicatum* spores regained the ability to germinate.

G. intraradices spores formed in media enriched in 0.1 mM Cd, 0.1 mM Pb, or 0.1 mM Zn (source medium) were plated on a control medium and on a medium enriched with the metal present in the source medium. Elevated concentrations of heavy metal ions in the target medium had significant effects on spore germination for all three metals ($P_{Cd} = 0.0489$, $P_{Pb} = 0.0012$, $P_{Zn} = 0.0028$). Spores formed under Cd or Zn metal stress did not differ from the control spores in germination ability when germinated either on the control medium or on the metal-amended media. Spores from the 0.1 mM Pb medium germinated under Pb stress at a significantly lower rate than the spores derived from a Pb-free environment ($P = 0.01$).

Presymbiotic hyphal extension. In *G. etunicatum*, presymbiotic hyphal extension was not affected by Cd at concentrations of 0.01 or 0.1 mM, nor by Zn at concentrations of 0.1 or 1.0 mM, even though these media inhibited spore germination in this species (Fig. 2A and C; Fig. 3A and C). A similar reaction was observed for *G. intraradices* at a Zn concentration of 10 mM, which also negatively affected spore germination (Fig. 3C).

G. etunicatum and *G. intraradices* differed in their presymbiotic hyphal extension responses to increasing Cd and Pb concentrations (Fig. 3). In *G. etunicatum*, presymbiotic hyphal extension did not vary with metal concentration, except for the inhibition of hyphal elongation at a 1.0 mM concentration of Pb, a level at which spore germination was also inhibited. Presymbiotic hyphal extension in *G. intraradices* was stimulated by increasing concentrations of Cd and Pb (Fig. 3A and B), including concentrations that were partially inhibitory to spore germination (Fig. 2A and B). No increase in presymbiotic hyphal extension was observed with *G. intraradices* in response to the elevated Zn concentrations (Fig. 3C).

Presymbiotic sporulation. In *G. intraradices*, hyphae that emerged from germinating spores formed a next generation of presymbiotic (secondary) spores. These spores were noticeably smaller (<30 μm in diameter) than the typical primary symbiotic spores. Presymbiotic spore formation was not observed in *G. etunicatum*. Presymbiotic spores in *G. intraradices* were found at all metal concentrations tested except 1.0 mM Cd and 10 mM Zn (Fig. 3). The average number of presymbiotic spores per parent spore was negatively correlated with the average presymbiotic hyphal extension across the tested gradients of Cd and Pb ($r_{Cd} = -0.59$, $P = 0.0004$; $r_{Pb} = -0.603$, $P = 0.0014$) but weakly positively correlated under Zn exposure ($r_{Zn} = 0.427$, $P = 0.0364$).

Comparison of metal sensitivity at various fungal life stages. Estimates of metal concentrations that would cause a 50% decrease in parameters characterizing different developmental stages of *G. intraradices* indicated that spore germination was dramatically less affected by an increase in metal concentration than presymbiotic sporulation, extraradical mycelium expansion, and symbiotic sporulation (Table 2).

DISCUSSION

The ecotypes of *G. etunicatum* and *G. intraradices* selected for this study were derived from relatively unpolluted environments and maintained under the same low-metal conditions for several generations of in vitro cultivation. Yet they differed markedly in the responses exhibited by their germ tube growth patterns to elevated Cd and Pb concentrations. In *G. etunicatum*, the extent of presymbiotic hyphal extension was generally constant, but presymbiotic hyphal extension in *G. intraradices* increased with Cd and Pb concentrations, even at concentrations that partially inhibited spore germination. We have not found reports of increased hyphal extension as a response to excessive heavy metal concentrations in other fungi, except for presymbiotic hyphal extension in a metal-sensitive culture of a mix of *Glomus* species exposed to moderately elevated concentrations of Zn (43) and stimulation of presymbiotic hyphal extension through an increase in Al soil saturation in an Al-tolerant ecotype of *Gigaspora gigantea* (5). The Al concentration that stimulated hyphal elongation in *Gigaspora gigantea* also partially inhibited spore germination. Our results suggest

TABLE 2. Cd, Pb, and Zn concentrations expected to cause a 50% decrease in parameters characterizing different developmental stages in *G. etunicatum* and *G. intraradices*[a]

Species and metal	Life cycle stage	EC_{50} (mM)	Lower 95% prediction limit (mM)	Upper 95% prediction limit (mM)
G. etunicatum				
Cd	SG	0.6	0.5	0.7
Pb	SG	1.2	0.7	3.1
Zn	SG	4.5	2.7	6.5
G. intraradices				
Cd	SG	2.4	1.6	4.3
	PS	0.5	0.06	1.1
	SEME	0.4	0	1.2
	SS	0.3	0	1.2
Pb	SG	3.1	1.9	7.5
	PS	0.6	0.04	1.4
	SEME	0.4	0	1.1
	SS	0.3	0	1.2
Zn	SG	17	12	28
	PS	2.9	0	9.6
	SEME	4.6	0	12
	SS	0.6	0	12

[a] EC_{50}, 50% effective concentration (50% decrease from the control value); SG, spore germination; PS, presymbiotic sporulation; SEME, symbiotic extraradical mycelium expansion; SS, symbiotic sporulation.

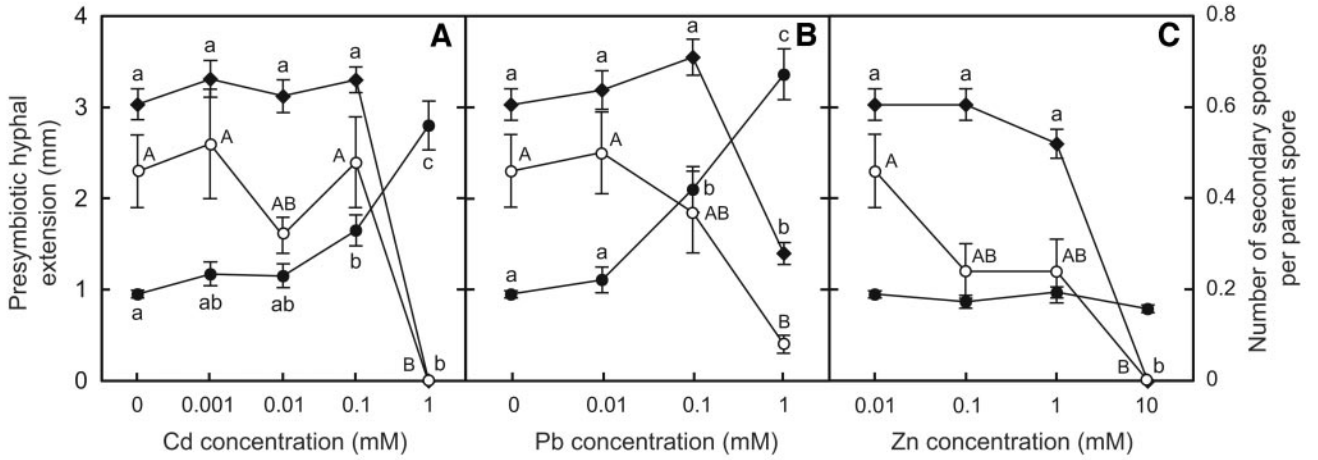

FIG. 3. Effects of Cd (A), Pb (B), and Zn (C) on the presymbiotic hyphal extension of *G. etunicatum* (solid diamonds; $n = 3$) and *G. intraradices* (filled circles; $n = 6$) and on the numbers of secondary spores per parent spore for *G. intraradices* (open circles; $n = 6$). Values are means ± SEM. Values indicated by different letters (uppercase letters for the secondary spores; lowercase letters for the hyphal extension) within a species are different (ANOVA and the Tukey procedure at $\alpha = 0.05$).

that the increase in germ tube length in response to environmental stress is a common response for AM fungi. This response parallels the metal avoidance strategy exhibited by plants and wood-rotting fungi at heavy metal-contaminated sites (41). For example, in *Filipendula ulmaria* from Cd-enriched meadow plots, where Cd contamination was most severe in the soil surface layers, the root growth inhibition in the surface layers was compensated for with more vigorous root growth in less-contaminated subsurface layers of the soil profile (4). Similarly, the ability to proliferate preferentially in uncontaminated zones of industrially contaminated soil was observed in roots of *Acer pseudoplatanus* seedlings (40). Tyler et al. (41) attributed the occurrence of wood-rotting fungi at metal-polluted sites to their ability to colonize plant substrates and thereby avoid exposure to the toxic metals in the soil at such sites. In AM fungi, increased hyphal elongation may be a means to escape local metal-enriched soil microenvironments and reach relatively less-contaminated pockets of the soil habitat.

Unlike Cd and Pb, Zn did not stimulate presymbiotic hyphal extension in *G. intraradices*. This difference may reflect an inhibitory effect of Zn on hyphal elongation similar to that observed with *Candida albicans*, where Zn stress inhibits hyphal growth without affecting spore germination (36).

Isolates of both *G. etunicatum* and *G. intraradices* had similar responses, in terms of spore germination and presymbiotic hyphal extension, to metal concentrations approximating those that occur naturally in low-metal environments. However, the two isolates differed dramatically when challenged by significantly elevated metal concentrations. Similar differences in metal sensitivities in closely related species derived from both unpolluted and contaminated environments are known for some fungi but not others (2, 25, 33). The lack of systematic differences in metal sensitivity suggests that the survival of some fungi in contaminated environments is a property of the organism's innate metal metabolism mechanisms rather than a specific environmental adaptation.

Germination rates of spores that had first failed to germinate at elevated Cd, Pb and Zn levels and were subsequently transferred to control media indicated that spores of *G. etunicatum* were more susceptible to metal-related irreversible damages, and that spores of *G. intraradices* were almost completely unaffected by the same metal concentrations. Hepper and Smith (22) reported a similar reactivation of the ability to germinate in *Glomus mosseae* spores that had failed to germinate under Zn and Mn stress. The preservation of germination ability despite a prolonged exposure to the inhibitory metal concentrations indicates that some AM fungi from generally low-metal environments may be able to survive temporary increases of metal ion concentrations and germinate when conditions in soil microhabitats become permissible. Such fungi also may be able to disperse into metal-polluted sites and germinate during ephemeral windows of favorable metal concentrations. However, spore germination is far less metal sensitive than other aspects of AM fungal life cycles, including symbiotic mycelial expansion or sporulation, so spore germination should be used with caution as a predictor of performance of a particular AM fungus under metal stress. Hyphal extension from colonized root fragments is probably a better predictor of performance (30, 31).

One of the most pronounced differences in developmental patterns between *G. etunicatum* and *G. intraradices* was the inability of *G. etunicatum* to form presymbiotic spores such as those produced by hyphae expanded from *G. intraradices* primary spores. A similar phenomenon of secondary spore formation has also been reported for other *Glomus* species (42). Secondary spores, as well as primary spores, allow spatial and temporal dispersal in instances when primary spore germination fails to result in an association with a host (7). Across the tested Cd and Pb concentrations, the average number of presymbiotic spores per parent spore was negatively correlated with the average presymbiotic hyphal extension, which indicates an altered resource allocation pattern under metal stress.

The limited ability of the *G. etunicatum* extraradical mycelium to expand from the host roots into the HC of the two-compartment culturing system is also an important difference between the two isolates. This and other disparities illustrate that substantial differences in mycelial architecture may be

present not only among different genera of AM fungi (13) but also between phylogenetically close taxa.

The compartmentalized in vitro culturing system is a useful tool for studying the effects of heavy metal stress on symbiotic extraradical mycelium and sporulation of *G. intraradices* without exposing the host roots to elevated metal concentrations, thereby avoiding host-mediated effects. In *G. intraradices*, as in other fungi (3), mycelium expansion and sporulation are differentially affected by heavy metal exposure. For example, while a concentration of 1.0 mM Zn had only a moderate effect on symbiotic extraradical mycelium expansion in the HC, it inhibited symbiotic sporulation in this compartment.

The localization of the negative metal effects on the *G. intraradices* extraradical mycelium and sporulation to the HC, accompanied by the absence of such effects from the RC, indicates that metal-sensitive AM fungi may be able to survive and propagate in metal-polluted environments by thriving in relatively uncontaminated soil microsites. Mycorrhizal colonization of host roots that proliferate preferentially in less-contaminated portions of the soil profile (4, 40) may additionally facilitate completion of the fungal life cycle. Otherwise, spores formed under metal stress may exhibit an elevated metal sensitivity as do *G. intraradices* spores derived from the Pb-rich medium. This increase in metal sensitivity in spores from the metal-rich medium may indicate that the cellular system(s) responsible for metal buffering is saturated with metal ions, and subsequent exposure to elevated metal concentrations results in toxicity.

In conclusion, our study, even though it was performed with only two isolates, suggests that AM fungi from low-metal environments differ in metal sensitivities and that some of these fungi may survive metal stress by avoiding soil microhabitats with toxic metal ion concentrations. This ability may be of particular importance for fungi introduced during restoration practices into habitats with a history of heavy metal pollution.

ACKNOWLEDGMENTS

We thank Andre J. Fortin for the *G. intraradices* inoculum and Guillaume Bécard and David Douds for the excised Ri T-DNA-transformed carrot roots (DC1 clone).

This research was funded by a University of Minnesota Graduate School Doctoral Dissertation Fellowship, by Minnesota Department of Transportation contract 72630 TOC 163, and by the Minnesota Legislature [ML 1995, chapter 220, section 19, subdivision 13(C)] as recommended by the Legislative Commission on Minnesota Resources from the Minnesota Environment and Natural Resources Trust Fund.

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