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Effects of Metal Phytoextraction Practices on the Indigenous Community of Arbuscular Mycorrhizal Fungi at a Metal-Contaminated Landfill

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Phytoextraction involves use of plants to remove toxic metals from soil. We examined the effects of phytoextraction practices with three plant species (*Silene vulgaris*, *Thlaspi caerulescens*, and *Zea mays*) and a factorial variation of soil amendments (either an ammonium or nitrate source of nitrogen and the presence or absence of an elemental sulfur supplement) on arbuscular mycorrhizal (AM) fungi (Glomales, Zygomycetes) at a moderately metal-contaminated landfill located in St. Paul, Minn. Specifically, we tested whether the applied treatments affected the density of glomalean spores and AM root colonization in maize. Glomalean fungi from the landfill were grouped into two morphotypes characterized by either light-colored spores (LCS) or dark-colored spores (DCS). Dominant species of the LCS morphotype were *Glomus mosseae* and an unidentified *Glomus* sp., whereas the DCS morphotype was dominated by *Glomus constrictum*. The density of spores of the LCS morphotype from the phytoremediated area was lower than the density of these spores in the untreated landfill soil. Within the experimental area, spore density of the LCS morphotype in the rhizosphere of mycorrhizal maize was significantly higher than in rhizospheres of nonmycorrhizal *S. vulgaris* or *T. caerulescens*. Sulfur supplement increased vesicular root colonization in maize and exerted a negative effect on spore density in maize rhizosphere. We conclude that phytoextraction practices, e.g., the choice of plant species and soil amendments, may have a great impact on the quantity and species composition of glomalean propagules as well as on mycorrhiza functioning during long-term metal-remediation treatments.

Health hazards posed by the accumulation of toxic metals in the environment accompanied by the high cost of removal and replacement of metal-polluted soil have prompted efforts to develop phytoremediation strategies that would utilize plants to extract excessive soil metals. Several plant species or ecotypes associated with heavy-metal-enriched soils accumulate metals in the shoots (15). These plants can be used to clean up metal-contaminated sites by extracting metals from soil and concentrating them in aboveground biomass (9, 34). The metal-enriched biomass can be harvested using standard agricultural methods and smelted to recover the metal.

Metal phytoextraction is not as extreme as conventional metal removal methods but still involves considerable alterations to the environment, including elimination of the existing vegetation cover and the application of fertilizers and soil amendments, such as synthetic chelates or sulfur, to increase metal availability to plants (14, 19, 38). Achieving a desired reduction in soil metal concentration may require cultivation of metal-accumulating plants at the remediated site for several cycles that include harvest and removal of metal-enriched biomass (16). The decontaminated area may be subsequently revegetated with a more appropriate and/or desirable plant cover.

Despite the important role that rhizosphere microorganisms play in plant interactions with the soil environment in general, and toxic metals in particular, relatively few studies have focused on the effects of these microorganisms on the metal remediation efforts (28). Moreover, the effects of phytoremediation practices on microbial communities indigenous to the remediated sites have been largely ignored. Yet, these microbes may be crucial for revegetation efforts following the removal of excessive soil metals.

Among the rhizospheric organisms involved in plant interactions with the soil milieu, the arbuscular mycorrhizal (AM) fungi (Glomales, Zygomycetes) deserve special attention. About 95% of the world's plant species belong to characteristically mycorrhizal families and potentially benefit from AM fungus-mediated mineral nutrition (37). Consequently, glomalean fungi are believed to play a fundamental role in biogeochemical element cycling (20). Early phytoextraction studies have focused on metallophytes from predominantly nonmycorrhizal plant families, e.g., *Brassicaceae* or *Caryophyllaceae*, so AM have not been considered an important component of phytoremediation practices (4, 24). Recently, plants capable of forming an association with AM fungi, including maize, have been shown to accumulate considerable amounts of metal (2, 3, 14, 19, 42). Unfortunately, the role that glomalean fungi play in plant interactions with soil metals is not fully understood (26), and little is known about mycorrhiza functioning under conditions imposed by metal remediation protocols and about the effects of phytoextraction on AM fungi. The latter is of particular importance, as the rate of site revegetation after metal removal may depend on AM inoculum present in soil (33).

The objective of this study was to determine the effects of phytoremediation on naturally occurring AM fungi at a metal-contaminated site. Specifically, we tested whether the applied

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phytoremediation treatments affected the density of glomalean spores and AM root colonization in maize. The study was performed at the Revival Field experimental area at the Pig's Eye Landfill, St. Paul, Minn., one of the first field trials of the phytoextraction technology (8). We demonstrated that the choice of plant species and soil amendments affected the quantity and species composition of glomalean propagules after a long-term metal remediation treatment.

MATERIALS AND METHODS

Site description. The Pig's Eye Landfill in St. Paul, Minn., has been closed since 1972; the Revival Field experimental area was established in 1991 (8). Landfill soil was highly calcareous, with total metal concentrations of 37 mg of Cd, 317 mg of Cr, 264 mg of Cu, 173 mg of Pb, and 530 mg of Zn kg of soil^{-1}. The site had a dense vegetation of ruderal species. The study area was treated with Round-Up herbicide and fertilized with superphosphate at 110 kg per ha and KCl at 110 kg per ha.

The experimental design involved factorial variation in sulfur addition to alter soil pH (no sulfur supplement or elemental sulfur supplement at 550 kg per ha) and in the form of nitrogen fertilizer (ammonium sulfate or calcium nitrate, both applied at 110 kg of N per ha), with comparison of three plant species. The tested plants included two metallophytes, *Thalaspi caerulescens* J. C. Presl, and *Silene vulgaris* (Moench) Garcke, from predominantly nonmycorrhizal families *Brassicaceae* and *Caryophyllaceae*, respectively, as well as a Cd-accumulating inbred maize line, *Zea mays* L. FR37, which was expected to become mycorrhizal (5). The treatments were replicated in four blocks. Sulfur and nitrogen fertilizer supplements were set up in each block as randomized combinations. Plant species was incorporated within each sulfur-nitrogen combination in a split-plot design. After applying the sulfur and fertilizer treatments to the plots, the entire experimental area was rototilled to the depth of 15 to 20 cm. Plot size was 80 cm^2, and plots were separated by a 15-cm border. *T. caerulescens* was introduced to the experimental plots in the form of seedlings, and plants of *S. vulgaris* and *Z. mays* were seeded 15 cm apart, at a density of 16 plants per experimental plot. *T. caerulescens* and *S. vulgaris* were introduced to the experimental area in 1991; the maize was reseeded in 1992 and 1993. Weeds were removed as needed.

Sampling. In fall 1993, three to four replicates of soil samples from the rhizospheres of *S. vulgaris*, *T. caerulescens*, and *Z. mays* plants, as well as soil samples from outside the experimental area, were collected. Depths of sampling corresponded to plant rooting depth and were up to 10 cm for *T. caerulescens* plants and up to 15 cm for *S. vulgaris* and *Z. mays* plants and the outside area. Collected root balls were subdivided into two parts. One part was used as a source of roots to assess the presence and extent of AM root colonization. The remaining portions of the root balls were air dried and stored at 4°C for use as inocula for AM fungal pot cultures and spore isolation. Soil pH (in 10 mM $CaCl_2$, 1:2 [wt/vol]) was assessed in all samples from maize rhizosphere.

Glomalean species identification. We established pot cultures with two host species, maize and *Andropogon gerardii* Vit., that were inoculated with the landfill soil containing glomalean propagules (spores, hyphae, and root fragments). The inoculum was placed as a 2-cm layer at a depth of 4.5 cm in 30-cm pots filled with steam-sterilized greenhouse soil mix combined with sand in a 1:1 proportion. One-week-old seedlings of maize (3 per pot) or *A. gerardii* plants (30 per pot) were planted in each pot. Cultures of each host species were replicated five times. Additionally, five noninoculated cultures of each host species were established as controls. All pots were covered with aluminum foil to prevent contamination with airborne AM inoculum. Cultures were maintained under greenhouse conditions and drip-irrigated with deionized water as needed. After 16 weeks, plants and soil were air dried and glomalean spores were isolated by wet sieving and decanting, followed by sucrose centrifugation (10). Voucher specimens were deposited in the I. Charvat laboratory at the University of Minnesota.

Spore viability. We grouped spores into two morphotypes, characterized by either light-colored spores (LCS) or dark-colored spores (DCS), and assessed their viability using a method modified from that described by Hepper (17). Twenty-five spores were sandwiched between two cellulose nitrate filters (Sartorius, Göttingen, Germany) held together by a photographic slide mount. These germination units were replicated six times for the LCS morphotype and nine times for the DCS morphotype and buried at a depth of 2 cm in steam-sterilized soil-sand mix (1:1). Twenty 2-week-old *A. gerardii* seedlings were planted above each germination unit. *A. gerardii* is an obligate mycorrhizal host and was expected to increase spore germination (11). Pots were maintained in the greenhouse and watered with deionized water as needed. Germination units were recovered after 2 weeks of incubation in *A. gerardii* rhizosphere, rinsed, stained in 0.05% (wt/vol) trypan blue in acid glycerol (23), opened, and examined for spore germination.

Assessment of AM colonization in plants introduced to the landfill site. Root samples were rinsed in tap water and fixed in 50% ethanol for storage (23). After clearing at 90°C in 10% KOH (wt/vol) for 60 min, roots were acidified in 1% HCl for 1 to 2 h and stained in 0.1% chlorazol black E at 90°C for 30 to 40 min (6). AM root colonization was assessed using a compound microscope after roots were mounted in polyvinyl alcohol-lactic acid-glycerine (30). Mycorrhizal arbuscules or coils were taken as evidence of root colonization. Maize roots were analyzed in more detail to assess the effects of soil amendments on AM colonization, which was estimated in six subsamples from four root systems per treatment by using a magnified intersection method (29). In particular, the proportion of root length containing arbuscules (arbuscular colonization) and the proportion of root length containing vesicles (vesicular colonization) were assessed.

Quantification of glomalean spores. Spores were extracted from the soil samples as described earlier (10). The density of viable glomalean spores, expressed as the number of spores per gram of dry soil, was assessed visually using a dissecting microscope.

Statistical analyses. Germination rates of the field-collected glomalean spores and spore densities in phytoremediated and untreated landfill areas were compared using an unpaired *t* test. Plant species and soil amendment effects on spore density and soil amendment effects on soil pH and mycorrhizal parameters of maize rhizosphere were evaluated using analysis of variance (ANOVA). All effects were treated as fixed. To assess variance homogeneity, data were analyzed by the equality of variance *F* test. Data normality was evaluated using a normal probability plot, and the data were transformed [$\log(1 + x)$] when necessary. Associations among soil pH and mycorrhizal parameters of corn rhizosphere were assessed using Pearson's correlation coefficient. Analyses were performed using StatView version 4.5 (Abacus Concepts, Inc., Berkley, Calif.) and SAS version 6.1 (SAS Institute Inc., Cary, N.C.). All results are presented as the mean ± standard error unless indicated otherwise.

TABLE 1. ANOVA of spore densities of the LCS and DCS morphotypes from different plant species and soil amendment treatments[a]

Source of variation	*df*	LCS morphotype[b]		DCS morphotype	
		MS	*F*	*MS*	*F*
Block	3	0.12		359	
N source	1	0.01	0.17 (ns)	42	1.2 (ns)
S supplement	1	0.24	4.8 (ns)	10	0.31 (ns)
N source-S supplement	1	0.01	0.12 (ns)	105	3.1 (ns)
Error 1	9	0.05		34	
Species	2	2.55	54*	37	1.1 (ns)
N source-species	2	0.02	0.29 (ns)	2.9	0.09 (ns)
S supplement-species	2	0.02	0.44 (ns)	24	0.73 (ns)
N source-S supplement-species	2	0.03	0.71 (ns)	31	0.95 (ns)
Error 2	13	0.05		33	

[a] ANOVA degrees of freedom (*df*), mean squares (*MS*), and *F* values are given. *, $P \leq 0.001$; ns, not significant.
[b] Data were transformed [$\log(1 + x)$] prior to analysis.

RESULTS

Glomalean fungi at the landfill site. Based on the spore specimens from pot cultures with maize and *A. gerardii* as hosts, dominant species of the LCS morphotype were determined to be *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe and an unidentified *Glomus* sp. The DCS morphotype was identified as *Glomus constrictum* Trappe. Field-collected spores of the LCS morphotype germinated at a rate of 38% ± 12%. For the DCS morphotype, the germination rate was 23% ± 7.4%. The germination rates were not significantly different ($P = 0.2828$).

Effects of phytoremediation practices on AM fungi. AM root colonization was found exclusively in maize. Nevertheless, spore density of the DCS morphotype was not affected by the plant species or any of the examined factors (Table 1). The average spore density in this morphotype was 10.5 ± 1.3 spores per g of dry soil. In contrast, the LCS morphotype was influenced by the plant species (ANOVA, $P = 0.0001$; Table 1). The spore density of the LCS morphotype decreased from 13.3 ± 2.9 spores per g of dry soil in maize rhizosphere to 1.7 ± 0.6 and 1.0 ± 0.2 spores per g of dry soil in rhizospheres of *S. vulgaris* and *T. caerulescens* plants, respectively. The spore density of the LCS morphotype was not affected by soil amend-

TABLE 2. ANOVA of the soil pH, percent vesicular root colonization, and glomalean spore density in the maize rhizosphere[a]

Source of variation	*df*	pH		% Vesicular root colonization		Spore density (no. of spores/g of dry soil)	
		MS	*F*	*MS*	*F*	*MS*	*F*
Block	3	0.04		50		320	
N source	1	0.02	1.9 (ns)	22	0.59 (ns)	34	0.31 (ns)
S supplement	1	0.57	62**	300	8.2*	580	5.3*
N source-S supplement	1	0.00	0.15 (ns)	1.6	0.04 (ns)	8.5	0.08 (ns)
Error	9	0.01		36		110	

[a] ANOVA degrees of freedom (*df*), mean squares (*MS*), and *F* values are given. *, $P \leq 0.05$; **, $P \leq 0.001$; ns, not significant.

ments and no plant species-soil amendment interactions were detected.

Supplementing soil with sulfur led to a significant decrease in soil pH ($CaCl_2$), from 7.2 ± 0.03 to 6.8 ± 0.05 (Table 2). Yet, soil amendments did not influence arbuscular root colonization in maize, which reached 40% of the analyzed root length. On the other hand, the elemental sulfur supplement affected the proportion of root length containing vesicles (Table 2). Vesicular colonization in maize roots increased from 5.6% ± 1.3% in plots without the sulfur supplement to 12% ± 1.8% in plots amended with sulfur. Moreover, vesicular colonization and soil pH were negatively correlated (Fig. 1). Glomalean spore density in the maize rhizosphere increased in the absence of sulfur supplement (Table 2) and was positively correlated with soil pH (Fig. 2). We also detected a trend towards a negative correlation between vesicular root colonization and spore density in maize rhizosphere ($r = -0.453$, $P = 0.0787$).

The spore density of the LCS morphotype was 23 ± 11 spores per g of dry soil in the untreated landfill area. It was significantly higher than in the experimental area, where there were 6.6 ± 1.6 spores per g of dry soil ($P = 0.0116$). No significant difference was observed in the spore density of the DCS morphotype, which averaged 16 ± 4.6 and 11 ± 1.3 spores per g of dry soil in the untreated and experimental areas, respectively ($P = 0.1797$).

DISCUSSION

G. constrictum, *G. mosseae*, and *Glomus* sp. were dominant species in the metal-contaminated soil of the Pig's Eye Landfill. These indigenous species could colonize roots of maize, a potentially mycorrhizal host that was introduced to the site as a metal extraction instrument. As expected, no evidence of AM was found in the roots examined from plots containing *S. vulgaris* and *T. caerulescens* plants. Absence of AM colonization in roots of *S. vulgaris* plants is consistent with a previous report based on specimens collected from a calamine spoil mound in Poland (32).

Glomalean fungi propagate by spores, hyphae, and colonized root fragments and the relative importance of these different propagules varies depending on the environmental conditions (37). Only spores can be used for species identification with confidence. Based on germination ability of the field-collected spores, we confirmed that spores were an effective source of inoculum at the landfill site. Germination rates that we observed were comparable to those reported by Weissenhornn and Leyval (41) for *G. mosseae* organisms isolated from metal-contaminated soil and germinated under various edaphic conditions. We concluded that spore frequency can be used to quantify the effects of phytoextraction practices on the composition of the glomalean community.

Species of plant used for phytoextraction affected both spore frequency and species composition of AM fungi in the Revival Field experiment. Significantly higher spore density of the LCS morphotype was recorded from the rhizosphere of mycorrhizal maize than from rhizospheres of nonmycorrhizal *S. vulgaris* or *T. caerulescens* plants. This decline in the LCS morphotype inoculum might be attributable to the lack of a suitable host. Recovery of low spore numbers of the LCS morphotype from the rhizospheres of nonhost species may indicate that the land-

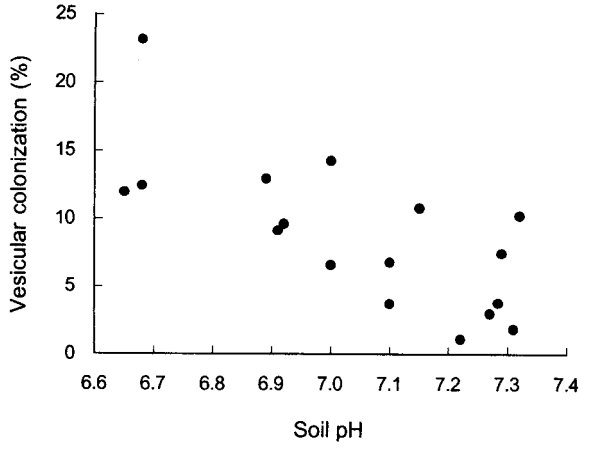

FIG. 1. Correlation between soil pH and vesicular root colonization in maize rhizosphere at the Revival Field experimental area ($r = -0.70$, $P = 0.0018$).

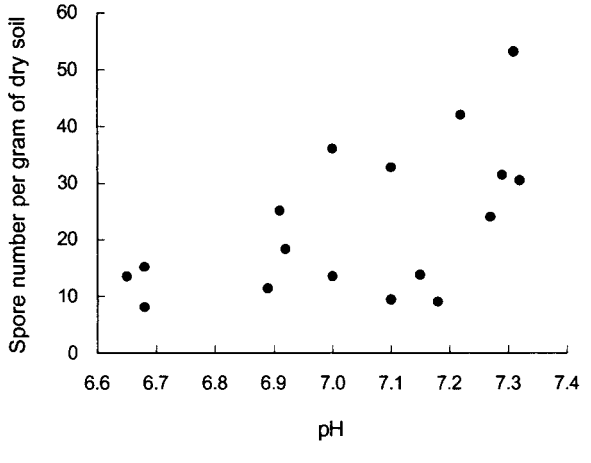

FIG. 2. Correlation between soil pH and density of glomalean spores in maize rhizosphere at the Revival Field experimental area ($r = 0.64$, $P = 0.0063$).

fill soil contained a spore bank that persisted in the absence of a suitable host. Also, spore dispersal from nearby mycorrhizal plots and surrounding landfill area cannot be excluded. In contrast to spores of the LCS morphotype, the host plant did not affect the spore density of the DCS morphotype. Furthermore, we did not detect any significant difference in the frequency of these spores between the untreated and the phytoremediated soil. The absence of the host effect on the spore density of the DCS morphotype might be explained by a preferential survival of the *G. constrictum* spores in comparison to the spores of the LCS morphotype in the absence of a suitable host. The similarity of spore germination rates observed for both morphotypes supports this hypothesis.

The LCS morphotype was not only affected by the plant species treatment within the Revival Field experiment but also decreased in spore frequency, when phytoremediated soil was compared to untreated landfill soil. Our data suggest that the plant species used for phytoextraction may have a great impact on propagule production and species composition of the glomalean community during long-term metal remediation treatments. Utilization of nonmycorrhizal metal-accumulating plant species, such as *T. caerulescens*, although superior for extraction of excessive soil metals (8), reduces the quantity of glomalean propagules. Since AM inoculum is known to accelerate the establishment of planted vegetation (25, 31, 36), glomalean propagule decline may necessitate reinoculation of the site with these fungi after completion of metal phytoextraction before the desired plant cover can be established.

As we demonstrated, reduction in glomalean inoculum may be avoided by including plants capable of mycorrhiza formation into the metal extraction protocols. Potentially mycorrhizal host species, including maize (19) and *Sonchus oleraceus* (42), can extract considerable amounts of lead from soil. Moreover, Ebbs and Kochian (14) reported that barley, which is also potentially mycorrhizal, can extract zinc as efficiently as can the nonmycorrhizal *Brassica juncea* aided by a synthetic chelate, EDTA, that mobilizes toxic elements but may also cause their leaching deeper into the soil profile. Unfortunately, these studies did not include evaluations of the mycorrhizal status of the roots and the effects of AM on metal accumulation in plants.

Although AM have been recovered from numerous metal-enriched habitats (13, 18, 32, 41), their role in plant interactions with toxic metals is not well understood (26). Glomalean hyphae may contribute directly to the uptake and translocation of metals to the host roots, including micronutrients such as Cu and Zn, as well as the toxic element Cd (7, 21, 27). AM fungi also may sequester toxic metals, thereby reducing their availability to the plant (22, 39). Such metal sequestration may alter metal translocation patterns in plants leading to metal accumulation in the mycorrhizal roots and reduced metal transfer to the aboveground biomass (12, 35). AM plants exhibiting this type of metal partitioning would be undesirable for phytoextraction, as metals would remain in unharvestable mycorrhizal biomass. In some species, however, including *Festuca arundinacea*, AM do not affect metal translocation patterns (35). Such species should therefore be targeted as tools for phytoextraction. Further research is needed to develop phytoextraction strategies that would balance the need for preservation of the rhizospheric organisms, including glomalean fungi, and facilitate metal uptake by the plant to the aboveground biomass.

Our data support the hypothesis that both the plant species and the soil amendments used in phytoextraction protocols may affect AM fungi and mycorrhiza functioning. We detected a negative correlation between vesicular colonization in maize roots and soil pH in maize rhizosphere, a positive correlation between spore density in maize rhizosphere and soil pH, and a trend towards a negative correlation between vesicular root colonization and spore density in maize rhizosphere. The observed changes indicated an alteration in the fungal resource allocation pattern in response to conditions imposed by soil amendments. They also confirm that AM fungi are sensitive to changes in soil pH, even within a relatively narrow pH range. A similar pH alteration in agricultural soils exposed to a long-term liming was reported to have no effect on the glomalean spore density and only a minor effect on percent AM root colonization in *Avena sativa* and *Solanum tuberosum* plants (40). However, as soil pH is a critical parameter affecting metal ion mobility, increased metal availability associated with the low soil pH may be detrimental to AM fungi. For example, Angle and Heckman (1) reported that a pH change from 6.2 to 5.6 in soil treated with metal-contaminated sewage sludge resulted in a dramatic decline of AM colonization in soybean roots. Attention, therefore, should be focused on the effects of pH-altering soil amendments on mycorrhiza-mediated metal uptake in AM plants used as phytoextraction instruments.

We examined the effects of phytoextraction practices with three plant species and a factorial variation of soil amendments on AM fungi at a moderately metal-contaminated landfill. Specifically, we tested whether the applied treatments affected the density of glomalean spores and AM root colonization in maize. The overall density of spores of the LCS morphotype from the phytoremediated area was lower than the density of these spores in the untreated landfill soil. Within the experimental area, the spore density of the LCS morphotype in the rhizosphere of AM maize was significantly higher than in rhizospheres of nonmycorrhizal *S. vulgaris* or *T. caerulescens* plants. Sulfur supplement increased vesicular root colonization in maize and reduced spore density in maize rhizosphere. We conclude that phytoextraction practices, e.g., the choice of plant species and soil amendments, may impact the quantity and species composition of glomalean propagules and the functioning of mycorrhiza during long-term metal-remediation treatments. Further studies are needed to assess the impact of AM fungi on metal uptake and accumulation in plants used for metal extraction.

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