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Diversity of Arbuscular Mycorrhizal Fungus Populations in Heavy-Metal-Contaminated Soils

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High concentrations of heavy metals have been shown to adversely affect the size, diversity, and activity of microbial populations in soil. The aim of this work was to determine how the diversity of arbuscular mycorrhizal (AM) fungi is affected by the addition of sewage-amended sludge containing heavy metals in a long-term experiment. Due to the reduced number of indigenous AM fungal (AMF) propagules in the experimental soils, several host plants with different life cycles were used to multiply indigenous fungi. Six AMF ecotypes were found in the experimental soils, showing consistent differences with regard to their tolerance to the presence of heavy metals. AMF ecotypes ranged from very sensitive to the presence of metals to relatively tolerant to high rates of heavy metals in soil. Total AMF spore numbers decreased with increasing amounts of heavy metals in the soil. However, species richness and diversity as measured by the Shannon-Wiener index increased in soils receiving intermediate rates of sludge contamination but decreased in soils receiving the highest rate of heavy-metal-contaminated sludge. Relative densities of most AMF species were also significantly influenced by soil treatments. Host plant species exerted a selective influence on AMF population size and diversity. We conclude based on the results of this study that size and diversity of AMF populations were modified in metal-polluted soils, even in those with metal concentrations that were below the upper limits accepted by the European Union for agricultural soils.

In recent years several studies have shown the harmful effects of metals on microbial diversity and activity in soil (8, 10, 28). The accumulation of metals in soils at high concentrations can be due to anthropogenic activities such as the application of sewage sludge. This practice has been widely used for nutrient recycling and is accepted for waste disposal in agricultural soils (32). However, the addition of sludge considerably increases the amount of heavy metals in soil, causing changes in soil properties which could be toxic for soil microorganisms (10). The primary chemical change in soil is acidification, which increases the availability of metal in the soil solution to toxic levels which can persist for extremely long periods of time. In spite of this, rates of 50 to 100 kg of dry matter per hectare per year are currently applied to agricultural soils. Thus, the contribution of sewage sludge to the overall input of heavy metals in soils is considerable. In this context, there is increasing concern about the possible side effects on microbial populations, especially after long-term sludge applications to acidic soils.

Soil microorganisms are known to play a key role in the mobilization and immobilization of metal cations, thereby changing their availability to plants (6). Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutual symbioses with the majority of higher plants, providing a direct physical link between soil and plant roots (3). AMF occur in almost all habitats and climates (4), including in disturbed soils such as those derived from mine activities (9), but soil degradation usually produces changes in the diversity and abundance of AMF populations (21, 23, 27). Mycorrhizal fungal populations are critical during and after soil disturbance because of their role in the establishment and survival of plants (18, 30). Thus, changes in the diversity of their population produced by the application of high amounts of metal are expected to interfere with the possible beneficial effects of this symbiotic association, since reestablishment of AMF populations is slow (12). However, only a few studies have been carried out involving interactions between AMF and metals as a source of soil disturbance. Most of the results already obtained derive from laboratory and pot experiments, with metal salts used as the source of heavy metals, which are not very representative of natural field conditions, under which metals usually accumulate in a less-available chemical form. Heavy metals can delay, reduce, and even completely eliminate AM colonization and AMF spore germination in the field (14), and a negative correlation between Zn concentrations and AM colonization has been reported in soil treated with urban-industrial sludge (7). In other studies, however, the addition of metal-containing sludge did not significantly affect AM development under field conditions (2), probably because different AMF ecotypes can exhibit different degrees of metal tolerance (26). Thus, a relatively high rate of mycorrhizal colonization can be found in plants growing in very polluted soils (31). A higher tolerance to Cu, Zn, Cd, and Pb of indigenous fungi from sludge-polluted sites in comparison to those of reference isolates from unpolluted soils has been described previously (13, 15). To our knowledge, no studies have been reported on the long-term effects of increasing concentrations of sewage sludge on the diversity of mycorrhizal propagules or on the influence of the host plant on AM fungal diversity in heavy-metal-polluted soils.

Our aim in this study was to determine how AM fungal diversity is affected by the addition of sewage-amended sludge in a long-term experiment compared with that of the appropriate noncontaminated control soils. Since the host plant can affect the structure and composition of the AMF population, four different plants with different life cycles and growth strategies were used.

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TABLE 1. Chemical and physicochemical characteristics of the test soils[a]

Sludge treatment	Organic matter (%)	NH_4NO_3 P[b] (mg/liter)	Total P[c] (mg/kg)	pH	Free-cation concn in the soil solution ($\mu g/liter^{-1}$)		Total metal concn[d] (mg/kg of soil)				
					Zn^{2+}	Cd^{2+}	Zn	Cd	Cu	Ni	Pb
Unsludged	1.5	0.9	462	6.0	105	1.5	43	0.3	9.8	8.6	29
100 m^3 ha^{-1} yr^{-1}, low metal	1.6	1.6	758	5.9	835	1.6	88	0.6	17	8.3	30
100 m^3 ha^{-1} yr^{-1}, high metal	1.8	1.7	822	6.0	1,940	9.3	163	1.4	37	12	55
300 m^3 ha^{-1} yr^{-1}, low metal	2.0	3.0	1,488	5.5	6,780	16	185	0.9	32	16	37
300 m^3 ha^{-1} yr^{-1}, high metal	2.3	3.4	1,762	5.3	22,700	90	295	2.8	92	29	111

[a] Adapted from reference 29 with permission from the publisher.
[b] NH_4NO_3-extracted P.
[c] Aqua regia-extracted P.
[d] Aqua regia-extracted metals.

MATERIALS AND METHODS

Experimental site. A long-term sewage sludge field experiment site located at the Federal Research Centre for Agriculture in Braunschweig (Germany) was established in 1980. The experimental site was formerly (40 years ago) a woodland and was later converted into arable soil. The soil (silty loam) contains 5% clay, 50% silt, and 45% sand, with a pH ranging from 5.3 to 6.0 (10). The sewage sludge was obtained from a local sewage works, but was rather low in heavy metal (low-metal sludge), as it was received from the treatment plant. Portions of the sludge were contaminated by adding water-soluble chlorides (10) of the heavy metals Pb, Cd, Cr, Cu, Ni, Hg, and Zn in order to obtain a higher metal concentration (high-metal sludge) and were incubated for 6 weeks. Five different treatments were applied to the experimental plots as follows: inorganic fertilizer at 180 kg of N ha^{-1} $year^{-1}$, low-metal sludge at 100 m^3 or 300 m^3 ha^{-1} $year^{-1}$, and high-metal sludge at 100 m^3 or 300 m^3 ha^{-1} $year^{-1}$. Each treatment was replicated four times. The sludges were applied annually from 1980 to 1990, and the changes induced in soil properties are recorded in the study of Chaudri et al. (10). However, the highest amounts of heavy metals in the experimental plots were still within the upper limits accepted by the European Union for metal concentrations in soils receiving sewage sludge. The main characteristics of the test soil, in which the range of heavy-metal concentrations was created by the combination of different doses and types of sludge, are shown in Table 1 (29).

Soil sampling. The study was carried out with soil from plots receiving the different treatments. At sampling (June, 1996), maize plants were growing at the experimental site. Spring rape had been grown before maize. Five cores of the arable-soil layer (0 to 20 cm) were randomly taken from maize rhizosphere in the central part of each replicate plot to avoid edge effects. Samples were then pooled, thoroughly mixed, sieved through 4-mm-pore-size mesh, and stored at 4°C until used.

Multiplication of AMF: establishment of trap cultures. The natural mycorrhizal potential in the soil samples in terms of both the number of AMF spores and the AM colonization levels was very low, making the study of diversity difficult. In order to increase the population of the indigenous AM fungi, trap cultures were established for all the different treatments and replicates. Four trap plants were used as follows: *Sorghum bicolor* (L.) Moench (sorghum), *Allium porrum* (L.) (leek), *Trifolium repens* (L.) (clover), and *Timus vulgaris* (L.) (thyme). Seeds of these test plants were surface sterilized, pregerminated, and transplanted after emergence into 2-liter pots containing soil from each experimental plot. Four replicates per soil treatment and host plant were established. Plants were grown in a greenhouse, with temperatures ranging from 18 to 25°C and relative humidity ranging from 80 to 60%. Plants were watered every 3 days with tap water and fertilized once a fortnight with Long Ashton nutrient solution lacking phosphorus (19). After 6 months, the pot cultures were sampled by taking up to 100 g of a soil and roots mixture that was stored at 4°C until used. The sample size was previously determined as the minimum amount required to study AMF diversity, since it contained a complete representation of all the different morphotypes present in the soil.

AMF extraction and identification. AMF spores were isolated from 100 g of soil by the wet sieving and decanting method, followed by sucrose centrifugation (34). After centrifugation, the supernatant was poured through 50-μm-pore-size mesh and quickly rinsed with tap water. Spores were counted with a Doncaster dish under the dissecting microscope and grouped according to morphological characteristics. Permanent slides were prepared for each different spore morphotype with both polyvinyl-alcohol and polyvinyl-alcohol plus Melzer's solution (1:1). After the uniformity of the morphological groups was confirmed under the optical microscope, the different morphotypes were identified to the genus level and, when possible, to the species level. Spore identification was based mainly on spore size and color, wall structure, and hyphal attachment (20, 33, 36). With the data obtained, several indices were calculated as follows: richness (R = number of species found in the sample), relative abundance of each species in each plot, calculated as $(n_i/N_j) \times 100$, where n_i = number of spores that belong to species i and N_j = total number of spores in the site. Mycorrhizal fungal diversity was calculated by using the Shannon-Wiener index, which combines two components of diversity, species richness and evenness of individuals among the species (24).

Statistical analysis. A two-way analysis of variance (ANOVA) was used to evaluate the effect of the different host species and soil treatments on total spore number, species richness, and diversity of the AM fungi. Relative densities were arcsine-square-root transformed before the two-way ANOVA was applied to evaluate the effects of the different treatments on the densities of the AM fungal species present in the plots. ANOVA was followed by Duncan's test when appropriate.

RESULTS

Six AM fungal species belonging to the genus *Glomus* were found in rhizosphere samples from the different experimental trap plants and soil treatments as follows: *G. claroideum*, *G. mosseae*, and four additional, unidentified species numbered III to VI. Total AMF spore number decreased significantly with increasing amounts of heavy metals in soil, from 550 spores (per 100 g of dry soil) in the control plot to 30 spores in the 300 m^3 ha^{-1} $year^{-1}$ contaminated sludge (Fig. 1). In the 100 m^3 ha^{-1} $year^{-1}$ uncontaminated and contaminated sludge plots, the number of spores averaged 330 and 230 per 100 g of soil, respectively. This value decreased to 110 and 30 spores in the soils added at a rate of 300 m^3 ha^{-1} $year^{-1}$. Host plant also had a significant effect on the total AMF spores produced in the rhizosphere, *Sorghum bicolor* being the trap plant that produced AMF spores most effectively (Fig. 1).

Both species richness and the Shannon-Wiener index rating increased at intermediate levels of soil contamination (100 m^3

TABLE 2. *F* and *P* values[a] from ANOVA tests of soil treatment, host plant effects, and their interaction on the relative spore densities of AMF species

Glomus species	Source of variation					
	Soil treatment		Host plant		Soil × plant interaction	
	F	*P*	*F*	*P*	*F*	*P*
Glomus sp. III	20.53	0.0001	31.77	0.0001	9.77	0.0001
G. mosseae	12.87	0.0001	4.30	0.082	4.90	0.0001
G. claroideum	3.94	0.0066	11.08	0.0001	7.56	0.0001
Glomus sp. IV	4.87	0.0018	0.91	0.44	0.83	0.62
Glomus sp. V	9.94	0.0001	5.12	0.0032	2.76	0.0047
Glomus sp. VI	9.20	0.0001	6.97	0.0004	7.46	0.0001

[a] *F* is the between treatment variance/within treatment variance ratio and provides a value for the equality of treatment means. The *P* value obtained for each *F*, according to the corresponding degree of freedom, as follows (soil treatment, 4; host plant, 3; soil × plant interaction, 12), indicates the probability that differences between treatment means are due to sampling error.

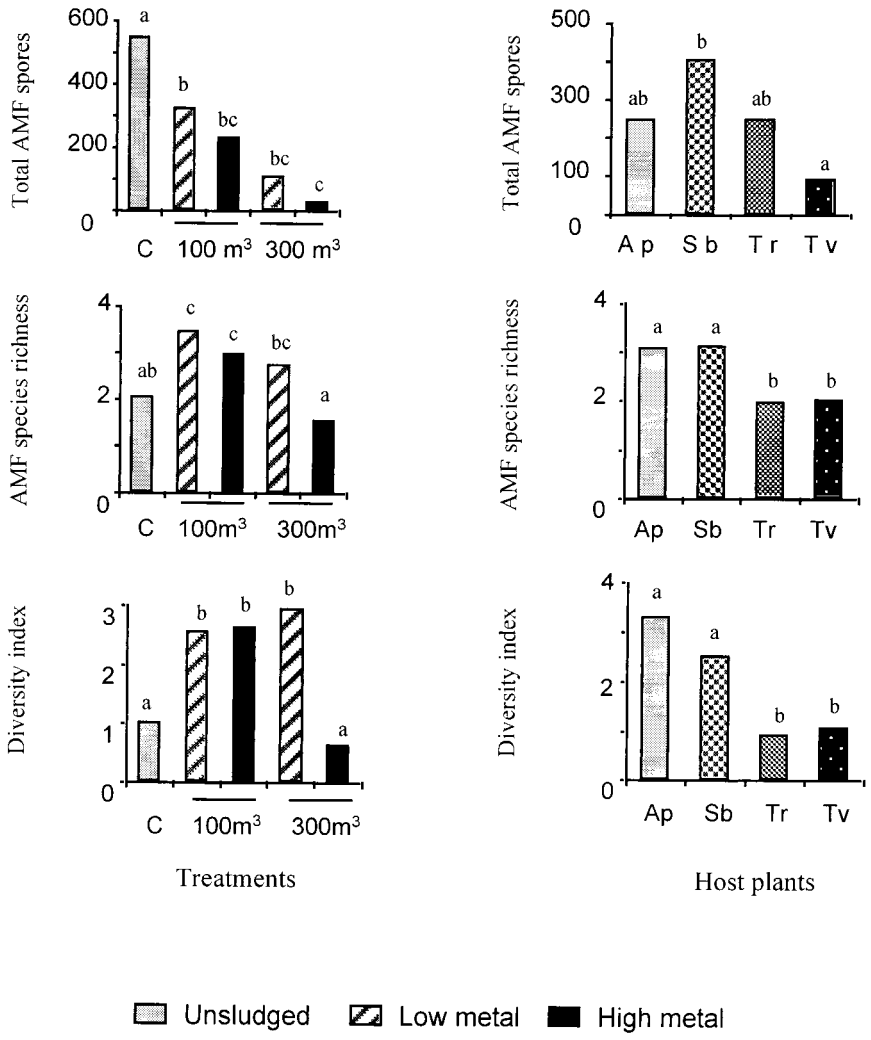

FIG. 1. Overall effect of soil treatments and host plants on the total number of AMF spores (per 100 g of dry soil), species richness, and diversity, measured by the Shannon Wiener index, of AMF populations. For each graph, bars with different letters indicate significantly different means ($P < 0.01$) by Duncan's test. Host plant abbreviations: Ap, *A. porrum*; Sb, *S. bicolor*; Tr, *T. repens*; Tv, *T. vulgaris*. Soil treatment abbreviations: C, control soil; 100 m^3 and 300 m^3 low-metal-sludge soil, 100 and 300 m^3 ha^{-1} $year^{-1}$ sludge-amended soil, respectively.

ha^{-1} $year^{-1}$ and 300 m^3 of low-metal sludge), decreasing at the highest contamination level (300 m^3 ha^{-1} $year^{-1}$ contaminated sludge) (Fig. 1). Host plants also exerted a differential effect on AMF diversity, with *A. porrum* and *S. bicolor* promoting significantly higher levels of diversity in their rhizospheres than those produced by *T. vulgaris* and *T. repens* (Fig. 1).

Relative densities of all AMF species were significantly influenced by soil sludge treatments. Four species, mainly *Glomus* sp. III, were significantly influenced by the host plant, and all *Glomus* species except *Glomus* sp. IV were significantly influenced by the soil × plant interaction (Fig. 2 and Table 2). The composition of the AM fungal population in the various host plants' rhizospheres, as affected by the different soil treatments, is recorded in Fig. 2. *Glomus* sp. III was the most common AMF species in the rhizospheres of *A. porrum* and *S. bicolor* in unpolluted soils, but its population decreased sharply with increasing metal content in soils. At higher rates of metal sludge contamination, *G. claroideum* seemed to be the AMF species with the best ability to sporulate, becoming the most common species in the rhizospheres of both plants. Both *T. repens* and *T. vulgaris* were colonized mainly by *G. claroideum*, even in the unpolluted soil. *G. claroideum* and *Glomus* sp. V were the most common fungi in the rhizospheres of all host plants growing in soils treated with 300 m^3 ha^{-1} $year^{-1}$ of contaminated sludge.

The effects of the interaction between soil treatment and host plant on the diversity of the AMF species and the richness of the AMF species, as measured by the Shannon-Wiener index, are recorded in Tables 3 and 4, respectively. Both response variables decreased significantly in the soil amended with the highest rate of contamination. Different host plants showed similar trends with regard to the changes they induced in species richness. The low diversity promoted by *T. repens* and *T. vulgaris* in their rhizospheres is remarkable, however.

The overall effects of soil contamination and host plant on the AMF population are illustrated in Fig. 2. As indicated above, *Glomus* sp. III was very sensitive to the presence of metals in soil, and its propagules practically disappeared in the most contaminated soil, while *G. claroideum* maintained a similar relative density in all soils independent of sludge treatment. *G. mosseae* showed another pattern, increasing its density at intermediate rates of contamination. It is noteworthy

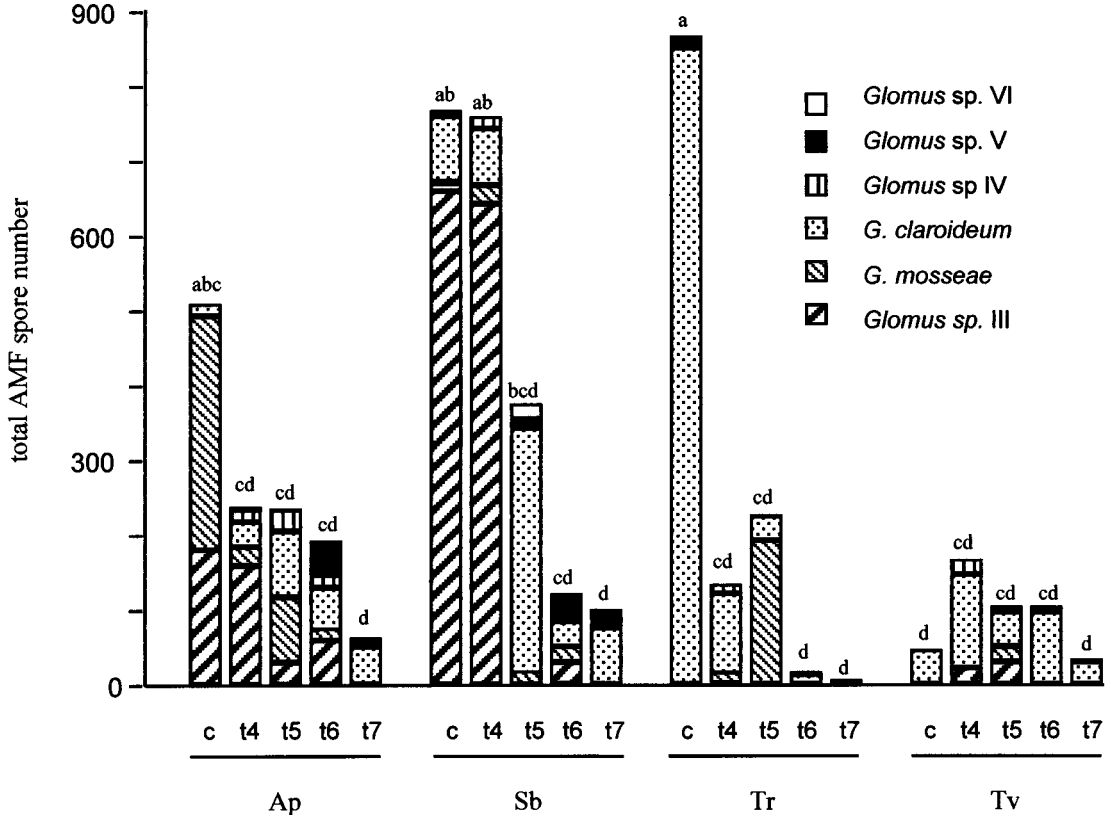

FIG. 2. Relative abundance of the various AMF species in the rhizospheres of different host plants grown in samples of field soil with different sludge application treatments. Bar height represents the mean of the total number of AMF spores for each treatment per 100 g of dry soil (the sum of the numbers of spores of each species in each treatment). Values for single fungal species, represented by various shading patterns within bars, are not cumulative. Bars with the same letter do not differ significantly ($P < 0.001$) by Duncan's test. Host plant abbreviations: Ap, *A. porrum*; Sb, *S. bicolor*; Tr, *T. repens*; Tv, *T. vulgaris*. Soil treatment abbreviations: c = control; t4 and t6, 100 and 300 m^3 ha^{-1} $year^{-1}$ of noncontaminated sludge, respectively; t5 and t7, 100 and 300 m^3 ha^{-1} $year^{-1}$ of contaminated sludge, respectively.

that *Glomus* sp. III was abundant in *A. porrum* and *S. bicolor* rhizospheres, since it was almost absent from the rhizosphere of *T. repens*.

A negative correlation ($P \leq 0.001$) was shown between the total number of AMF spores and soil metal content (Table 5). For metals, both the total amount of Zn and the free Zn cation concentration determined in the soil solution gave the lowest correlation coefficients with total number of spores, which also correlated negatively with the P content of the soil (both total and available) and positively with soil pH. *Glomus* sp. III was negatively correlated with the total content of all the metals studied (Ni, Cu, Cd, Pb, and Zn), corroborating its sensitivity to the presence of heavy metals (Table 5). *Glomus* sp. V, however, did not show a significant correlation with the content of the metals studied, except for the free Zn cations in the soil solution (Table 5).

DISCUSSION

Long-term sludge application with increasing concentrations of heavy metals produced a significant decrease in both the size and diversity of AMF populations in soil.

The total number of AMF spores strongly decreased with the addition of increasing amounts of heavy metals, but the AMF propagules never disappeared completely in soils amended with the highest rates of sludge, suggesting a certain adaptation of these indigenous AMF to such environmental stress. Notably, the total number of AMF spores correlated

TABLE 3. Diversity of AMF populations by the Shannon-Wiener index as affected by soil amendment and different types of host plants

Variable	Mean for indicated host plant[a]			
	A. porrum	*S. bicolor*	*T. repens*	*T. vulgaris*
Unsludged	2.13 bcd	1.61 bcd	0.39 d	0.00 d
Low metal, 100 m^3 ha^{-1} yr^{-1}	4.28 ab	2.03 bcd	1.77 bcd	2.25 bcd
High metal, 100 m^3 ha^{-1} yr^{-1}	4.05 ab	2.69 abcd	1.23 cd	2.63 abcd
Low metal, 300 m^3 ha^{-1} yr^{-1}	5.32 a	5.29 a	0.93 d	0.33 d
High metal, 300 m^3 ha^{-1} yr^{-1}	0.99 d	1.08 d	0.32 d	0.25 d

[a] Means followed by the same letter did not differ significantly ($P \leq 0.01$) by Duncan's test.

TABLE 4. Species richness index scores of AMF populations as affected by soil amendment and different types of host plants

Variable	Mean for indicated host plant[a]			
	A. porrum	*S. bicolor*	*T. repens*	*T. vulgaris*
Unsludged	3.0 abcd	3.0 abcd	1.3 ef	1.0 f
Low metal, 100 m^3 ha^{-1} yr^{-1}	4.0 a	3.8 a	3.0 abcd	3.3 abc
High metal, 100 m^3 ha^{-1} yr^{-1}	3.0 abcd	3.0 abcd	2.8 abcde	3.3 abc
Low metal, 300 m^3 ha^{-1} yr^{-1}	3.5 ab	4.3 a	1.8 cdef	1.5 def
High metal, 300 m^3 ha^{-1} yr^{-1}	2.0 bcdef	1.8 cdef	1.3 ef	1.3 ef

[a] Means followed by the same letter did not differ significantly ($P \leq 0.01$) by Duncan's test.

negatively with the metal (Zn, Cu, Cd, and Ni) content of the soils, but the correlation coefficient was higher for the concentration of free cations (Zn^{2+} and Cd^{2+}) in the soil solution. When present in excess, these ions are generally assumed to be the chemical species that are taken up by and are toxic to soil microbes (16). In previous studies on AMF and Cu (17) and Zn and Cd (39), no correlation was found between the concentration of these metals in sludge-amended agricultural soils and AMF populations. Despite their availability, methods to measure free-ion activity in the soil solution have rarely been used in studies relating to heavy metal and AMF. The use of such methods as a reference for comparison would probably help elucidate the reason for the discrepancies found between different studies. AMF population size also correlated negatively with the P content of the soil, a result that is well documented (35).

Species richness and diversity as measured by the Shannon-Wiener index increased at moderate levels of soil contamination. This increase in AM propagule diversity could be a fungal stress response whereby fungal ecotypes better adapted to unpolluted soil but affected at intermediates rates of contamination allow other fungi, probably less competitive in nonstressed soils but better adapted to heavy metals, to colonize the roots and complete their life cycles. Thus, the number of fungal ecotypes in these soils can be increased. However, at the highest levels of soil pollution, both indices diminished sharply. This may have resulted from a fungitoxic effect of metals, causing certain AMF species' inability to colonize the root system and/or to multiply in the rhizosphere. Only AMF species better adapted to the disturbance produced by the addition of metals would overcome the stress situation and complete their life cycles. A similar response model concerning diversity, suggested for other microbial groups (37), may hold true for *Rhizobium leguminosarum* bv. *trifolii* from the same experimental field in Braunschweig; the relationship between genetic diversity within populations and heavy-metal stress in soils may lead to an increase in diversity with a moderate metal loading, followed by a sharp decrease at higher levels of stress (16). These changes in genetic diversity may be crucial in determining the response of a population to changing conditions. However, genetic diversity studies have not yet been described for AM fungi; thus, it is not possible to relate the phenotypical changes found in the present study to changes in the genetic structure of the AMF population.

Glomus sp. III spore density was very much influenced by both soil treatments and host plant species. This fungal ecotype appears to be very sensitive to increasing concentrations of metals in soil, disappearing almost completely in the most polluted soil. At intermediate rates of contamination, *Glomus* sp. III was replaced by other species, such as *G. claroideum*. In fact, *G. claroideum* maintained similar relative density levels in all treatments, showing a higher degree of tolerance to heavy metals than the other fungal species present in the soil, as has been described for other AMF ecotypes (13, 15, 25, 38). *Glomus* sp. V was preferentially found in soils with the highest level of contamination, indicating a low competitiveness of this fungus in the absence of the stress situation to which it is well adapted. Major differences among the species in terms of both numbers of spores and tolerance to metals suggest that fungi follow different strategies to establish symbiosis and probably reveal differences in functioning.

The host plant-mediated effect on the composition and diversity of the AM fungal community is noteworthy. In particular, the high diversity promoted by *S. bicolor* and *A. porrum* contrasts with the poor levels induced by *T. repens* and *T. vulgaris. S. bicolor* appeared to be a good host for spore production, possibly because of the higher root growth rate of this plant species, which can facilitate further contact with most AM fungi present in the soil. In contrast, in the rhizosphere of *T. vulgaris*, a species with a very slow growth rate and a poorly developed root system, AMF population size and diversity were very low. These results corroborate the key role of the host plant as a selective force in maintaining specific populations of these ecologically obligate fungal symbionts (1, 5, 22). The root growth rate seems critical to allow colonization by certain AM fungi; thus, the present results provide new insights into the specificity concept in arbuscular mycorrhizas.

The reasons underlying stress-related changes in the diversity of AMF populations, particularly those due to the presence of heavy metals, are not completely understood. It is well known that heavy metals cannot be chemically degraded. Therefore, remediation of metal-polluted soils is limited mainly to immobilization, for example by phytostabilization,

TABLE 5. Correlation coefficients[d] for several parameters of the test soils and some characteristics of the AMF spore populations

Variable	Total no. of AMF spores	Spore density	
		Glomus sp. III	*Glomus* sp. V
pH	0.939**	NS	−0.942**
P_{total}[a]	−0.971**	NS	0.910*
$P_{available}$[b]	−0.959**	NS	0.886*
NO_3-N	NS	NS	−0.970**
Zn_{total}[a]	−0.978***	−0.931**	NS
Zn^{2+c}	−0.957***	−0.884*	0.890*
Cd_{total}[a]	−0.905*	−0.951**	NS
Cd^{2+c}	−0.922**	−0.894*	NS
Ni_{total}[a]	−0.897*	−0.971**	NS
Pb_{total}[a]	NS	−0.941**	NS
Cu_{total}[a]	−0.944**	−0.952**	NS

[a] Extracted with aqua regia.
[b] Extracted with NH_4NO_3.
[c] Free in soil solution (29).
[d] *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; ***, significant at $P \leq 0.001$; NS, not significant.

which consists of promoting plant growth to reduce or eliminate the bioavailability of metals (11). In this context, AMF constitute an important functional component of the soil-plant system that is critical for sustainable productivity in stressed soils (3). A better understanding of the mechanisms behind these changes in AMF diversity, and particularly of those on which AMF adaptation and tolerance to metals are based, is important, since such an understanding could facilitate the management of these soil microorganisms for a restoration and/or bioremediation program.

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