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The application of an organic amendment modifies the arbuscular mycorrhizal fungal communities colonizing native seedlings grown in a heavy-metal-polluted soil

M.M. Alguacil [a,*], E. Torrecillas [a], F. Caravaca [a], D.A. Fernández [a], R. Azcón [b], A. Roldán [a]

[a] CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, P.O. Box 164, Campus de Espinardo, 30100 Murcia, Spain
[b] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda no 1, 18008 Granada, Spain

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ABSTRACT

A mesocosm experiment was conducted to investigate whether communities of arbuscular mycorrhizal (AM) fungi associated with roots of native (*Piptatherum miliaceum*, *Retama sphaerocarpa, Psoralea bituminosa, Coronilla juncea*, and *Anthyllis cytisoides*) and for comparison (*Lolium perenne)* seedlings in a heavy-metal-contaminated, semiarid soil were affected by the application of composted sugar beet waste. We also investigated whether there were relation between AMF diversity and metal concentration (Al, Cd, Cu, Fe, Mn, Pb and Zn) and total P in shoot as well as some soil parameters (total organic carbon and total N) when the SB waste was added to the soil. We analyzed a portion of approximately 795 base pairs of the small-subunit (SSU) rRNA gene by nested PCR, cloning, sequencing, and phylogenetic analyses. Twelve different AMF sequence types were distinguished: seven of these belonged to *Glomus* group A, one to *Glomus* group B, one to *Diversispora,* one to *Archaeospora*, and two to *Paraglomus*. The AM fungal populations colonizing roots in a heavy-metal-polluted soil were quite dependent on the host plant, the highest diversity values being obtained in authochtonous plants recognized as metallophytes, such as *P. bituminosa*, and in an allochtonous, invasive species (*L. perenne*). No significant correlation was found between AMF diversity and plant metal concentration and soil parameters. Excepting *P. bituminosa*, when sugar beet waste was added to soil, the populations of AM fungi in roots increased and the shoot metal concentrations decreased in all host plant species studied. Therefore, the addition of sugar beet waste can be considered a good strategy for the remediation and/or phytostabilization of mine tailing sites.

1. Introduction

The waste materials generated by mining activities may contain great quantities of heavy metals, which can accumulate in the soil and affect microbial diversity and activity (Brooks et al., 1986; McGrath et al., 1995; Del Val et al., 1999). Among the soil microorganisms are the arbuscular mycorrhizal fungi (AMF), which belong to the phylum Glomeromycota (Schüβler et al., 2001) and form mutualistic symbioses with the majority of known terrestrial plant species (Smith and Read, 1997). Besides an improvement in the plant growth, due to enhanced water and mineral nutrient supply, and a supply of carbon compounds for the fungus, this symbiotic relationship protects plants against diverse biotic and abiotic stresses (Smith and Read, 1997), such as those produced in sites contaminated by heavy metals (Leyval and Joner, 2001). The AM fungi colonize the roots of plants growing on heavy-metal-contaminated soils and play an important role in metal tolerance, sequestration, and accumulation (Gaur and Adholeya, 2004), helping in the revegetation of mine areas or trace-element-contaminated soils (Göhre and Paszkowski, 2006; Hildebrandt et al., 2007). It has been reported that different AMF ecotypes can confer differing degrees of metal tolerance to their host plant (Del Val et al., 1999; Gaur and Adholeya, 2004; Zarei et al., 2008; Khade and Adholeya, 2009) and that AM fungal species differ in their capacity to affect heavy metal uptake by plants (Leyval et al., 1997). Thus, the establishment and survival of plants in these polluted sites might be governed by the communities of AMF. In fact, the presence, abundance, and composition of the AMF community are related strongly to the composition of the plant community (Van der Heijden et al., 1998).

Previous molecular studies have reported different AMF taxa in different plant species, showing host plant preference and/or specificity (Helgason et al., 2002; Vandenkoornhuyse et al., 2002, 2003; Alguacil et al., 2009b). Also, other environmental factors,

* Corresponding author. Tel.: +34 968396337; fax: +34 968396213.
E-mail address: mmalguacil@cebas.csic.es (M.M. Alguacil).

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such as site (Öpik et al., 2003), soil fertilization (Santos et al., 2006), or the agricultural management and ecosystem type (Hijri et al., 2006), have been found to influence the AMF community structure. Therefore, for the reclamation of heavy-metal-contaminated sites, it is very important to know the dynamics of the AMF communities in these soils and to have a good selection of tolerant plant species that can establish, grow, and survive under these conditions.

Organic amendments have been used widely to facilitate the revegetation of contaminated soils. In previous studies it has been showed that in soils affected by heavy metals, treated sugar beet (SB) waste improved the plant growth (Medina et al., 2006; Azcón et al., 2009) and structural stability (Carrasco et al., 2009). Immobilization and/or biosorption of heavy metals in soil after SB amendment have been attributed to the presence of carboxyl groups and strong complexes among its constituents (Reddad et al., 2002). Several studies have demonstrated the beneficial effect of the application of various organic amendments to soil, with regard to increasing the proliferation and development of natural AM fungal populations in crop systems (Harinikumar et al., 1990; Jacquot-Plumey et al., 2001) or degraded, semiarid soils (Alguacil et al., 2009a). However, to the best of our knowledge, no studies have been reported on the effects of organic amendments on the diversity of the AMF colonizing plant rhizospheres in a heavy-metal-polluted, Mediterranean soil.

Therefore, the objectives of this work were: 1. To assess whether the application of a composted SB waste could influence in the diversity of AM fungal populations detected in different plant species in a heavy-metal-contaminated soil. 2. To investigate whether there were relations between AMF diversity and metal concentration in shoot as well as soil parameters when the SB waste was added to the soil.

The information gained here will give us a better understanding of the interaction between plants and AMF, for future revegetation and phytoremediation studies in polluted sites.

2. Materials and methods

2.1. Study site

Soil used in this experiment comes from the La Unión mine district (Southeast Spain). The climate is semiarid Mediterranean with an annual rainfall around 250–300 mm and a mean annual temperature of 17.5 °C; the potential evapo-transpiration reaches 1000 mm year^{-1}. This zone constituted an important mining nucleus for more than 2500 years. The ore deposits of this zone have iron, lead and zinc as the main metal components. Iron is present in oxides, hydroxides, sulfides, sulfates, carbonates and silicates; lead and zinc occur in galena, sphalerite, carbonates, sulfates, and lead- or zinc-bearing (manganese, iron) oxides (Oen and Fernández, 1975). In this area a mine tailing with an age of about 50 years called "Gorguel" (UTM X687480 Y4162800 Z135, length: 200–300 m, width: 95 m, height: 25 m, volume: 750,000 m^3, IGME, 1999) was selected. Three soil samples were taken, each one consisted of a mixture of six subsamples randomly taken from the top 20 cm depth of soil. The analytical characteristics of the mine tailing are shown in the Table 1.

2.2. Materials

Sugar beet residue (SB), a lignocellulosic material was dried at 60 °C and then ground to pass a 2-mm-pore sieve. Portions of 15 g of SB were mixed with 40 mL of Czapek solution (agar 15.0 g L^{-1}; di-potassium hydrogen phosphate 1.0 g L^{-1}; iron (II) sulfate heptahydrate 0.01 g L^{-1}; potassium chloride 0.5 g L^{-1}; magnesium sulfate heptahydrate 0.5 g L^{-1}; sodium nitrate 3.0 g L^{-1}; sucrose 30.0 g L^{-1}; pH = 7.3) for static fermentation in 250 mL Erlenmeyer flasks. The mixture was allowed to ferment at 30 °C for 20 days without shaking. The characteristics of the SB after fermentation were: pH, 5.3; total P, 224 μg mL^{-1}; total N, 1.2%; cellulose, 11.3%; hemicellulose, 3.1%; lignin, 4.1% and reducing sugar, 0.25 g L^{-1}.

Table 1
Chemical, biochemical, microbiological and physical characteristics of the soil used in the experiment ($n = 3$).

pH (H_2O)	7.67 ± 0.03[a]
Electrical conductivity (1:5, dS m^{-1})	1.3 ± 0.7
Glomalin-related soil protein (μg g^{-1})	523.2 ± 24.8
Total organic C (g kg^{-1})	10.5 ± 0.1
Total N (g kg^{-1})	1.33 ± 0.05
Total Al (mg kg^{-1})	14.500 ± 300
Total Cd (mg kg^{-1})	36.8 ± 1.1
Total Cr (mg kg^{-1})	91.2 ± 2.8
Total Cu (mg kg^{-1})	163.1 ± 5.8
Total Fe (mg kg^{-1})	190.300 ± 5.100
Total Ni (mg kg^{-1})	15.3 ± 0.3
Total P (mg kg^{-1})	6.400 ± 200
P available (mg kg^{-1})	7.0 ± 0.5
Total Pb (mg kg^{-1})	6.900 ± 0
Total S (mg kg^{-1})	12.700 ± 300
Total Zn (mg kg^{-1})	12.000 ± 300

[a] Mean ± standard error.

For this study we selected five plant species which naturally grow in the nearby areas of the mine tailing, mature seeds of *Piptatherum miliaceum* (L.) Coss, *Retama sphaerocarpa* L., *Psoralea bituminosa* L., *Coronilla juncea* L. and *Anthyllis cytisoides* All plant species are drought resistant and form arbuscular mycorrhizal symbiosis, from them only *P. bituminosa* has been reported as heavy metal tolerant plant species (Poschenrieder et al., 2001; Walker et al., 2007). For comparison purposes, we selected *Lolium perenne* L. which do not naturally thrive in the area but is also mycorrhizal dependant and easy to grow. *L. perenne* has never been reported as a drought or heavy metal tolerant plant species. The seeds were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) for 5 min and subsequently rinsed thoroughly with sterilized water prior to a wetting treatment with sterilized water for 2 h.

2.3. Experimental design

The experiment was conducted as a completely randomized two factor factorial with six replicates. The first factor was the addition or not of fermented SB residue to the soil. The second factor was the plant species with six levels (*P. miliaceum*, *R. sphaerocarpa, P. bituminosa, C. juncea*, *A. cytisoides* and *L. perenne*).

Five-hundred grams of air-dried soil were placed in 600 mL pots, where seeds of the selected plants were sowed. The fermented SB was mixed manually with the experimental soil at a rate of 2.5% (w/w). The experiment was conducted as a mesocosm assay in a greenhouse, located in the Campus of Espinardo (Murcia, Spain). During the experiment, the average maximum temperature reached 22 °C. Plants were watered regularly with sterile water to a 60% of field capacity, without any fertilizer treatment. Eight months after sowing, the plants were sampled (a total of 72 plants). Plants, including root systems, were collected and placed in polyethylene bags for transport to the laboratory, where fine roots were separated from the soil. Roots were then briefly rinsed, quickly dried on paper and used partly for morphological and partly for molecular analysis. Samples of rhizospheric soil were also collected. The soil samples were sieved through 2-mm pores to eliminate large particles and stored in plastic bags at −20 °C until processed.

2.4. Mycorrhizal determinations

The percentage of mycorrhizal root colonization was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

2.5. Soil parameters measured and total P and metals concentrations in plant

The total N was determined with the Kjeldahl method, consisting of titration after distillation and sample digestion.

Total organic carbon content was determined with an automatic Carbon Elemental Analyzer (Flash EA 1112 Series-Leco Truspec) after pre-treatment with HCl to eliminate carbonates and combustion at 1020 °C.

P, Al, Fe, Cu, Mn, Pb, Zn and Cd were quantified using an Inductively Coupled Plasma Mass Spectrometry (ICP–MS) (Thermo electron corporation Mod. IRIS intrepid II XDL).

2.6. Roots and soil DNA extraction and PCR

For each sample (total 72), total DNA was extracted from root material (representing approx. 5–8 cm root length) using a DNeasy plant mini Kit following the manufacturer's recommendations (Qiagen). The roots samples were placed into a 2-mL screw-cap propylene tube together with two tungsten carbide balls (3 mm) and ground (3 min, 13,000 r.p.m.) using a mixer mill (MM 400, Retsch, Haan, Germany). The extracted DNA was resuspended in 20 μL of water.

Soil from each plant species and treatment were pooled into twelve soil samples, and then genomic DNA was extracted from 0.5 g of soil using a FastDNA™ Spin kit for soil according to the recommendations of the manufacturer (Q-BIOgene, Heidelberg, Germany).

Several dilutions of extracted DNA (1/10, 1/50, 1/100) were prepared. Partial ribosomal small-subunit (SSU) DNA fragments were amplified using nested PCR with the universal eukaryotic primers NS1 and NS4 (White et al., 1990). PCR was carried out in a final volume of 25 μL using the "ready to go" PCR beads (Amersham Pharmacia Biotech), 0.2 μM dNTPs and 0.5 μM of each primer (PCR conditions: 94 °C for 3 min, then 30 cycles at 94 °C for 30 s, 40 °C for 1 min, 72 °C for 1 min, followed by a final extension period at 72 °C for 10 min). As a template, 2 μL of extracted DNA was used in all reactions.

Several dilutions (1/10, 1/20, 1/50 and 1/100) were used as template DNA in a second PCR reaction performed using the specific primers AML1 and AML2 (Lee et al., 2008). PCR reactions were carried out in a final volume of 25 μL using the "ready to go" PCR beads (Amersham Pharmacia Biotech), 0.2 μM dNTPs and 0.5 μM of each primer (PCR conditions: 94 °C for 3 min, then 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C and 1 min extension at 72 °C, followed by a final extension period of 10 min at 72 °C). As a template, 2 μL of extracted DNA was used in all reactions. Positive and negative controls using PCR positive products and sterile water respectively were also included in all amplifications. DNA extracts were stored at −20 °C. All the PCR reactions were run on a Perkin Elmer Cetus DNA Thermal Cycler. Reactions yields were estimated by using a 1.2% agarose gel containing ethidium bromide.

2.7. Cloning and sequencing

The PCR products were purified using a Gel extraction Kit (Qiagen) cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* (Xl1 blue). Forty putative positive transformants were screened in each resulting SSU rRNA gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and a re-amplification with AML1 and AML2 primers with the same conditions described above. Product quality and size were checked in agarose gels as described above. All clones having inserts of the correct size in each library were sequenced.

They were grown in liquid culture and the plasmid extracted using the QIAprep Spin Miniprep Kit (Qiagen). The sequencing was done by Laboratory of Sistemas Genómicos (Valencia, Spain) using the universal primers SP6 and T7. Sequence editing was done using the program Sequencher version 4.1.4 (Gene Codes Corporation). Sequences of the clones generated in this study have been deposited at the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers FN869698 to FN869856.

A search for chimeric sequences was performed using the program CHIMERA_Check 2.7 of the Ribosomal Database Project (http://rdp.cme.msu.edu/html/analyses.html) (Maidak et al., 2001).

2.8. Phylogenetical analysis

Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool (Altschul et al., 1997) provided by GenBank. Phylogenetic analysis was carried out on the sequences obtained in this study and those corresponding to the closest matches from GenBank. Sequences were aligned with other published glomeralean sequences using the program ClustalX (Thompson et al., 1997) and the alignment was adjusted manually in GeneDoc (Nicholas and Nicholas, 1997). Neighbor-joining (NJ) phylogenetic analyses (Saitou and Nei, 1987) was performed with the program PAUP4.08b (Swofford, 2002) and using the default parameters. *Endogone pisiformis* Link and *Mortierella polycephala* Coem were used as the out-groups.

2.9. Statistical analysis

Treatment effects on measured variables (mycorrhizal determinations, soil chemical and biological parameters and metals concentrations) were tested by analysis of variance, and comparisons among means were made using the Duncan's test calculated at $P < 0.05$. Sugar beet addition, type of plant species and their interactions effects on measured variables were tested by a two-way analysis of variance. Correlation analysis between the soil biological and chemical parameters measured, colonized root length, metals concentrations in plant and the AM diversity were carried out using Pearson's rank correlation coefficients. All statistical procedures were carried out with the software package SPSS 17.0 for Windows.

A log-linear saturated model that contained all the independent variables as well as combinations of the variables was constructed to examine the relationships among the variables. General log-linear modelings were carried out using SPSS. The saturated model reproduced the observed data exactly. The relative importance of a given interaction term was estimated after having removed this term from the saturated model. The overall goodness-of-fit statistics were calculated using likelihood radio chi-square to determine the significance levels and deduce whether this new unsaturated model fit the data. In the models were analyzed the relationships between explanatory variables (plant species and SB addition) and presence of AMF sequence types. The five less frequent AM fungal types were not taken into account in this analysis in order to limit a possible bias in likelihood ratio estimates.

The influence of environmental factors (plant species and SB addition) on the distribution of the AMF sequence types in the root samples was analyzed by ordination analysis conducted in CANOCO for Windows, version 4.5 (Ter Braak and Smilauer, 2004), using the presence/absence data for each root sample. Initial detrended correspondence analysis suggested a lineal character of the data response to the sample origin (the lengths of gradients were <3); therefore, the multivariate redundancy analysis (RDA) was used. The variance-partitioning method with permutations in blocks defined by the covariables was used to compare the influence of host plants with that of treatments. Monte Carlo permutation tests were conducted using 499 random permutations. The subsequent forward-selection procedure ranked the environmental variables according to their importance and significance for the distribution of the AMF sequence types.

The presence or absence of AMF phylotypes in each root sample was used to construct the sampling effort curves (with 95% confidence intervals) using the software EstimateS 8.00 (Colwell, 2005). The sample order was randomized by 100 replications.

The Shannon-Weaver (H′) index was calculated as an additional measure of diversity, as it combines two components of diversity, i.e., species richness and evenness. It is calculated from the equation $H' = -\sum p_i (\ln p_i)$, where p_i is the proportion of individuals found in the ith species (in a sample, the true value of p_i is unknown but is estimated as n_i/N, [here and throughout, n_i is the number of individuals in the ith species]).

3. Results

3.1. Soil chemical parameters

Of the soil parameters shown in Table 1, only the total organic carbon and total N in the soil were affected by the addition of the SB (data not shown): their contents were increased significantly by the SB addition for all six plant species tested (Table 2).

3.2. Morphological analyses

The colonized root length was affected only by the type of plant species (Table 3). *A. cytisoides* had the highest value of mycorrhization in non-amended soil (68.20%) with respect to the rest of plant species. There were not differences in the percentage root colonization values for any of the plant species due to SB addition (Table 2).

Table 3
Two factor ANOVA (SB addition and plant species) for the chemical properties measured in the soil, degree of AM fungal colonization in roots and Total P and metals concentrations in plant.

	SB addition (A)	Plant species (P)	Interaction (A × P)
TOC	<0.001	NS	NS
Total N	<0.001	NS	NS
Colonized root length	NS	<0.001	NS
Al	<0.001	<0.001	<0.001
P	<0.001	<0.001	<0.001
Cd	<0.001	<0.001	<0.001
Cu	<0.001	<0.001	<0.001
Fe	<0.001	<0.001	<0.001
Mn	<0.001	<0.001	<0.001
Pb	<0.001	<0.001	<0.001
Zn	<0.001	<0.001	<0.001

P significance values. NS: not significant. TOC: Total organic carbon.

3.3. Total P and metal concentrations in the plants

The total P concentration in the plants was affected significantly by the SB addition, the type of plant species, and the interaction between these two factors (Table 3). The shoot P concentrations of all the plant species were enhanced by the SB addition, *A. cytisoides* and *L. perenne* showing the largest increases (around 200%) (Table 4). However, in *P. miliaceum* seedlings the SB addition enhanced the total P concentration by only 28%, not significantly different from the value of the control plants. In non-amended soil, *R. sphaerocarpa* showed the highest total shoot P concentration and *L. perenne* the lowest.

The concentrations of all the metals measured in the plants were also affected greatly by the experimental factors as well as the interaction between them (Table 3). Thus, in amended soil, *P. miliaceum*, *A. cytisoides*, and *L. perenne* seedlings showed significantly lower concentrations of Al, Fe, Cu, Mn, Pb, and Zn than control plants (except for Cu in *A. cytisoides* and Zn in *L. perenne*). Solely for *P. bituminosa* did the SB addition not significantly change the shoot concentrations of heavy metals (Table 4).

3.4. Molecular analysis of plant roots

We extracted DNA from 72 root samples (six plants for each plant species and treatment). All root samples gave a PCR product for which the size of the amplified fragment was around 795 bp. From the 72 clone libraries, a total of 2880 clones were screened by PCR (40 clones/sample), of which 2676 contained the SSU rRNA

Table 2
Chemical properties of the soil, degree of AM fungal colonization in roots, Shannon diversity index and total number of AMF sequence types of the six plant species studied under control and SB addition treatments ($n = 6$).

Plant Species	Treatment	TOC (g kg^{-1})	Total N (g kg^{-1})	Colonized root length (%)	Shannon diversity index (H')	No of AMF sequence types
Piptatherum miliaceum	Control	11.1 ± 0.1a	1.4 ± 0.1a	53.0 ± 3.0abcd	0.69	3
	SB addition	17.8 ± 0.8b	2.28 ± 0.3b	78.8 ± 0.5d	1.26	6
Retama sphaerocarpa	Control	11.7 ± 0.1a	1.50 ± 0.1a	44.6 ± 2.8abc	1.38	6
	SB addition	17.4 ± 0.2b	2.22 ± 0.3b	38.0 ± 3.1a	1.15	6
Psoralea bituminosa	Control	11.0 ± 0.1a	1.41 ± 0.0a	56.8 ± 2.8abcd	1.94	9
	SB addition	17.6 ± 0.2b	2.25 ± 0.2b	72.8 ± 1.2d	1.29	5
Coronilla juncea	Control	10.2 ± 0.2a	1.30 ± 0.2a	57.0 ± 2.9abcd	1.16	4
	SB addition	16.3 ± 0.1b	2.08 ± 0.0b	50.8 ± 2.9abcd	1.57	6
Anthyllis cytisoides	Control	9.5 ± 0.1a	1.22 ± 0.1a	68.2 ± 2.0bcd	1.05	4
	SB addition	17.1 ± 0.3b	2.19 ± 0.2b	70.0 ± 1.7cd	1.46	6
Lolium perenne	Control	12.0 ± 0.3a	1.54 ± 0.1a	41.8 ± 1.1ab	1.81	8
	SB addition	18.0 ± 0.5b	2.30 ± 0.1b	37.6 ± 1.1a	1.94	9

TOC: Total organic carbon. Mean ± standard error for each measure is given. Values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by the Duncan test.

Table 4
Total P and metals concentrations in shoot of the six plant species studied under control and SB addition treatments ($n = 6$).

Plant Species	Treatment	Total P (μg g^{-1})	Al (μg g^{-1})	Fe (μg g^{-1})	Cu (μg g^{-1})	Mn (μg g^{-1})	Pb (μg g^{-1})	Zn (μg g^{-1})	Cd (μg g^{-1})
Piptatherum miliaceum	Control	568 ± 37bc	518 ± 16e	410 ± 13e	11.4 ± 0.3e	91 ± 1h	49 ± 1e	284 ± 6g	0.29 ± 0.02ab
	SB addition	727 ± 16cde	60 ± 2ab	40 ± 2ab	4.6 ± 0.2a	20 ± 0bc	5 ± 0ab	72 ± 2b	0.19 ± 0.01a
Retama sphaerocarpa	Control	836 ± 16e	37 ± 1a	49 ± 3ab	10.1 ± 0.4d	44 ± 3e	15 ± 1bc	236 ± 5f	0.61 ± 0.01e
	SB addition	1983 ± 152i	25 ± 1a	34 ± 4a	8.5 ± 0.3c	27 ± 1cd	0.1 ± 0a	113 ± 4c	0.37 ± 0.01bc
Psoralea bituminosa	Control	608 ± 5bcd	63 ± 2ab	65 ± 2abc	5.4 ± 0.1ab	8 ± 0a	15 ± 1bc	66 ± 1b	0.35 ± 0.02bc
	SB addition	1085 ± 41f	72 ± 4ab	92 ± 7abcd	5.0 ± 0.1a	14 ± 0ab	14 ± 1bc	43 ± 1a	0.38 ± 0.01bc
Coronilla juncea	Control	735 ± 15de	111 ± 9bc	144 ± 14cd	9.9 ± 0.4d	35 ± 2d	40 ± 1de	150 ± 5de	0.39 ± 0.02bc
	SB addition	1663 ± 27h	86 ± 7ab	124 ± 13bcd	8.6 ± 0.3c	23 ± 1bc	21 ± 1c	116 ± 5c	0.25 ± 0.01a
Anthyllis cytisoides	Control	499 ± 7ab	315 ± 6d	503 ± 15f	8.6 ± 0.2c	64 ± 2f	115 ± 4f	248 ± 5f	0.52 ± 0.01d
	SB addition	1470 ± 14g	113 ± 11bc	177 ± 17d	8.2 ± 0.2c	27 ± 1cd	37 ± 1de	127 ± 3cd	0.43 ± 0.02cd
Lolium perenne	Control	383 ± 4a	343 ± 17d	382 ± 20e	9.0 ± 0.1c	98 ± 1h	34 ± 2d	171 ± 5e	0.67 ± 0.03e
	SB addition	1193 ± 37f	156 ± 11c	145 ± 14cd	6.1 ± 0.2b	82 ± 2g	11 ± 1abc	152 ± 3e	0.43 ± 0.01cd

Mean ± standard error for each measure is given. Values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by the Duncan test.

gene fragment. From six to ten clones per library (i.e., root sample) were sequenced, producing a total of 470 clones. BLAST searches in the GenBank database showed that 370 clones (78.72%) had high similarity to sequences from AM fungi and belonged to members of the phylum Glomeromycota (Table 5). The 100 remaining clones (21.28%) were identified as non-AMF, since they were similar to sequences belonging to the six host plant species (16%) and/or other eukaryotes (5.28%). We did not detect chimeric sequences in this study.

3.5. Molecular analysis of soil

The DNA from all soil samples gave PCR products of the expected size (approximately 795 bps). A library was created for each soil sample. From the 12 clone libraries, a total of 480 clones were screened for the presence of the insert and 440 clones resulted positive. The BLAST search of the first sequences analyzed (100 clones) showed that only 2% of sequences belonged to AMF; the rest (98%) showed high similarity with sequences of zygomycetes, ascomycetes, chytridiomycetes, basydiomycetes, and other, uncultured eukaryotes. Therefore, due to the low specificity of this set of primers for the DNA from the soil samples, we decided not to perform more sequencing of clones from soil samples.

3.6. Phylogenetic analysis of AMF sequences

Neighbor-joining (NJ) analyses of 157 different glomalean SSU sequences obtained from plant root samples, two obtained from soil samples, and 70 sequences that were downloaded from GenBank made possible the recognition of 12 sequence groups or phylotypes as separate clades, with support in the bootstrap analysis of ≥80% (Fig. 1). The pairwise sequence similarities within the clades ranged from 98 to 100%. Since identical sequences were detected, the clones producing the same sequence for each host plant species and treatment were represented once in the alignment for clarity (see supplemental material for a detailed description of the number of clones of each AMF sequence type that were recovered from each host plant species, soil, and treatment).

The 12 AMF sequence types detected in this study covered four families of Glomeromycota, the Glomaceae, Diversisporaceae, Archaeosporaceae, and Paraglomeraceae. Seven of these sequence types belonged to *Glomus* group A, one to *Glomus* group B (Schüßler et al., 2001), one to *Diversispora*, one to *Archaeospora*, and two to *Paraglomus* (Table 5; Fig. 1).

Three sequence types clustered with previously-identified AMF sequences: the sequence type Glo G1 clustered together with *Glomus intraradices* sequences from GenBank, the Glo G6 showed high similarity to *Glomus mosseae* sequences and Glo G8 to *Glomus lamellosum*. The fungal type Para 1, although showing 95% homology with *Paraglomus occultum*, seem to be a novel taxon within the genus *Paraglomus* since it formed a different clade with a high bootstrap value (100%). The same occurred for the fungal type Arch with *Archaeospora trappei*. The Glo G3 and Para 2 sequence types received very strong support in the phylogenetic analysis and did not cluster with any known *Glomus* or *Paraglomus* sequences, respectively. The remaining sequence types were related to previously-described, root-derived sequences in GenBank belonging to unknown glomalean species and are, therefore, not characterized taxonomically (Fig. 1).

3.7. The AM fungal community composition

The most-abundant AM fungal types in this study were Glo G1 (34.32% of AMF clones analyzed), Div (19.73%), Glo G4 (11.08%), and

Table 5
Number of clones of arbuscular mycorrhizal fungal (AMF) sequence types detected in the six plant species studied under control and SB addition treatments.

Plant species	Treatment	Glo G1	Glo G2	Glo G3	Glo G4	Glo G5	Glo G6	Glo G7	Glo G8	Div	Arch	Para 1	Para 2	Total
Piptatherum miliaceum	Control	17	–	–	–	–	–	1	–	5	–	–	–	23
	SB addition	17	–	–	1	–	12	4	1	1	–	–	–	36
Retama sphaerocarpa	Control	2	4	–	5	1	–	–	–	16	–	2	–	30
	SB addition	20	–	–	6	–	1	–	1	3	–	1	–	32
Psoralea bituminosa	Control	2	5	2	9	1	–	–	2	4	2	1	–	28
	SB addition	9	–	–	8	–	3	1	–	–	–	1	–	22
Coronilla juncea	Control	–	–	5	–	–	6	–	4	19	–	–	–	34
	SB addition	6	–	–	5	–	2	1	10	11	–	–	–	35
Anthyllis cytisoides	Control	23	1	–	–	–	–	–	8	8	–	–	–	40
	SB addition	9	–	3	2	–	–	–	5	3	–	–	–	22
Lolium perenne	Control	11	7	1	5	2	–	2	2	–	–	3	–	33
	SB addition	11	2	2	–	–	1	–	4	3	2	8	3	36
Total	*n*	127	19	13	41	4	25	9	37	73	4	16	3	370
	%	34.32	5.14	3.51	11.08	1.08	6.76	2.43	10.00	19.73	1.08	4.32	0.81	100

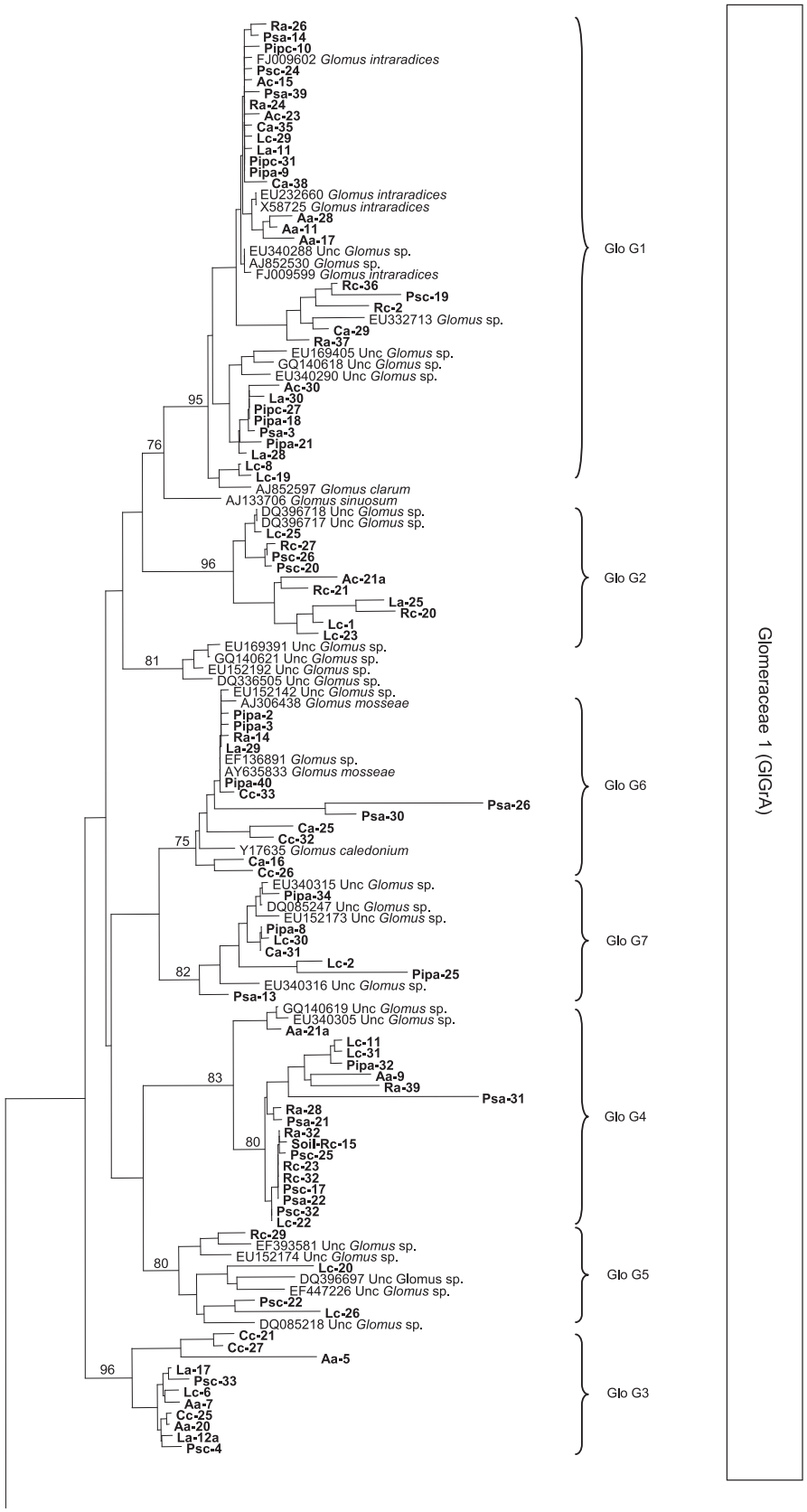

Fig. 1. Neighbor-Joining (NJ) phylogenetic tree showing examples of the AM fungal sequences types isolated from roots of *L. perenne*, *C. juncea, P. bituminosa, P. miliaceum, R. sphaerocarpa* and *A. cytisoides* and rhizospheric soil under control and SB addition and reference sequences from GenBank. All bootstrap values >75% are shown (100 replicates). Sequences obtained in the present study are shown in bold type. They are labeled with the host plant (*L. perenne* = L., *C. juncea* = C., *P. bituminosa* = Ps., *P. miliaceum* = Pip., *R. sphaerocarpa* = R. and *A. cytisoides* = A), soil, treatment (Control = c., SB addition = a) and the clone identity number from which they were obtained. Group identifiers (for example Glo G1) are AM fungal sequences types found in our study. See the supplemental material for a detailed description of the all clones obtained in the present study for each group. *Endogone pisiformis* and *Mortierella polycephala* were used as out-groups.

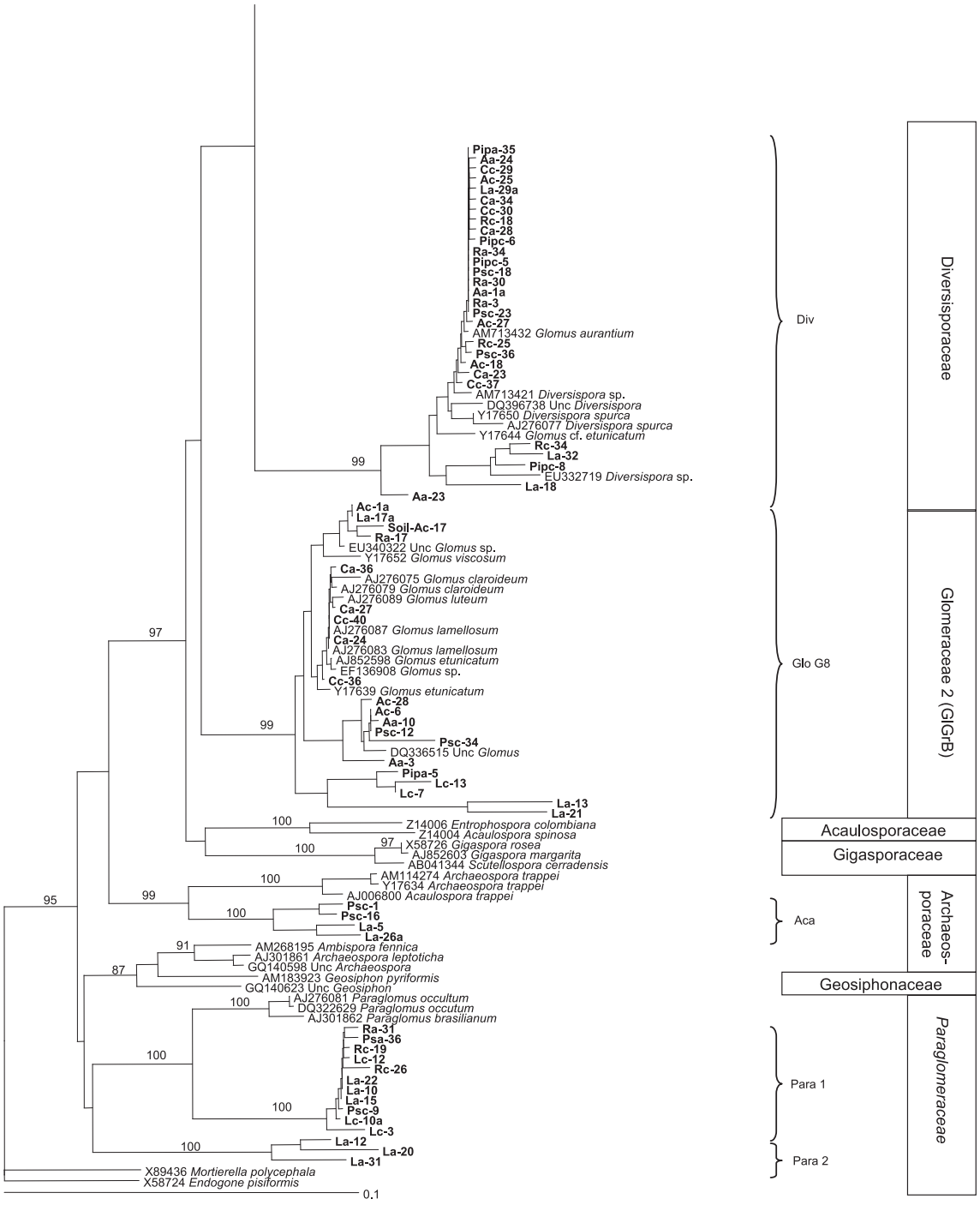

Fig. 1. (*continued*).

Glo G8 (10%). They occurred in the roots of all six plant species studied (Table 5). Glo G1 was the most widespread and, except for *C. juncea* in the control treatment, the only fungal type found in all six plant species under both control and SB addition treatments. Div, related closely to sequences of uncultured *Diversispora* sp. showed higher richness under control conditions, except in the case of *L. perenne.* There were sequence types that occurred exclusively in some plant species and treatments; for example, Glo G5 seemed to be specific for *R. sphaerocarpa*, *P. bituminosa*, and *L. perenne* under control conditions. Also, with the exception of *L. perenne*, Glo G2 appeared exclusively in *R. sphaerocarpa*, *P. bituminosa*, and *A. cytisoides* in the absence of the SB addition. Another AM fungal type, Para 1, accounting for 4.32% of the clones, was found in *R. sphaerocarpa*, *P. bituminosa*, and *L. perenne* in both the control and SB addition treatments. Glo G6 seemed to be specific to *P. miliaceum*, *R. sphaerocarpa*, *P. bituminosa*, and *L. perenne* when SB waste was added to the soil. The Para 2 sequence types occurred exclusively in *L. perenne* in the presence of the SB waste. The rest of the AM fungal types (Glo G3, Glo G7, and Arch), representing 7.02% of the AMF sequences, did not have a clear distribution or behavior, being distributed haphazardly in the different host plants and treatments.

3.8. The AMF communities affected by the plant species and SB addition

The general log-linear analysis demonstrated that the AMF communities and their diversity varied by plant species. In fact, the variable plant species identity was found to have a significant effect on the composition of the AM fungal community ($\chi^2 = 12.87$, 5 df, $P < 0.05$) whereas the variable SB addition did not ($\chi^2 = 1.53$, 1 df, $P < 0.22$). All 12 AM fungal types detected in this study were hosted by *L. perenne* (Tables 2 and 5), *P. bituminosa* was the plant species hosting the second-highest number of AMF sequence types (11), and the remaining species (*P. miliaceum*, *R. sphaerocarpa*, *C. juncea*, and *A. cytisoides*) showed similar numbers of AMF sequence types (between 6 and 8) although the AMF communities were different in the different plant species. Only *P. miliaceum* and *C. juncea* had the most-similar AMF community, since these two plant species shared the same AMF sequence types.

When SB addition was added to soil, the AMF diversity in roots of *P. miliaceum*, *C. juncea*, *A. cytisoides*, and *L. perenne* was increased and the AMF community composition changed with respect to control soil (Table 5). This effect could be observed clearly in the highly-significant host–treatment interaction regarding the AMF community composition ($\chi^2 = 18.43$, 5 df, $P < 0.01$). The greatest influence of the SB addition was on *P. miliaceum*, since the AMF diversity was doubled (from $H' = 0.69$ to $H' = 1.26$) with three new AMF sequence types appearing. *R. sphaerocarpa* showed the same number of AMF sequence types under both treatments (control and SB addition), four of six AMF sequence types being common to both treatments, while *P. bituminosa* was the only plant species for which the SB addition decreased the AMF diversity (from $H' = 1.94$ to $H' = 1.29$).

In the RDA ordination diagram (Fig. 2), we can observe the results described above. Thus, the symbols representing the control and SB addition are close to each other, indicating that the SB addition did not affect significantly the composition of the AMF community. However, the symbols representing different plant species are distant to each other, which demonstrates that the identity of the plant species had a significant effect on the AMF diversity, with the different species hosting distinct AM sequence types. This diagram also shows the AMF sequence types found in each host plant species. When an AM fungal type was common to several plant species, the proximity of these AM fungal types to each plant species depended on its richness.

Sampling effort curves (Fig. 3) showed that for *P. miliaceum*, *A. cytisoides*, *C. juncea*, and *L. perenne* the number of samples analyzed was sufficient to detect the majority of AMF sequence types present in their roots, since the curve leveled off. However, for *P. bituminosa* and *R. sphaerocarpa*, although the curves showed a decreasing rate of accumulation of AMF sequence types they did not approach saturation. Therefore, an increased number of samples of these plant species could have revealed additional AMF sequence types.

3.9. Relationship between the biodiversity of AMF, soil chemical properties, total metal concentrations in plants, and colonized root length

We could not detect any significant correlation between the biodiversity of the AMF, measured as the Shannon–Weaver index, the soil parameters measured, the plant metal and total P concentrations, and the colonized root length.

4. Discussion

Here, we show that the application of an organic amendment, such as sugar beet waste, to semiarid, Mediterranean soils contaminated by heavy metals increased and modified the diversity and composition of the community of arbuscular mycorrhizal fungi and that this depended on the plant species.

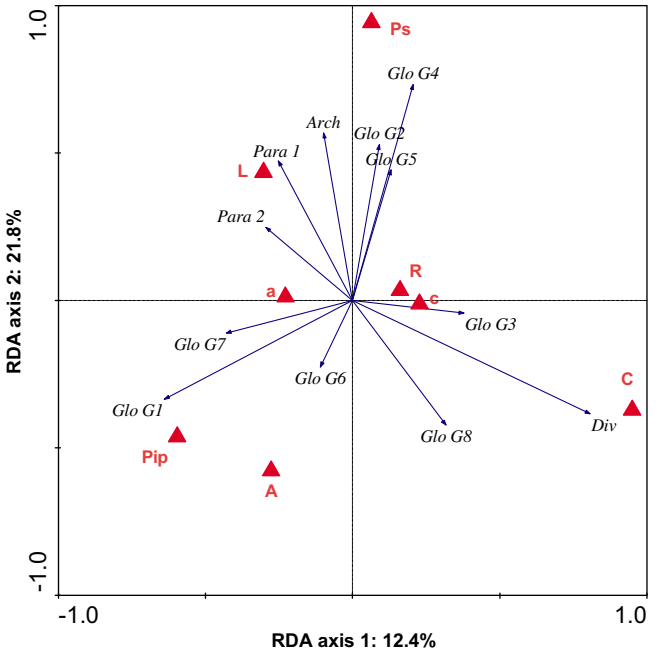

Fig. 2. RDA biplot of the AMF sequence types and environmental factors. Host plant species are represented by *L. perenne* = L., *C. juncea* = C., *P. bituminosa* = Ps., *P. miliaceum* = Pip., *R. sphaerocarpa* = R. and *A. cytisoides* = A and the treatments are represented by Control = c., SB addition = a. All environmental variables explained 66.8% of the total variance. The significance (according to the Monte Carlo permutation test) of all canonical axes was $P = 0.012$, which means that the environmental factors have a significant influence on the distribution of the AMF sequence types. The percentages shown on the first and second axes correspond to the percentages of variance of AMF sequence type data explained by the particular axis.

We detected 12 different AMF sequence types in total, in accordance with the study of Vallino et al. (2006), who found 14 AM fungal types in a polluted, Italian soil. In other molecular work with heavy-metal-polluted soils, Whitfield et al. (2004) and Zarei et al. (2008) detected nine and seven AMF sequence types, respectively, when analyzing the AM fungal community from one host plant species. The AMF diversity found in our study can be considered high if we bear in mind that the total soil content of

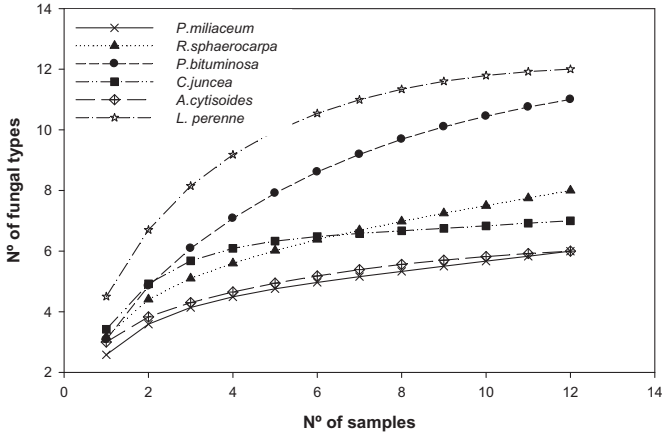

Fig. 3. Sampling effort curves for *L. perenne*, *C. juncea*, *P. bituminosa*, *P. miliaceum*, *R. sphaerocarpa* and *A. cytisoides*. The sample order was randomized by 100 replications in EstimateS, version 8.0 (Colwell, 2005).

heavy metals such as Pb, Zn, and Cd was at least twice as high as that of the most-contaminated site of the above-cited studies. In this regard, Zarei et al. (2008) detected only two AMF types in the most-polluted site of their study. Also, it is important to note that the use of different primers targeting the SSU rDNA, as in our case, and different criteria to define sequence types as well as the higher number of plant species sampled here could mean that the AMF diversities are not directly comparable.

A fungal type possibly adapted to heavy metal stress is Glo G8, identified as *Glomus claroideum*, since besides being found in all the plant species in our study it was one of the dominating fungi in previous surveys of polluted soils (Del Val et al., 1999; Turnau et al., 2001). In contrast, Zarei et al. (2008) and Khade and Adholeya (2009) found this fungus only at non-polluted sites.

Glo G1, which can be assigned to *G. intraradices* can be considered the generalist fungal species "par excellence" since it is present in all the places around the world investigated so far (Öpik et al., 2006; Helgason et al., 2007).

The dominance of *G. mosseae* in disturbed habitats is well documented (Chen et al., 2007). It was the only fungus always present in plant roots in polluted soils in the studies of Turnau et al. (2001) and Whitfield et al. (2004) and the most-abundant fungus in the most-heavy-metal-contaminated sites examined by Vallino et al. (2006). Also, Zarei et al. (2008), using molecular and morphological analyses, found this fungus to be the most-frequent and abundant in all four sites of their study. In contrast, in our work, Glo G6, identified as *G. mosseae*, represented only 6.76% of the total AMF sequences analyzed and was present in the roots of five plant species studied, exclusively with the SB addition treatment (with the exception of *C. juncea* in the control).

In our study, we did not observe a clear relationship between the AMF diversity composition and the degree of root colonization by AMF for the different plant species studied, although we found a good percentage of colonization in all seedlings (mean value around 56%), in accordance with previous work on heavy-metal-polluted sites (Khan et al., 1998; Leung et al., 2007; Zarei et al., 2008; Ortega-Larrocea et al., 2009; Khade and Adholeya, 2009). However, Pawlowska et al. (2000) and Regvar et al. (2006) found that AMF colonization of pioneer plants in mine tailings was low or zero. Also, Vallino et al. (2006), in spite of reporting AMF diversity similar to ours, found colonization in roots of *Solidago gigantea* to be four-fold lower.

The present results show a clear pattern of host preference, since the AMF community composition differed significantly depending on the plant species. Host preference has been reported in other ecosystems (Helgason et al., 2002; Öpik et al., 2003; Sýkorovà et al., 2007; Alguacil et al., 2009b). We used five drought-resistant native plant species belonging to the families Leguminosae and Graminaceae and only one species for comparison, the grass *L. perenne*. Surprisingly, of all the species studied, *L. perenne* roots yielded the highest number of AMF phylotype, hosting all 12 AM fungal types detected. This could be because seedlings growing away from their natural habitat have a greater probability of encountering the mycorrhiza most beneficial for survival (Husband et al., 2002), developing in this way different strategies for survival in contaminated sites with the help of indigenous AMF (Leung et al., 2007). Of the native plant species, *P. bituminosa* was the one that hosted the highest number of AMF types in non-amended soils and is the only metallophyte, plants characterized by their ability to hyper-accumulate heavy metals (Regvar et al., 2006). Therefore, this species might be able to extract heavy metals from the soil, producing a more-suitable environment for the proliferation of the majority of AM fungal types present in its rhizosphere. The remaining species (*P. miliaceum*, *R. sphaerocarpa*, *C. juncea*, and *A. cytisoides*) had a similar number of fungal types (between 6 and 8), Glo G1, Glo G4, Glo G8, and Div. being common to all species.

Between all the plants species studied we could observe that each plant species showed different metal tolerance depending on plant species–AMF combination. That is; the AM fungal types found in plant under non-amended soils are different and host-plant specific. When the soil was amended, the AMF diversity changed and was different depending of plant species. Thus, AMF diversity increased for *P. miliaceum*, *C. juncea*, *A. cytisoides and L. perenne* and decreased for *P. bituminosa*. Although it is to note that AMF diversity in *L. perenne* was the less affected by the SB addition. Moreover, no clear relation was found between metal availability and AM fungal type for each plant species.

Although no correlation was found also between AMF diversity and the soil parameters measured, we found that the addition of SB waste to the soil affected the behavior of the plant–AMF association, modifying the composition of the AM fungal community according to the plant species studied. For the majority of plant species, except *P. bituminosa* and *R. sphaerocarpa*, the SB addition increased and changed the AMF diversity. This may be explained by the supplementary nutrients added to the soil via the SB addition. It has been suggested that nutrient availability affects the composition of AMF communities in the soil (Johnson, 1993; Ezawa et al., 2000; Jacquot-Plumey et al., 2001). We found a generalized increase in the contents of total organic carbon, total N and available P after the SB addition in the rhizospheres from all six plant species studied. Also, the new C input provided by the SB addition could have been used as an energy source by soil microorganisms (such as bacteria and saprophytic fungi) and have stimulated the microbial activity. So, the changes in the AMF community composition might have been mediated by changes in the microbial communities. Alguacil et al. (2009a) showed that application of an urban refuse increased soil microbial activities and the AMF diversity when applied in a semiarid area for restoration purposes.

Heavy metals can inhibit soil organic matter decomposition and N mineralization processes, causing changes in soil properties which can affect negatively the activity and diversity of soil microorganisms, such as AMF (Del Val et al., 1999; Misra, 2000). It has been shown that organic amendments, including SB waste, are able to reduce the solubility and therefore the availability of the metals in the soil and consequently their accumulation in plants (Azcón et al., 2009). In our study, we observed that the addition of SB waste (with some exceptions) reduced metal concentrations in the shoot tissue for five of the six plant species studied. The SB addition increased shoot contents of Al, Fe, Mn and Cd, only for *P. bituminosa*, which was related to the decreased AMF diversity found for this plant species in the amended soil. *P. bituminosa* was the only plant species in our study considered to be a metallophyte, having the ability to colonize heavy-metal-contaminated soils. Thus, since this species has basic strategies to deal with high metal concentrations, such as accumulation involving several mechanisms to immobilize and compartmentalize metals in vacuoles (Becerril et al., 2007), the addition of an organic amendment could produce the opposite effect – reducing and altering the AMF species diversity, as observed in our results.

Therefore, the change produced in the soil microbial status or soil microbiota by the SB addition could have given rise to a microhabitat more suitable for the proliferation of new AM fungal types, which were unable to colonize the root system and/or multiply in the rhizosphere due to fungitoxic effects of heavy metals, thus producing a shift in the AMF diversity.

5. Conclusions

Our study demonstrates that the AM fungal populations colonizing roots in a heavy-metal-polluted soil depend quite strongly on the host plant, the highest diversity values being obtained in an authochtonous, metallophyte species and in an allochtonous

species. The application of SB waste was very efficient with regard to increasing the AMF communities in the majority of the host plant species assayed and decreasing the concentration of metals in shoot tissues. Therefore, the SB addition could be a good strategy for the remediation and/or phytostabilization of mine tailing sites, although its beneficial effects on the survival and proliferation of the majority of AM fungal species detected appear to be restricted to non-metallophyte species.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.soilbio.2011.03.026.

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