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Increased Diversity of Arbuscular Mycorrhizal Fungi in a Long-Term Field Experiment via Application of Organic Amendments to a Semiarid Degraded Soil▽†

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In this study, we tested whether communities of arbuscular mycorrhizal (AM) fungi associated with roots of plant species forming vegetative cover as well as some soil parameters (amounts of phosphatase and glomalin-related soil protein, microbial biomass C and N concentrations, amount of P available, and aggregate stability) were affected by different amounts (control, 6.5 kg m^{-2}, 13.0 kg m^{-2}, 19.5 kg m^{-2}, and 26.0 kg m^{-2}) of an urban refuse (UR) 19 years after its application to a highly eroded, semiarid soil. The AM fungal small-subunit (SSU) rRNA genes were subjected to PCR, cloning, single-stranded conformation polymorphism analysis, sequencing, and phylogenetic analyses. One hundred sixteen SSU rRNA sequences were analyzed, and nine AM fungal types belonging to *Glomus* groups A and B were identified: three of them were present in all the plots that had received UR, and six appeared to be specific to certain amendment doses. The community of AM fungi was more diverse after the application of the different amounts of UR. The values of all the soil parameters analyzed increased proportionally with the dose of amendment applied. In conclusion, the application of organic wastes enhanced soil microbial activities and aggregation, and the AM fungal diversity increased, particularly when a moderate dose of UR (13.0 kg m^{-2}) was applied.

The semiarid Mediterranean areas of Southeastern Spain are affected by environmental degradation and erosive processes due to the fact that they are characterized by a set of climatic conditions that includes irregular and scarce rainfall and long, dry, and hot summers. Under these conditions, the soil organic matter content decreases, and the availability of nutrients and water for plants is reduced. Consequently, soil productivity decreases, levels of below-ground microbially diverse populations decline, and the water deficit limits plant growth so that the vegetation cover of natural soils cannot be sustained. Therefore, the development of revegetation techniques to reduce erosion, to remediate the effects of degradation, and, thus, to allow the restoration of biodiversity is needed. It was previously demonstrated that the application of organic amendments, such as urban refuse (UR), to soil increases the organic matter content of soil and improves the quality and productivity of degraded soils (17, 44, 57). Also, it was previously shown that the organic residues yield an improvement in levels of microbially diverse populations in the soil (43).

A substantial part of the soil microbial communities belongs to the arbuscular mycorrhizal (AM) fungi, an ancient group of fungi belonging to the phylum *Glomeromycota* (49), which form mutualistic associations with the roots of the majority of land plants. These fungi have a variety of beneficial effects on their host plants, such as increasing the uptake of mineral nutrients, particularly phosphorus and nitrogen (41, 52); reduction of pathogen infections (7); improvement of water relations (12) and soil stability (58); and the limitation of heavy metal uptake (34). It is evident that AM fungi are an important factor contributing to the maintenance of terrestrial ecosystem functioning. Studies have shown that the diversity of AM fungal populations in the soil can affect plant diversity and productivity and ecosystem stability (62). Therefore, information on the species composition of the AM fungal community in roots is important for an understanding of mycorrhizal function as well as for the effective management and preservation of the diversity of AM fungal populations in ecological field studies.

Thanks to advances in molecular techniques in recent years, it is possible to apply PCR-based molecular methods in order to analyze the diversity of AM fungi colonizing the roots of an individual plant at any given time. Traditional identification based on spore morphology is often problematic, and the abundance of spores in the soil may not accurately reflect AM fungal community composition and dynamics (8). The single-stranded conformation polymorphism (SSCP) approach is a very sensitive and reproducible technique for analyzing the sequence diversity of AM fungi within roots (30). This method is based on nucleotide differences between homologous sequence strands, which are detected by electrophoresis of single-stranded DNA under nondenaturing conditions (38).

It is known that the application of organic amendments can have a positive effect on the proliferation of natural AM fungi in crop systems (20, 26). The stimulatory effects of the addition

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of organic matter on the development of AM fungi could be related to an improvement in the extensive network of AM fungal mycelium in the soil. In this way, the colonized plants are able to effectively exploit nutrients and water from soil (52). Moreover, AM fungi are able to exploit nutrients released by the mineralization of organic matter due to the activities of mineralizing microorganisms (28). However, there are many previous reports that showed a strong negative impact on the presence of AM fungal populations and mycorrhizal colonization when composted urban waste was added to the soil (19, 46). Also, research using trap cultures of host plants showed a decrease in the level of diversity of AM fungal species in soils amended with sewage sludge (25, 61).

In a previous study carried out in 1992 at the site that is also the subject of the current work, Roldán and Albaladejo (43) found that the application of UR decreased levels of AM fungal populations in the first year after amendment; however, they observed an increase in levels of these populations 3 years after the addition. We hypothesized that after a long period of time, the application of UR could alter the diversity of AM fungal populations in a highly eroded, semiarid soil and that this effect could be influenced by the refuse application rate. In order to verify this hypothesis, we studied the diversity of the AM fungi associated with the roots of plant species forming the vegetative cover of five plots that received different amounts of UR 19 years after the amendment. Also, we determined whether there was an improvement in soil quality parameters related to soil microbial activity.

MATERIALS AND METHODS

Study site. The experimental area was located in Abanilla (30 km north of Murcia, Southeast Spain [1°50′W, 38°11′N]), one of the most degraded areas along the Spanish Mediterranean coast. The climate is arid to semiarid Mediterranean: the annual rainfall averages 200 to 300 mm, mostly in autumn and spring. The mean annual temperature is 19.2°C, potential evapotranspiration reaches 900 mm year^{-1}, and the drought period can last for 11 to 12 months. The natural vegetation of the area is mainly slow-growing, low shrubs with only 2 to 4% canopy cover. The soil in the experimental area is a xeric torriorthent (53), formed from marls, with only an ochric epipedon as a diagnostic horizon. The rate of soil degradation is very high because of the lack of vegetal cover and the nature of the parent material (2). The low organic matter content and silty clay texture facilitate the deterioration of the soil structure and surface sealing and, ultimately, the intensity of degradation. The addition of urban solid refuse (USR) has been shown to be very effective in preventing erosion and physical soil degradation (1) and increased fertility and vegetal biomass and plant cover relative to those of the control plot (13).

Experimental design. In October 1988, a set of experimental plots consisting of five 90-m^2 rectangles was established on a hillside with a 10% slope and northwesterly orientation in a homogeneous area. To improve soil quality, different doses of USR were added to the top 30-cm layer with a rotovator. A control plot was also tilled by the rotovator. The amounts of USR added were as follows: plot 0, control (no addition of USR); plot 1, 6.5 kg m^{-2}; plot 2, 13.0 kg m^{-2}; plot 3, 19.5 kg m^{-2}; plot 4, 26.0 kg m^{-2}. The USR amendment levels were designed to raise the soil's organic matter content by 0.5, 1.0, 1.5, and 2.0% relative that of to the control plot. The USR used in this experiment was fresh material that had been allowed to mature naturally for 10 to 15 days after technical removal of the larger inorganic components (plastic and glass, etc.). Only one application of refuse was carried out at the beginning of the experiment. The analytical characteristics of the USR are shown in Table 1.

Sampling procedures. Soil samplings from the five plots were carried out in early December 2007 after the autumn rainy season (19 years after), when the highest microbial activity could be expected (33). The dominant plant species in the plots were *Phagnalon rupestre* (L.) D.C., *Piptatherum miliaceum* (L.) Cosson., *Stipa parviflora* Desf., and *Plantago lagopus* L. Plant cover was a measuring of the percentage of ground covered by vegetation. This measure is usually used for trend comparisons or for calculations of species composition. Cover is thought to be more ecologically significant than density or frequency because it is an estimate of how much a plant dominates an ecosystem (is more highly related to biomass than density or frequency). Plant cover was estimated by direct estimates of cover using three replication plots (1 m^2). The plots did not show differences in plant community composition; however, the differences between plots were due to the percentages of plant cover. The plant cover in the different treatments was as follows: plot 0, 5%; plot 1 and plot 2, 15%; plot 3 and plot 4, 40%.

TABLE 1. Analytical characteristics of the USR (dry weight) used in the experiment

Parameter	Value
Water content (%)	45.4
Ash (%)	40.7
pH (1:20)	6.5
Electrical conductivity (1:20) (μS cm^{-1})	4220
Total organic carbon (g kg^{-1})	230
Total N (g kg^{-1})	12.7
Total P (g kg^{-1})	2.6
Available P (μg g^{-1})	500
Polysaccharide (g kg^{-1} glucose)	130
Total Cu (μg g^{-1})	237
Total Zn (μg g^{-1})	650
Total Cr (μg g^{-1})	365
Total Cd (μg g^{-1})	2
Total Ni (μg g^{-1})	328
Total Pb (μg g^{-1})	235
Na^+ (g kg^{-1})	6.8
C^- (g kg^{-1})	8.8
K^+ (g kg^{-1})	5.5
SO_4^{2-} (g kg^{-1})	14.0

A within-plot sampling design was established. Samples were taken from the upper, middle, and lower parts of each plot (each treatment was divided into three subplots, equal rectangles of 5 by 5 m) in triplicate from rhizospheric soil from the surface horizon (0 to 25 cm) from each of the three subplots. For each subplot, the samples were combined into bulk composite samples of approximately 2 kg. These samples containing rhizospheric soil and roots were stored in polyethylene bags for transport to the laboratory.

The roots were separated from the soil in the laboratory, washed, quickly dried on paper, and used partly for morphological and partly for molecular analyses.

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% (vol/vol) trypan blue in lactic acid, according to methods described previously by Phillips and Hayman (39). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (18).

Soil biological, biochemical, and physical analyses. Concentrations of soil microbial biomass C and N were determined using a fumigation-extraction method (60). Ten grams of soil at 60% of its field capacity was fumigated in a 125-ml Erlenmeyer flask with purified $CHCl_3$ for 48 h. After the removal of residual $CHCl_3$, 40 ml of a 0.5 M K_2SO_4 solution was added, and the sample was shaken for 1 h before filtration of the mixture. The concentrations of K_2SO_4-extracted C and N were determined with an automatic carbon analyzer for liquid samples (Shimadzu TOC/TN), and microbial biomass C and N values were calculated as the difference between fumigated and nonfumigated samples.

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (0.115 M) as a substrate. Two milliliters of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid (36) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37°C for 90 min. The reaction was stopped by cooling the mixture at 2°C for 15 min. Next, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4,000 rpm for 5 min. The amount of *p*-nitrophenol formed was determined by spectrophotometry at 398 nm (55). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

The percentage of stable aggregates was determined according to a method described previously by Lax et al. (32). A 4-g aliquot of sieved (0.2- to 4-mm sieve) soil was placed onto a small 0.250-mm sieve and wetted by spray. After 15 min, the soil was subjected to an artificial rainfall of 150 ml with an energy of 270 J m^{-2}. The remaining soil on the sieve was placed into a previously weighed capsule (T), dried at 105°C, and weighed (P1). The soil was then soaked in distilled water and, after 2 h, passed through the same 0.250-mm sieve with the

assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105°C and weighed (P2). The percentage of stable aggregates with regard to the total aggregates was calculated as follows (P1 − P2) × 100/(4 − P2 + T).

Glomalin-related soil protein was extracted from soil samples sieved between 0.2 and 4 mm with 20 mM sodium citrate (pH 7.0) at a rate of 250 mg of aggregates in 2 ml of buffer and autoclaved at 121°C for 30 min (66). The supernatant was removed, and two additional sequential 1-h extractions were performed. All supernatants from a sample were combined, the volume was measured, an aliquot was centrifuged at 10,000 × *g* for 15 min to remove soil particles, and the amount of Bradford-reactive total protein was measured.

Available P, extracted with 0.5 M sodium bicarbonate (pH 8.5), was determined by colorimetry according to a method described previously by Murphy and Riley (35).

DNA extraction and PCR. For each sample, total DNA was extracted from root material (representing an approximately 5- to 8-cm root length) using a DNeasy plant minikit according to the manufacturer's recommendations (Qiagen). The roots samples were placed into a 2-ml screw-cap propylene tube, and the DNA extracts were obtained by disrupting roots with a sterile disposable micropestle in liquid nitrogen. The DNA was resuspended in 20 μl of water. Several dilutions of extracted DNA (1/10, 1/50, and 1/100) were prepared. Partial ribosomal small-subunit (SSU) DNA fragments were amplified using two different sets of primers. One set consisted of universal eukaryotic primer NS31 (51), used as a forward primer, and a mixture of equal amounts of primers AM1 (21), AM2, and AM3 (48), which were used as the reverse primer combination. PCRs were carried out in a final volume of 25 μl using Ready To Go PCR beads (Amersham Pharmacia Biotech), 0.2 μM deoxynucleoside triphosphates, and 0.5 μM of each primer (PCR conditions were 95°C for 3 min; 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min 30s; and 72°C for 8 min). As a template, 2 μl of extracted DNA was used in all reaction mixtures. Positive and negative controls using PCR-positive products and sterile water, respectively, were also included for all amplifications. DNA extracts were stored at −20°C.

Concerning the other primer set, a two-step procedure (nested PCR) was conducted. The first amplification with primers NS31 and NS41 (51) was performed as described above but with an annealing temperature of 50°C. Aliquots of 5 μl were run on an agarose gel to estimate the quantity of PCR product. Several dilutions (1/10, 1/20, 1/50, and 1/100) were used as a template for the second PCR step. The second step was conducted with specific primer ARCH1311 (42) in combination with NS8 (64). Reactions were performed under the same conditions as described above for AM1 to AM3/NS31.

All the PCRs were run on a Perkin-Elmer Cetus DNA thermal cycler. Reaction yields were estimated by using a 1.2% agarose gel containing ethidium bromide.

Cloning, generation of SSCPs, 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 units of Red *Taq* DNA polymerase (Sigma) and reamplification with primers NS31/AM1, AM2, and AM3 or ARCH1311/NS8 under the same conditions described above.

Positive clones from each sample were analyzed by SSCP. Samples for SSCP were prepared by mixing 5 μl of the clone PCR product with 5 μl loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.5 M EDTA). The samples then were denatured at 95°C for 5 min, placed on ice to stabilize single strands, and then loaded onto a 20- by 20-cm by 0.75-mm gel containing 0.6× MDE gel (BMA, Rockland, ME), 10% ammonium persulfate, 10× Tris-borate-EDTA buffer, and 1% *N*-tetramethylethylenediamine. Electrophoresis was performed using 0.6× Tris-borate-EDTA buffer and was run at 20°C at a constant voltage of 150 V for 16 h in a D-Code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). Bands were visualized by silver staining using a DNA silver staining kit provided by Bio-Rad.

The SSCP banding patterns obtained from different root samples were compared by eye, and the bands were grouped according to similar mobilities. Examples of each SSCP pattern were chosen for sequencing. The samples were grown in liquid culture, and the plasmid was extracted using the QIAprep Spin miniprep kit (Qiagen). Sequencing was done by the Molecular Biology Laboratory (SACE—Murcia University, Spain) using universal primers SP6 and T7. Sequence editing was done using the program Sequencher, version 4.1.4 (Gene Codes Corporation). Sequences were screened for a possible chimeric origin using the chimera detection function of the Ribosomal Database Project Internet site (httpt://rdp.cme.msu.edu).

Phylogenetic analysis. Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool (5) provided by GenBank. Phylogenetic analysis was carried out for the sequences obtained in this study and those corresponding to the closest matches from GenBank. Sequences were aligned with other previously published glomalean sequences using the program ClustalX (56), and the alignment was adjusted manually using GeneDoc (version 2.6.002; K. B. Nicholas and H. B. Nicholas, Jr. [http://www.psc.edu/biomed/genedoc]). Neighbor-joining (NJ) and maximum parsimony phylogenetic analyses (47) were performed with the program PAUP4.08b (54) using the default parameters. *Paraglomus occultum* and *Paraglomus brasilianum*, ancient taxa of the phylum *Glomeromycota*, were used as the outgroup. Fungal types, equivalent to operational taxonomical units, were defined as groups of closely related sequences, usually with a high level of bootstrap support in the phylogenetic analysis.

Diversity of the AM fungal community. The Shannon-Weaver index (H') 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 $H' = -\sum pi(\ln pi)$, where pi is the number of sequences belonging to each fungal type relative to the total number of sequences.

Statistical analysis. Treatment effects on measured variables (colonization and soil properties) were tested by analysis of variance, and comparisons among treatment means were made using a least-significant-difference test calculated at a P value <0.05. Correspondence analysis (CA) with presence and/or absence data for all fungal types at all plots was performed, and the results were summarized in an ordination diagram. CA is a multivariate statistical method that allows comparisons of AM fungal community compositions between all plots. Statistical procedures were carried out with the software package SPSS 15.0 for Windows (16).

The presence or absence of fungal types in each root sample was used to construct the sampling-effort curves (with 95% confidence intervals) using the software EstimateS 8.00 (9). The sample order was randomized by 100 replications.

Nucleotide sequence accession numbers. Sequences of the clones generated in this study have been deposited in the EMBL database under accession numbers FM876854 to FM876969.

RESULTS

Soil biological, chemical, and physical properties. The soil from plots 3 and 4 (where higher doses of UR were added) had significantly higher levels of acid phosphatase activity and available phosphorus and a higher percentage of stable aggregates than did soil from plots 1 and 0 (the lowest dose of UR and the control, respectively) (Table 2).

The addition of the highest amendment dose (26 kg m^{-2}) to plot 4 significantly increased the concentrations of microbial biomass C with respect to plots 0, 1, 2, and 3; these showed increases which were proportional to the UR dose but which did not differ significantly.

The concentration of N in the microbial biomass and levels of the glomalin-related soil protein also showed increases that were dependent on the amendment dose, with plot 4 showing the highest values and the control plot showing the lowest values.

The percentages of colonization of plant roots did not differ significantly among plots 0, 1, 3, and 4, ranging from 45.3% to 52.3% of the root length (Table 2); however, plot 2 showed the highest value, which differed significantly from that of the control plot. Microscopic analysis showed structures characteristic of AM fungi such as arbuscules and vesicles as well as the presence of dark septate fungi colonizing the plant roots examined.

PCR and SSCP type analysis. All DNA extract preparations consisted of pooled roots of individual plants. The occurrence of AM fungi in 15 root samples (three root samples for each plot) was monitored. Template DNA was amplified success-

TABLE 2. Biological, chemical, and physical properties of soil for the five plots of study ($n = 3$) and degree of AM fungal colonization in roots of cover plants ($n = 6$)[a]

Plot	Mean phosphatase level (μmol *p*-nitrophenol g^{-1} h^{-1}) ± SE	Mean level of GRSP (mg g^{-1} soil) ± SE	Mean concn of MBC (mg kg^{-1}) ± SE	Mean concn of MBN (mg kg^{-1}) ± SE	Mean concn of P available (mg kg^{-1}) ± SE	Mean aggregate stability (%) ± SE	Mean colonization (%) ± SE
0	0.38 ± 0.07b	327 ± 27b	258 ± 23b	12 ± 1b	6.6 ± 0.5b	36.5 ± 2.2b	45.3 ± 5.9b
1	0.52 ± 0.02b,c	519 ± 44c,d	320 ± 29b	15 ± 1b,c	7.0 ± 0.3b	42.3 ± 2.0b,c	52.3 ± 3.2b,c
2	0.74 ± 0.05c,d	395 ± 29b,c	362 ± 23b	18 ± 1c	7.2 ± 1.0b	47.6 ± 1.5c,d	58.4 ± 3.3c
3	0.78 ± 0.05d	458 ± 31b,c	380 ± 19b	21 ± 0c,d	15.2 ± 0.2c	52.2 ± 0.6d	46.2 ± 4.51b
4	0.93 ± 0.01d	654 ± 29d	505 ± 14c	25 ± 1d	14.4 ± 1.5c	54.9 ± 1.8d	46.6 ± 5.2b

[a] GRSP, glomalin-related soil protein; MBC, microbial biomass C; MBN, microbial biomass N. Values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by the least-significant-difference test. Plot 0, control; plot 1, 6.5 kg m^{-2} USR added; plot 2, 13.0 kg m^{-2} USR added; plot 3, 19.5 kg m^{-2} USR added; plot 4, 26.0 kg m^{-2} USR added.

fully with the combination of primers AM1, AM2, and AM3/NS31. All samples generated PCR products of the expected band of 550 bp, which were used for cloning and creating a clone library. From the 15 clone libraries, a total of 600 clones were screened by PCR (40 clones by sample); out of these, 536 contained the SSU rRNA gene fragment (see Table S1 in the supplemental material). All of these 536 clones were subjected to SSCP analysis. Representative clones for each pattern were sequenced, for a total of 170 sequences, while the remaining 366 clones were classified by the SSCP pattern. After preliminary BLAST searches, a total of 213 clones (39.7%) had a high degree of similarity to sequences from taxa belonging to the phylum *Glomeromycota*, while the 323 remaining clones (60.3%) were identified as being non-AM fungi since they showed a high level of similarity to sequences from the *Ascomycota* (Fig. 1). Sequences belonging to the *Ascomycota* were detected in all clone libraries. The band patterns that produced sequences of nonglomalean origin in the SSCP gels were easily distinguished, as they migrated far in the gel and showed migration patterns different from those of glomalean bands.

With respect to the other set of primers used, ARCH1311/NS8, with an aim to analyze whether AM fungi belonging to the *Paraglomeraceae* or *Archaeosporaceae* were present inside the roots grown in each of the five investigated plots, no PCR products or only non-AM fungal amplicons were obtained. This was probably because of the absence of these fungi in our samples (data not showed).

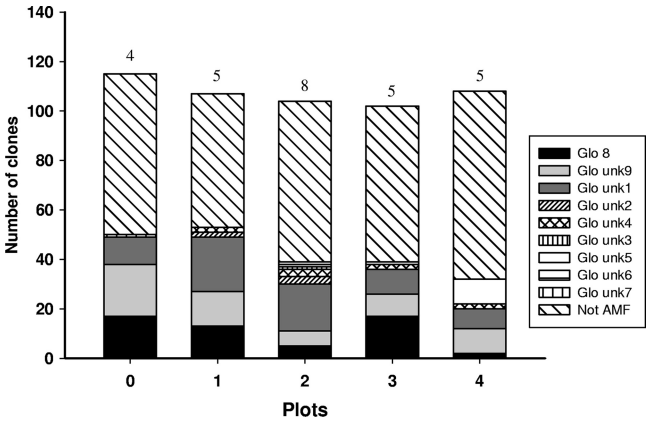
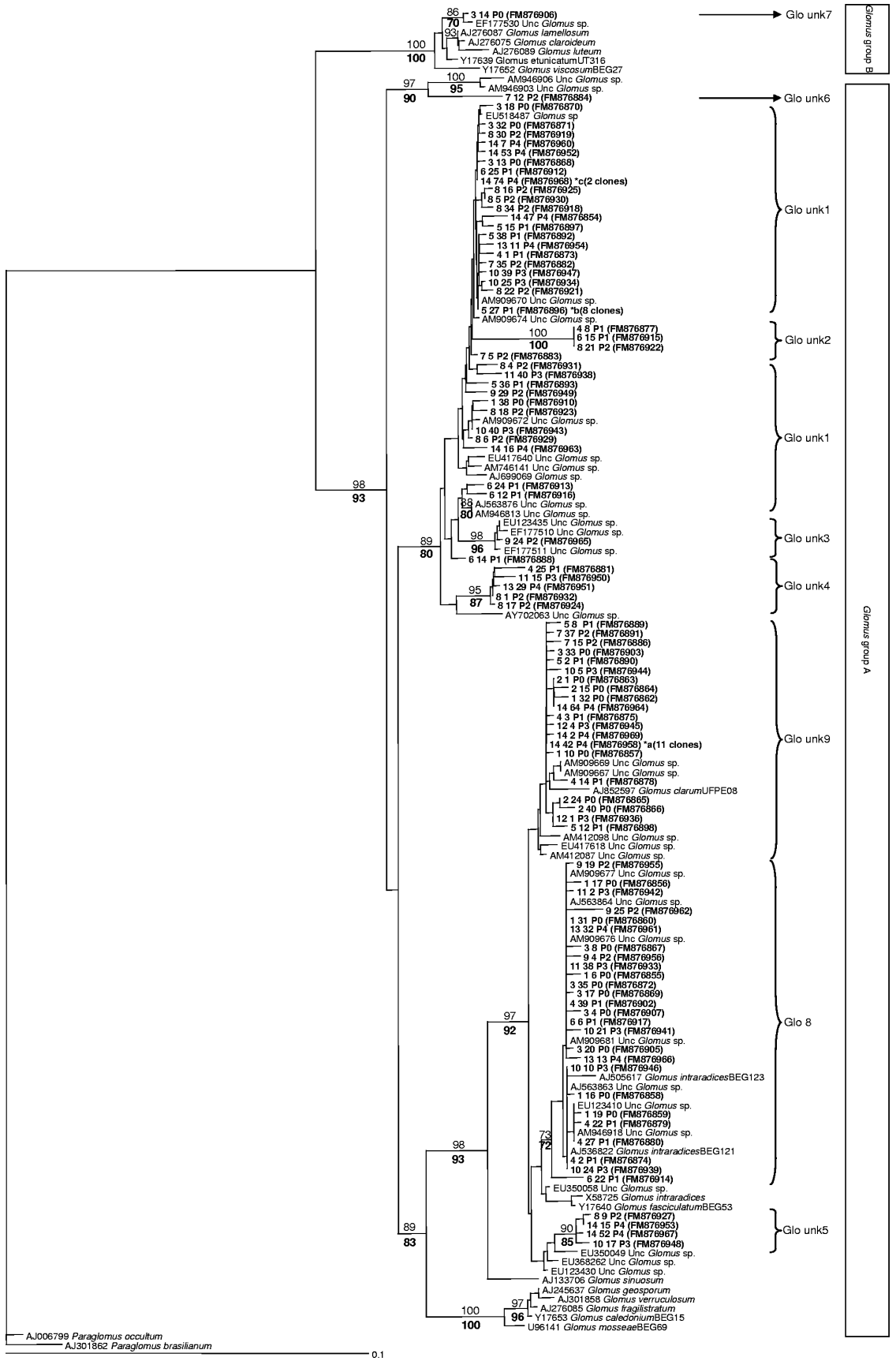


FIG. 1. Proportional distribution of the total number of clones detected for each fungal type according to SSCP patterns in the roots of plant cover in the five plots analyzed (plot 0, control; plot 1, 6.5 kg m^{-2} USR added; plot 2, 13.0 kg m^{-2} USR added; plot 3, 19.5 kg m^{-2} USR added; plot 4, 26.0 kg m^{-2} USR added). The richness of AM fungal types is indicated at the tops of the respective bars.

Phylogenetic analysis of AM fungal groups. All sequences had high levels of similarity (98 to 100% identity) to AM fungi and belonged to members of the phylum *Glomeromycota* (Fig. 2). The constructed alignment included 50 sequences that were downloaded from GenBank and the 95 different glomalean sequences that were recovered in this study. Some clones produced the same sequence and are represented just once in the alignment (see the supplemental material for a detailed description of the clone groups with identical sequences). The sequences obtained in this study were clustered into nine discrete groupings or fungal types with support in the maximum parsimony and NJ bootstrap analyses of ≥70%. The nine sequence groups were mainly consistent with SSCP patterns and were grouped into the family *Glomeraceae*. Eight of these sequence groups belonged to *Glomus* group A, which was the most frequently represented group. *Glomus* group B was represented by only one fungal type. The majority of AM fungal types showed a high level of similarity to previously described, root-derived sequences in GenBank belonging to uncultured glomalean species. Three clusters (Glo unk2, Glo unk4, and Glo unk5) did not seem to be related to any sequences of AM fungi in culture or previously found in the database; thus, they could be species unique to this environment. Only clade Glo 8 included sequences which were 99% identical to the sequences of *Glomus intraradices* BEG121 and BEG123. Clade Glo unk9 showed a high level of similarity to *Glomus clarum*, but this relationship was not well supported by the analysis.

Analysis of AM fungal community distribution. The three most common fungal types (Glo unk1, Glo 8, and Glo unk9) accounted for 86.5% of the AM fungal clones analyzed and were present in all five plots (Fig. 1; see also Table S2 in the supplemental material). Glo unk3 and Glo unk6 seemed to be specific to plot 2, which had received 13 kg m^{-2} of UR. Glo unk7 was found exclusively in plot 0 (no addition of UR). The remaining AM fungal types (Glo unk2, Glo unk4, and Glo unk5) were found principally in plots which had received different amounts of UR. Plot 2 had the most diverse AM fungal community, hosting eight of the nine fungal types found in this study, and exhibited the highest Shannon-Weaver diversity index ($H' = 1.58$). The rest of the plots which had received UR (plots 1, 3, and 4) showed similar Shannon diversity indices (between 1.30 and 1.40), and each one harbored five fungal



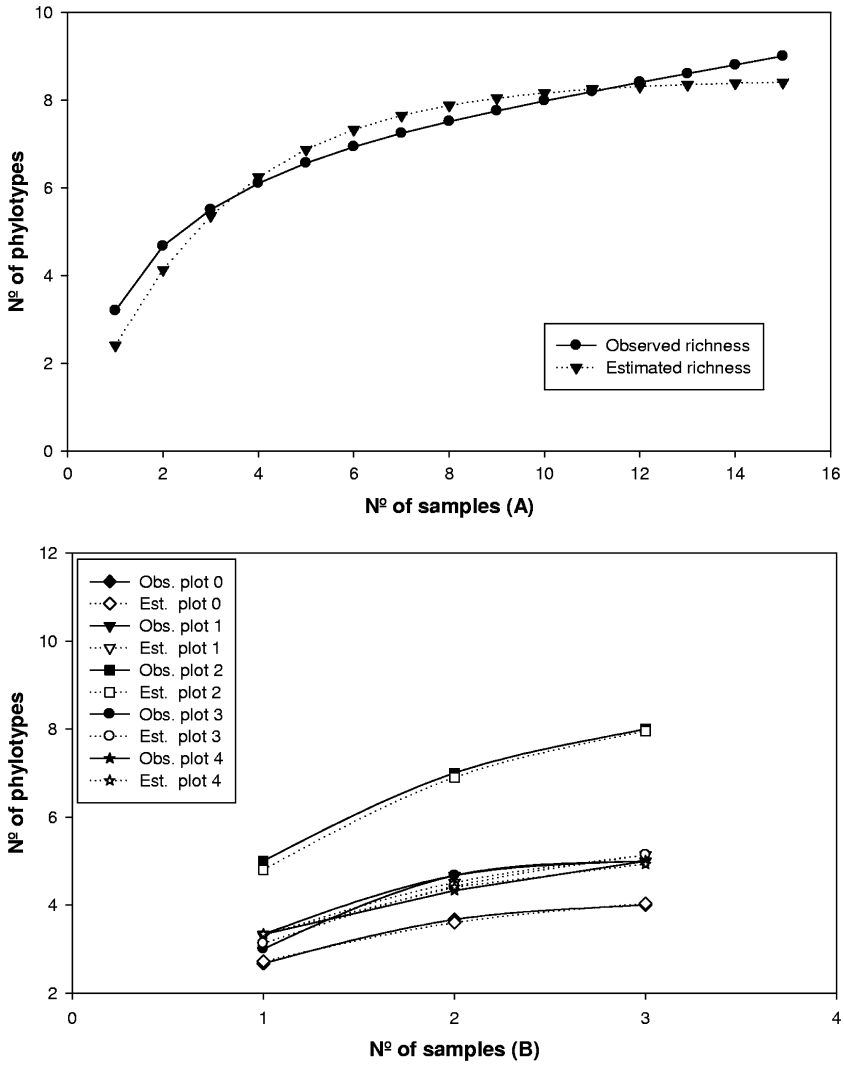


FIG. 3. Sampling-effort curves for the AM fungal community representing the observed (Obs.) and the estimated (Est.) richnesses for the whole sampling (A) and for each UR dose (B). The sample order was randomized by 100 replications in EstimateS, version 8.0 (9).

types; however, the plot that did not receive UR (plot 0) had the lowest AM fungal species diversity ($H' = 1.14$).

The sampling-effort curve (Fig. 3) showed that the number of analyzed root samples was sufficient to detect the majority of fungal types present in the roots as the curve approaches saturation. The estimated-richness curve showed the same tendency as the observed-richness curve, and its slope had a value of 0.018, which was lower than 0.05 (slopes lower than a cutoff value such as 0.05 are complete enough to consider a taxonomic unit or phylotype to be well sampled for studies of biodiversity) (23).

The distribution given in the CA biplot (Fig. 4) clearly showed which fungal types occurred in which of these five plots. Thus, the diagram showed that a higher level of AM fungal diversity was found in plot 2, harboring all the fungal types found except Glo unk7. Plots 2, 4, and 5 harbored five fungal types, and plot 0 showed low-level AM fungal diversity, with four fungal types.

FIG. 2. NJ phylogenetic tree showing AM fungal sequences isolated from roots of cover plants in the five plots analyzed and reference sequences from GenBank. Numbers above branches indicate the bootstrap values (above 70%, 100 replicates) of the NJ analysis; numbers below branches indicate the bootstrap values of the maximum parsimony analysis. Sequences obtained in the present study are shown in boldface type. They are labeled with the clone identity number, the number of plots from which they were obtained (plot 0 [P0], plot 1, plot 2, plot 3, and plot 4), and the database accession number in parentheses (e.g., EMBL accession number FM876927). Identical sequences are grouped and are represented by letters, followed by the numbers of clones having that particular sequence. See the supplemental material for a detailed description of these clones' identifiers. The brackets show the delimitation of the fungal types found in this study. Group identifiers (for example, Glo unk1) are AM fungal sequence types found in our study. *Paraglomus occultum* and *Paraglomus brasilianum* were used as outgroups. Unc, uncultured.

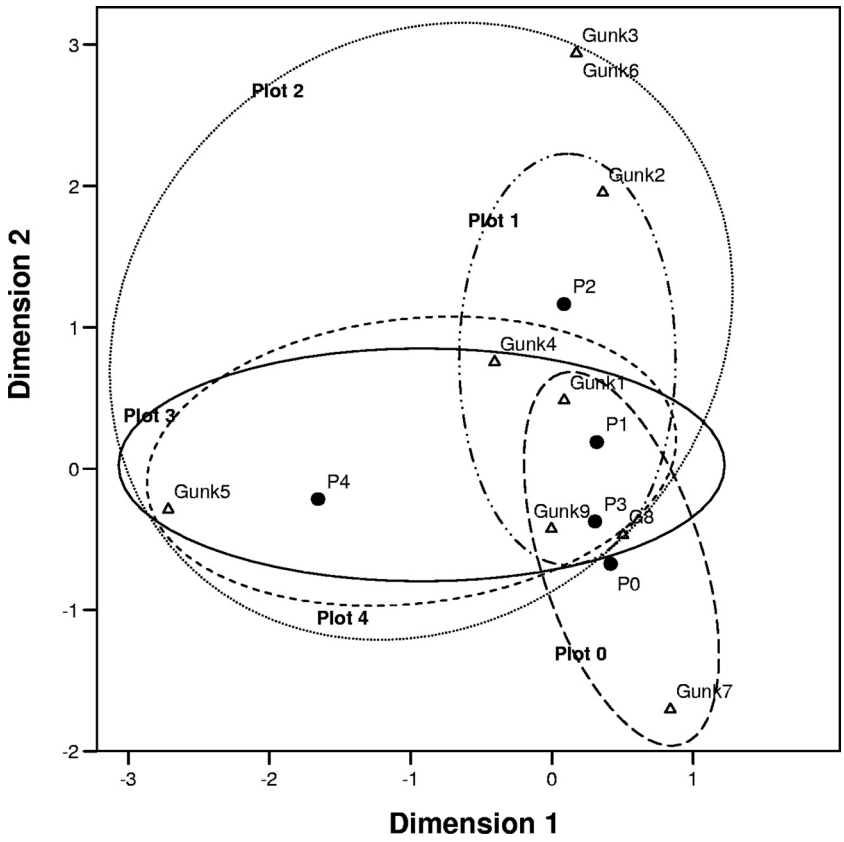


FIG. 4. CA of the AM fungal communities found in the roots of plant cover for the five different plots. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as follows: dimension 1, 0.50; dimension 2, 0.40. Circles represent the respective plots, and the triangles represent the fungal types. Ovals with dashed, dotted-dashed, dotted, full, and small dashed lines represent the distributions of diversity of AM fungal populations in plot 0 (P0), plot 1, plot 2, plot 3, and plot 4, respectively.

DISCUSSION

This study is the first to use PCR-SSCP to characterize the molecular diversity of AM fungi in a highly eroded, Mediterranean, semiarid soil a long time (19 years) after the application of different doses of UR. A previously reported study on this topic used spore numbers, root colonization percentages, and infective propagules in order to evaluate the native mycorrhizal populations during the first and third years after amendment (43).

We used a reverse-primer combination composed of a mixture of primers AM1, AM2, and AM3 (48), since AM1 exhibits some mismatches in the annealing sites with taxa belonging to *Glomus* groups B and C (50). These primers amplified an AM fungal consortium, colonizing the roots of a vegetation cover, composed of various taxa (eight) within *Glomus* group A and only one taxon belonging to *Glomus* group B, both from the family *Glomeraceae*. However, we did not detect the presence of fungal sequences belonging to the families *Acaulosporaceae*, *Diversisporaceae*, *Gigasporaceae*, and *Pacisporaceae*, which can also be detected with this set of primers, in spite of employing this multiple-primer strategy. Also, no sequences from representatives of the families *Paraglomeraceae* and *Archaeosporaceae* were detected using the other primer combination, ARCH1311/NS8. This could be caused by their absence in our samples.

As in previously reported studies carried out for other ecosystems such as tropical forests (24), agricultural sites (3, 22), wetland soils (65), gypsum soil (4), or polluted soils (59), we also found an AM fungal community dominated by *Glomus* species. They survive and propagate more easily thanks to the high sporulation rate and the ability to colonize via pieces of mycelium or mycorrhizal root fragments of this genus (11). These attributes might explain why *Glomus* species are better adapted to disturbed environments.

Out of the nine fungal types detected, three (Glo unk1, Glo 8, and Glo unk9) were the most abundant sequence groups in our study, representing 86.5% of all the AM fungal sequences found, and were present in the five plots studied (Fig. 1 and 4). These fungal types were already detected in previously reported molecular field studies with different hosts and ecosystems (4, 6, 65), suggesting a certain adaptation of these fungal types to a wide range of harsh conditions. It is worth noting that the community most similar to that in our study in terms of sequence similarity of fungal types is that in gypsum soils described previously by ourselves (4), which, apart from being a very similar ecosystem (semiarid Mediterranean), is also the closest geographically. The sequences clustering in the Glo unk2, Glo unk 4, and Glo unk5 fungal types were found only in the plots amended with different doses of UR and were detected for the first time; i.e., no sequences from these fungal

types have been published previously. Therefore, they could be novel fungal groups that possess some functional mechanism allowing them to adapt to and proliferate in soils treated with UR. As the fungal type Glo unk7 was detected only in plot 0 (no amendment), we might conclude that this sequence type possesses a higher degree of sensitivity to soil amendment with UR. Finally, Glo unk3 and Glo unk6 were related closely to sequences of uncultured *Glomus* spp. and were found exclusively in plot 2, suggesting that the soil conditions resulting from the addition of 13 kg m^{-2} of UR are suitable for the proliferation of the majority of AM fungal species detected in this study.

Interestingly, the same tendency shown by the percentage of colonization was observed for the diversity of the AM fungal community in the roots growing in the five plots sampled. Plot 0 (no addition of UR) showed the lowest level of colonization and fewer AM fungal species than the other plots (Table 2 and Fig. 1 and 4). However, the plot that received 13 kg m^{-2} of UR had the largest number of AM fungal species, and the roots of its plant cover showed a significant percentage of colonization (58%). Interestingly, the percentage of colonization increased by 10% for all treatments with respect to data for the year 1993.

In this study, the changes in the AM fungal diversity in response to the application of different doses of UR that we observed might be due to several changes: in the soil fertility due to increases in the nutrient content and organic matter of the soil, in weed populations, or in microbial activity.

Previous studies that reported the use of organic amendments in eroded soils show that they can improve soil productivity, increasing the soil nutrient status (10). It was previously proposed that nutrient availability affects the composition of AM fungal communities in the soil (15, 27). In the present study, the addition of UR to soil increased the available P content relative to that in the control plot, with the content increasing with the dose of UR (Table 2). The occurrence of more fungal groups in the roots from the amended soils shows that these fungi were more tolerant to these doses.

On the other hand, the increased level of diversity of the AM fungal community in the roots of amended soils, compared with that of the control soil, might be due to the changes in the composition of the weed plant community in response to different doses of UR and differences in infection among species. At this site, Roldán and Albaladejo (43) found that during the first year after the addition of UR, plots were covered by pioneer plant species from genera that did not form mycorrhizas; later, these weed populations were replaced progressively by mycorrhizal genera (13). At the time of sampling, plant communities in the experimental plots did not differ in species composition but in cover percentage.

The level of soil microbiological activity, measured as the C and N biomass and phosphatase activity (17), was also increased by the addition of UR (Table 2). The improvement of soil biological fertility promotes soil microbial populations such as AM fungi (43); thus, it is possible that the effects of the UR on the composition of the AM fungal community might have been mediated by changes in the microbial communities.

We cannot discount the possibility of seasonal variations in the communities of AM fungi colonizing the roots, although we selected the sampling date where the highest level of microbial activity is expected (33). In fact, Roldán and Albaladejo (43) found seasonal variations in colonization and chemical properties of the soil in the same plots 19 years ago, although they did not study AM fungal biodiversity. However, other studies of the seasonal variations in species compositions of the AM fungal communities colonizing roots did not reveal any significant changes (45, 48).

As in other studies carried out using primer AM1 with different host plants and ecosystems (3, 4, 14, 42) and in the work of Santos-González et al. (48) using the same two additional primers as those used by us, we observed that the roots showed a surprisingly large number of *Ascomycetes* sequences. These are another group of root-colonizing fungi that coexist with the AM fungi and whose function is not well known in most cases (29), ranging from pathogenic to beneficial. In the literature, they are referred to as dark septate endophytes, and it was previously reported that they colonize as many plant species and are as abundant as AM fungi (31), in accordance with our results. It was previously suggested that in arid ecosystems, dark septate endophytes contribute to plant drought resistance and facilitate nutrient acquisition (37, 63); thus, they possess an important ecological role in the maintenance of plant community structure and in the function of these ecosystems (40). In our case, we detected approximately the same numbers of clones corresponding to *Ascomycetes* in all plots sampled (Fig. 1), except in plot 4, where an increase of about 17% was observed. We suggest that under the conditions which developed after the addition of the highest doses of amendment, there was a change in the pattern of the fungal community due to the higher level of microbial activity.

In conclusion, the addition of different doses of UR improved a number of soil properties related with microbial activities when measured 19 years later, with the increase being proportional to the applied dose. Also, the community of AM fungi was more diverse after the application of this amendment, with the plot that received 13 kg m^{-2} of UR being the most suitable for the survival and proliferation of the majority of AM fungal species detected in this study. Therefore, the application of organic amendments can help to mitigate the degradation of semiarid degraded lands, improving soil aggregation and microbial activities, and this technique increased AM fungal diversity even on a long-term basis.

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