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Host Preferences of Arbuscular Mycorrhizal Fungi Colonizing Annual Herbaceous Plant Species in Semiarid Mediterranean Prairies

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In this study, we have analyzed and compared the diversities of the arbuscular mycorrhizal fungi (AMF) colonizing the roots of five annual herbaceous species (*Hieracium vulgare*, *Stipa capensis*, *Anagallis arvensis*, *Carduus tenuiflorus*, and *Avena barbata*) and a perennial herbaceous species (*Brachypodium retusum*). Our goal was to determine the differences in the communities of the AMF among these six plant species belonging to different families, using *B. retusum* as a reference. The AMF small-subunit rRNA genes (SSU) were subjected to nested PCR, cloning, sequencing, and phylogenetic analysis. Thirty-six AMF phylotypes, belonging to *Glomus* group A, *Glomus* group B, *Diversispora*, *Paraglomus*, and *Ambispora*, were identified. Five sequence groups identified in this study clustered to known glomalean species or isolates: group *Glomus* G27 to *Glomus intraradices*, group *Glomus* G19 to *Glomus iranicum*, group *Glomus* G10 to *Glomus mosseae*, group *Glomus* G1 to *Glomus lamellosum/etunicatum/luteum*, and group *Ambispora* 1 to *Ambispora fennica*. The six plant species studied hosted different AMF communities. A certain trend of AMF specificity was observed when grouping plant species by taxonomic families, highlighting the importance of protecting and even promoting the native annual vegetation in order to maintain the biodiversity and productivity of these extreme ecosystems.

Arbuscular mycorrhizal fungi (AMF) form associations with the majority of terrestrial plant species. Among their beneficial effects, they improve the growth and nutrition of individual plants (41, 52), thus promoting plant performance and enhancing the sustainability of ecosystems (54).

Plant diversity and productivity in ecosystems are influenced significantly by the AM fungal diversity in the soil (50). In the Mediterranean semiarid areas of southeast Spain, the acceleration of soil degradation due to the climatic characteristics of this area has become an important problem. Both the rainy season, with scarce and irregular rainfall, and the dry summer contribute to this process. Under these conditions, besides a loss of vegetation cover and degeneration of the physical, chemical, and biological soil properties, a reduction in the amount, diversity, and activity of AMF is produced (9, 34). It has been shown that an increase in the number of shrub species used to restore degraded lands increases the AMF diversity in the soil and, thus, the below-ground, positive interactions (5). Previous studies have revealed that AMF stimulate the growth of shrubs and improve their drought tolerance (15, 33), while at the same time, the shrubs exert a selective pressure on the AMF species (2). Thus, it is important to obtain a better understanding of the composition of the AMF community in these ecosystems.

Recent reports have shown that AMF-plant associations may not always be species specific (31, 37) and that factors such as soil properties can determine the AMF-plant interactions (24, 26, 38).

The application of molecular techniques in recent years to identity AMF in the field, particularly in plant roots, has revealed a high diversity, including many sequences that cannot be related to known taxa (19, 30).

Until now, all the molecular studies carried out in these areas have been on woody (shrub) perennial plant species (6, 4), but little is known about the annual plant species that grow exclusively in semiarid prairies when the conditions are favorable (rainy periods during winter and spring) (47) and their relationships with AMF.

In this study, we have analyzed and compared the diversities of the AMF colonizing the roots of five annual herbaceous species (*Hieracium vulgare*, *Stipa capensis*, *Anagallis arvensis*, *Carduus tenuiflorus*, and *Avena barbata*) and a perennial herbaceous species (*Brachypodium retusum*). Our goal was to determine the differences in the communities of the AMF among these six plant species belonging to different families in order to ascertain a possible effect of host life strategy. Achieving this objective can provide insight into the strategies for conservation of the diversity in semiarid ecosystems.

MATERIALS AND METHODS

Study site. The experimental area was located in the natural ecological park Vicente Blanes in Molina de Segura, Province of Murcia (Southeastern Spain) (coordinates, 38°12′N, 1°13′W; altitude, 393 m). The climate is semiarid Mediterranean with an absence of a frost period, the average annual rainfall is lower than 270 mm, the mean annual temperature is 19.2°C, and the potential evapotranspiration (PET) reaches approximately 1,000 mm. The soil in the experimental area is a Typic Torriorthent (42) with low organic matter content and a silty clay texture. The vegetation in the zone was dominated by *Piptatherum miliaceum* (L.) Cosson and some shrubs of *Thymus vulgaris* L. and *Rosmarinus officinalis* L. growing with patchy distribution.

Experimental design. The field sampling was conducted using a factorial design with four replication blocks (4 m by 4 m) separated by 20 m. Six plant species growing in this area were selected: *Hieracium vulgare* Tausch (Asteraceae), *Anagallis arvensis* L. (Primulaceae), *Stipa capensis* Thunb (Poaceae), *Carduus tenuiflorus* Curtis (Asteraceae), *Avena barbata* Brot. (Poaceae), and *Brachypodium retusum* (Pers.) P. Beauv. (Poaceae). Excepting *B. retusum*, a perennial herbaceous species, all were annual herbaceous species. *B. retusum* was the only perennial species in the area

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which had a high enough abundance to include it in the block experimental design.

Sampling. In the second half of April 2010, plants including root systems of the six plant species selected were harvested and placed in polyethylene bags for transporting them to the laboratory, where they were stored at 4°C prior to their examination. Roots were then briefly rinsed, quickly dried on paper, and used for molecular analysis. Four plants each of the six selected species were collected per replication block (a total of 96 plants).

Root DNA extraction and PCR. DNA extractions from 24 root samples (one root sample from each replication block for each plant species) were carried out. The root 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 rpm) using a mixer mill (MM 400; Retsch, Haan, Germany). For each sample (total of 24), total DNA was extracted from 0.1 g (fresh weight) root material using a DNeasy plant minikit following the manufacturer's recommendations (Qiagen). The extracted DNA was resuspended in 20 μl of water. Two microliters was used as the template for PCR amplifications. Partial small-subunit (SSU) rRNA gene fragments were amplified using nested PCR with the universal eukaryotic primers NS1 and NS4 (53). PCR was carried out in a final volume of 25 μl using the ready-to-go PCR beads (Amershan Pharmacia Biotech), 0.2 mM deoxynucleoside triphosphates (dNTPs), and 0.5 mM each primer (PCR conditions, 94°C for 3 min and then 30 cycles at 94°C for 30 s, 40°C for 1 min, and 72°C for 1 min, followed by a final extension period at 72°C for 10 min). Two microliters of several dilutions (1/10, 1/20, 1/50, and 1/100) from the first PCR was used as the template DNA in a second PCR performed using the specific primers AML1 and AML2 (25). PCRs were carried out in a final volume of 25 μl using the ready-to-go PCR beads (Amershan Pharmacia Biotech), 0.2 mM dNTPs, and 0.5 mM each primer (PCR conditions, 94°C for 3 min and 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). Positive and negative controls using PCR-positive products and sterile water, respectively, were also included in all amplifications. 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 GelRed (Biotium).

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* XL2-Blue. Thirty-two positive transformants were screened in each resulting SSU rRNA gene library, using 0.8 units of RedTaq DNA polymerase (Sigma) and a reamplification with the 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.

Clones were grown in liquid culture, and the plasmid was extracted using the QIAprep spin miniprep kit (Qiagen). The sequencing was done by the 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).

Phylogenetic analysis. Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) (8) provided by NCBI.

Phylogenetic analysis was carried out on the sequences obtained in this study and those corresponding to the closest matches from GenBank as well as sequences from cultured AMF taxa, including representatives of the major taxonomical groups described by Schüßler et al. (40). Sequences were aligned using the program ClustalX (46), and the alignment was adjusted manually in BioEdit software version 7.0.4.1 (18). A phylogenetic tree based on maximum parsimony (MP) that was implemented in MEGA version 4.0.2 (44) was constructed. The MP tree was obtained using the close-neighbor-interchange algorithm. The MP bootstrap values were calculated with 1,000 replicates. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 663 positions in the final data set, out of which 187 were parsimony informative. *Endogone pisiformis* Link and *Mortierella polycephala* Coem. were used as the outgroups.

Different sequence types or phylotypes were defined as groups of closely related sequences, usually with a high level of bootstrap support in the phylogenetic analyses (higher than 80%) and with pairwise similarity (higher than 97%). The pairwise analysis within clusters was carried out using BioEdit software version 7.0.9.0 (18).

Diversity analysis. To investigate the influence of environmental factors (host plant species) on the distribution of the AMF phylotypes in the root samples, ordination analyses were conducted in CANOCO for Windows, version 4.5 (45), using the presence/absence data for each root sample. 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 sequence types.

The Shannon (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 $H' = -\Sigma p_i(\ln p_i)$, 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]).

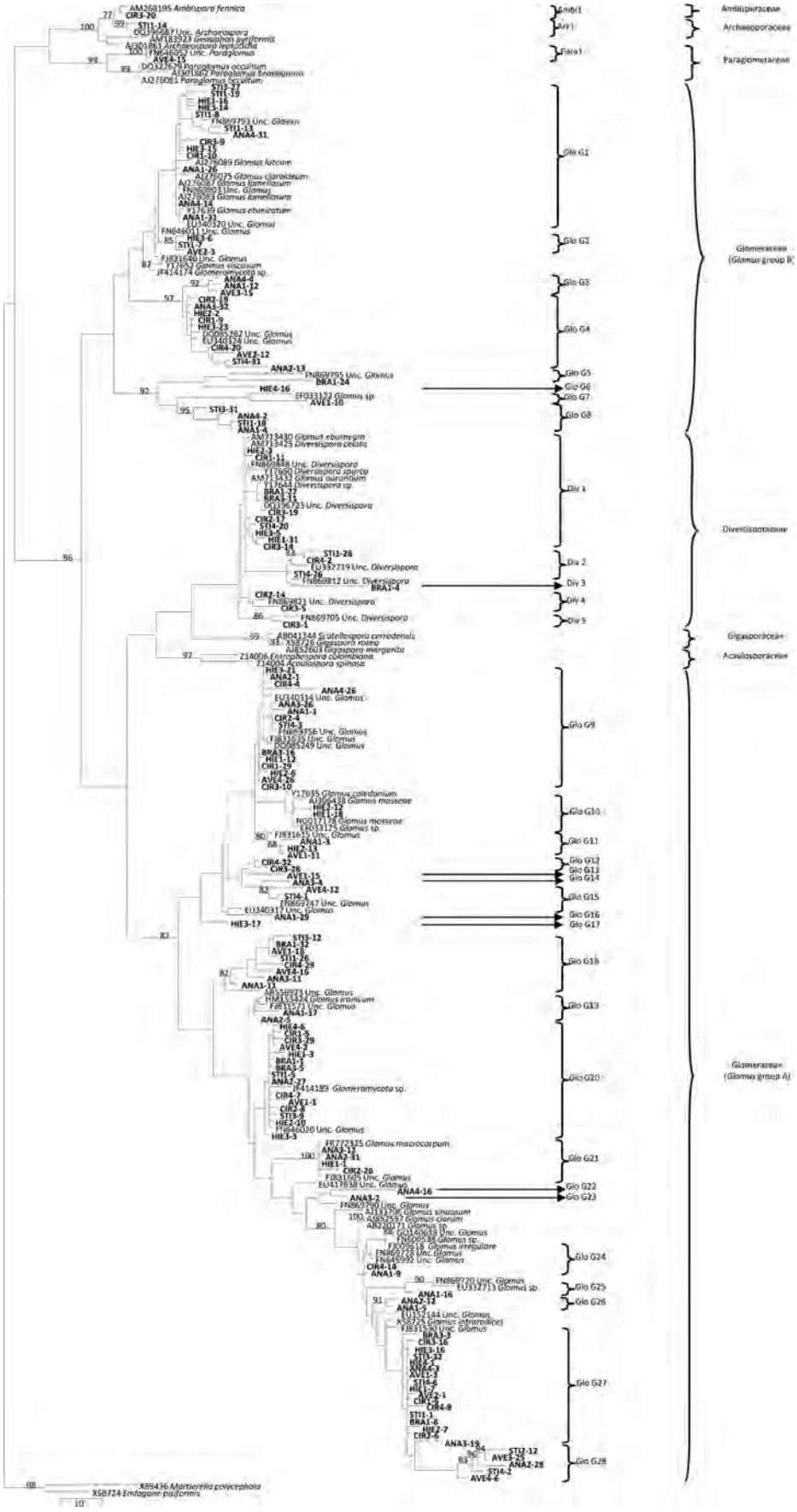
The presence or absence of AMF phylotypes in each soil sample was used to construct the sampling effort curves (with 95% confidence intervals) using the software EstimateS 8.0 (12). The sample order was randomized by 100 replications.

Nucleotide sequence accession numbers. A total of 138 representative sequences of the clones generated in this study have been deposited at the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers HE614945 to HE615082.

RESULTS

PCR and sequence analysis. All the root samples extracted were amplified successfully by nested PCR and generated PCR products of the expected band of approximately 795 bp which were used for cloning and for creating a clone library. From the 24 clone libraries, a total of 768 clones were screened by PCR (on average, 32 clones were analyzed per sample/library); out of these, 526 clones contained an insert of the right length, and subsequently, all clones were sequenced. The BLAST search revealed that 463 sequences (88%) had a high degree of similarity (97 to 100% similarity) to sequences from taxa belonging to the phylum *Glomeromycota*. The rest of the sequences were erroneous.

Phylogenetic analysis of AMF groups. The phylogenetic analyses of 463 glomalean sequences obtained in this study from plant roots and 75 sequences downloaded from GenBank made possible the recognition of 36 phylotypes as separate clades on the basis of bootstrap values of ≥80%. The sequence groups or phylotypes covered five families of *Glomeromycota*: the *Glomeraceae* (*Glomus* groups A and B), *Diversisporaceae*, *Paraglomeraceae*, *Ambisporaceae*, and *Archaeosporaceae*. The pairwise sequence similarities within the clades ranged from 97 to 100%. The 138 clones that produced different sequences were represented in the MP tree. Of all the AMF sequence types, 20 belonged to *Glomus* group A, eight to *Glomus* group B, five to *Diversispora*, one to *Paraglomus*, one to *Ambispora*, and one to *Archaeospora* (Fig. 1). Five sequence groups identified in this study clustered to known glomalean species or isolates: group *Glomus* G27 to *Glomus intraradices*, group *Glomus* G19 to *Glomus iranicum*, group *Glomus* G10 to *Glomus mosseae*, group *Glomus* G1 to the *Glomus lamellosum/etunicatum/luteum* group, and group *Ambispora* 1 to *Ambispora fennica*. There were other groups, such as *Glomus* G2, *Glomus* G3, *Glomus* G6, *Glomus* G8, *Glomus* G12, *Glomus* G13, *Glomus* G14, *Glomus* G16, *Glomus*



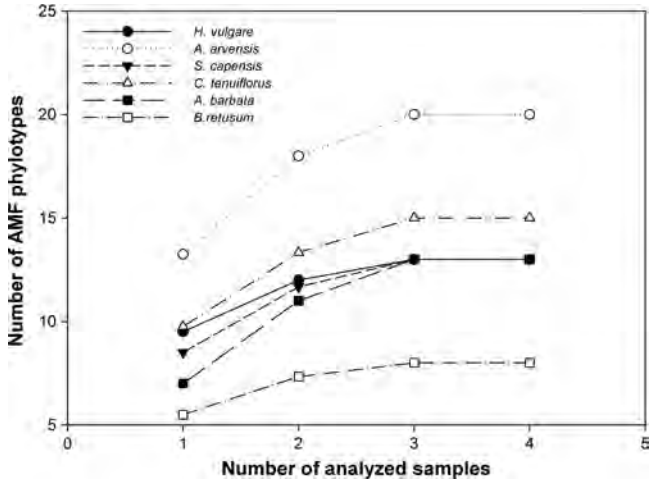


FIG 2 Sampling effort curves for the AM fungal community in the roots and rhizosphere soil of *H. vulgare*, *A. arvensis*, *S. capensis*, *C. tenuiflorus*, *A. barbata*, and *B. retusum*. The sample order was randomized by 100 replications in EstimateS, version 8.0 (12).

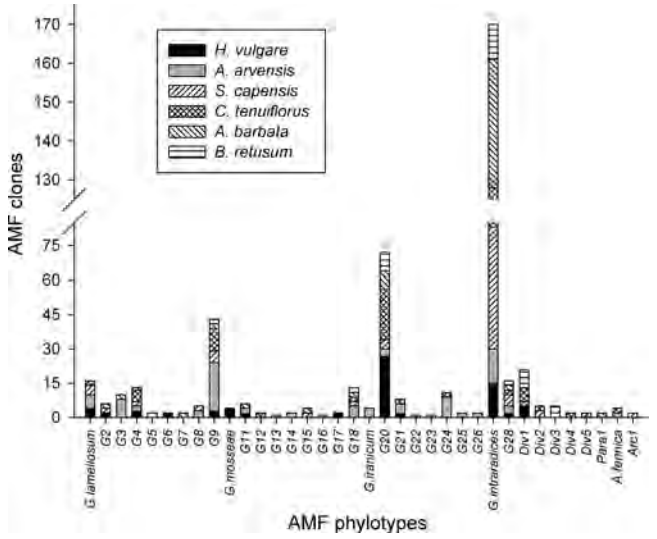


FIG 3 Bar plot showing the number of clones detected for each phylotype in the roots of *H. vulgare*, *A. arvensis*, *S. capensis*, *C. tenuiflorus*, *A. barbata*, and *B. retusum*.

G17, *Glomus* G18, *Glomus* G22, *Glomus* G23, *Glomus* G26, *Glomus* G28, and *Diversispora* 3, that did not seem to be related to any sequences of AMF in the database since these phylotypes did not cluster closely with any sequences of AM fungi maintained as reference AMF isolates in international culture collections. Thus, they may be unique to this study. The remaining groups clustered with root-derived sequences of uncultured AMF species.

To determine whether the number of clones sequenced was sufficient to represent the AMF diversity in the roots, sampling effort curves were constructed (Fig. 2). The results indicate that the number of sequences analyzed per sample was sufficient to provide coverage of the AM fungal diversity in roots from the six plant species studied. In fact, all the curves reached the plateau.

AMF phylotype distribution. The Shannon diversity index was calculated in order to compare the diversities of the AMF in the plant roots. The number of phylotypes found in the roots of different plant species was 13 in the case of *H. vulgare*, 13 in *S. capensis*, 15 in *C. tenuiflorus*, 13 in *A. barbata*, 8 in *B. retusum*, and 20 in *A. arvensis*, with the last species showing the greatest richness. Also, the Shannon diversity index for *A. arvensis* corresponded to the highest diversity (H_{HIE} = 2.05, H_{ANA} = 2.59, H_{STI} = 1.63, H_{CAR} = 1.95, H_{AVE} = 1.74, and H_{BRA} = 1.88, with the subscript of each variable giving the first three letters of the genus of the indicated plant species).

The most-abundant phylotypes in this study were *Glomus* G27 (36.7% of the clones belonged to this group), *Glomus* G20 (15.5%), and *Glomus* G9 (9.3%), which occurred in all the plant species, followed by *Diversispora* 1 (4.5%), present in all roots except those of *A. barbata* and *A. arvensis*. The phylotypes *Glomus* G5, *Glomus* G6, *Glomus* G10, *Glomus* G12, *Glomus* G13, *Glomus* G14, *Glomus* G16, *Glomus* G17, *Glomus* G19, *Glomus* G22, *Glomus* G23, *Glomus* G25, *Glomus* G26, *Diversispora* 3, *Diversispora* 4, *Diversispora* 5, *Paraglomus* 1, and *Archaeospora* 1 were found exclusively in the roots of one plant species. *H. vulgare* harbored all the clones of the groups *Glomus* G6, *Glomus* G10, and *Glomus* G17. In the same way, *A. arvensis* harbored all the clones of seven phylotypes (*Glomus* G14, *Glomus* G16, *Glomus* G19, *Glomus* G22, *Glomus* G23, *Glomus* G25, and *Glomus* G26), *S. capensis* harbored all clones of the phylotype *Archaeospora* 1, *C. tenuiflorus* harbored all clones of the phylotypes *Glomus* G12, *Diversispora* 4, and *Diversispora* 5, *A. barbata* harbored all clones of the phylotypes *Glomus* G13 and *Paraglomus* 1, and *B. retusum* harbored all clones of the phylotypes *Glomus* G5 and *Diversispora* 3. Four phylotypes (*Glomus* G9, *Glomus* G18, *Glomus* G20, and *Glomus* G27) were found in all six plant species. Therefore, the phylotype *Glomus* G27, corresponding to the known sequence group *G. intraradices*, was present in all the plant species, while *Glomus* G10, corresponding to *G. mosseae*, and *Glomus* G19, corresponding to *G. iranicum*, were exclusive to one plant species: *H. vulgare* and *A. arvensis*, respectively (Fig. 3).

The influence of the host plant species on the distribution of AMF sequence types was investigated using a multivariate statistical approach. The analysis explained 62.4% of the whole variance, with the influence of the host plants being statistically significant (P = 0.002). According to the forward-selection output, *A. arvensis* (P = 0.002), *S. capensis* (P = 0.002), *H. vulgare* (P = 0.002), *C. tenuiflorus* (P = 0.002), *B. retusum* (P = 0.004), and *A. barbata* (P = 0.004) were the six variables with significant contributions. These results indicate that these six plant species hosted

FIG 1 Maximum parsimony (MP) phylogenetic tree showing AM fungal sequences isolated from roots of *H. vulgare*, *A. arvensis*, *S. capensis*, *C. tenuiflorus*, *A. barbata*, and *B. retusum* and reference sequences from GenBank. All bootstrap values of >80% are shown (1,000 replicates). Numbers above branches indicate the bootstrap values of the MP analysis. Sequences obtained in the present study are shown in bold type. They are labeled with the host plant from which they were obtained (HIE, roots of *H. vulgare*; ANA, roots of *A. arvensis*; STI, roots of *S. capensis*; CAR, roots of *C. tenuiflorus*; AVE, roots of *A. barbata*; BRA, roots of *B. retusum*) and the clone identity number. Group identifiers (for example, Glo G1) are phylotypes found in our study. *Endogone pisiformis* and *Mortierella polycephala* were used as outgroups.

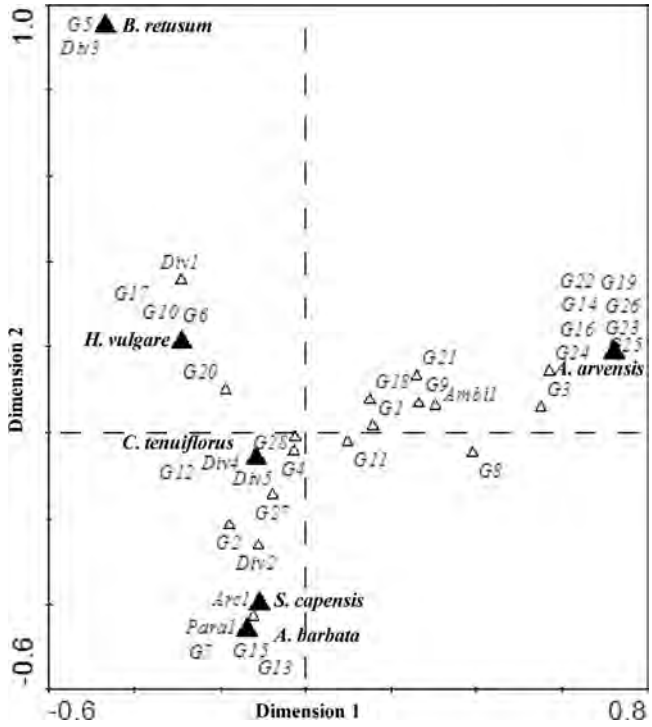


FIG 4 Canonical correspondence analysis (CCA) of the AM fungal community composition found in the roots of *H. vulgare*, *A. arvensis*, *S. capensis*, *C. tenuiflorus*, *A. barbata*, and *B. retusum*. The eigenvalues of the *x* and *y* axes in the two-dimensional ordination diagrams are as follows: dimension 1, 0.39; dimension 2, 0.26. Open triangles indicate the respective phylotypes, and the filled triangles indicate the plant species.

different AMF communities. The biplot diagram of the canonical correspondence analysis (CCA) (Fig. 4) supports these results; the centroids representing different host plant species are distant to each other, indicating that they harbored distinct AMF, and this was particularly evident for *B. retusum*.

DISCUSSION

In this study, we analyzed the composition of the community of AMF in the roots of six plant species growing under natural conditions in a semiarid Mediterranean ecosystem; of these, five are annual herbaceous plants and one is a perennial herbaceous plant.

The number of phylotypes detected, 36, gives an idea of the high diversity of AMF in these ecosystems, and it is probably even higher, but we are limited by the fact that the molecular delimitation of an AMF species is problematic due to sequence heterogeneity (36). Twenty-one of the 36 groups of sequences described have been identified in previous studies. Fifteen groups of sequences (*Glomus* G2, *Glomus* G3, *Glomus* G6, *Glomus* G8, *Glomus* G12, *Glomus* G13, *Glomus* G14, *Glomus* G16, *Glomus* G17, *Glomus* G18, *Glomus* G22, *Glomus* G23, *Glomus* G26, *Glomus* G28, and *Diversispora* 3) have been described for the first time. The most abundant phylotype—with almost 37% of the sequences—was *Glomus* G27, which showed very high similarity (99% identity) to the described species *Glomus intraradices*, which appeared in all the plant species. This demonstrates once again the widespread nature of this AM fungal species, which exhibits high local abundance and low host specificity. *Glomus intraradices* has been found in a wide range of environments (10, 19, 21, 26, 29, 30, 43, 55). This coincides with other results obtained in previous work in the Mediterranean area, although the plants studied were shrubs and crops (4, 11, 28).

The only perennial plant species considered, *B. retusum*, harbored the fewest number of phylotypes, only eight, while the other species were colonized by 13, 15, or 20 fungal types. Also, this species was separated from the annual species in the CCA biplot diagram. According to Hooper and Vitousek (20), the roots of annual plants seem to be active earlier than those of perennial plants every season and differ in the timing and extent of their activity during the year (47). If the roots of *B. retusum* take more time to become active than those of the other species in this study, this may be one of the reasons why it was the species which harbored the lowest number of AMF sequence types. Other reports suggest that AMF associations with plants may occur at a higher level, such as the plant functional type or ecological group (31), and not necessarily at the species level (51).

It has been shown that different groups of plant species that differ in their morphofunctional traits may show distinct patterns of responses to mycorrhiza (13, 32). Plants belonging to different life form groups have differing mycorrhizal responsiveness (32, 47). However, plants select for the AMF that benefit them most in local environments (22). Thus, in contrast to our results, Alguacil et al. (7) showed that perennial plant species from semiarid gypsum soils supported higher AMF diversity in their roots than did annual plants. Therefore, as has been reported already in several studies, soil characteristics, such as moisture content (56), structure (26), fertility (3, 14, 24), and disturbance (35), may have influenced the composition and distribution of the AMF community.

Our results reveal that the AMF communities harbored were different in all the plant species studied, as expected. Not only did each plant species have its own AMF population, as reported in other studies in different ecosystems (1, 5, 16, 27, 29, 39, 43, 48, 49), but the phylotypes shared between plant species also had different frequencies of occurrence, which shows that some types of AMF have host plant preferences (17, 27, 48). More evidence of this specificity of AMF is the fact that 18 of the 36 groups of sequences identified were specific to only one plant species, including three phylotypes specific to *H. vulgare*, seven to *A. arvensis*, one to *S. capensis*, three to *C. tenuiflorus*, two to *A. barbata*, and two to *B. retusum*.

Of the six plant species studied, three belong to the family *Poaceae*, two to the family *Asteraceae*, and one to the family *Primulaceae*. A certain trend of AMF specificity was apparent when the plant species were grouped according to their taxonomic families. There were two groups, *Glomus* G20 and *Glomus* G21, which showed a clear trend toward plants belonging to the *Asteraceae*. The group *Glomus* G21 was detected only in the plants of the families *Asteraceae* and *Primulaceae* and not in those of the *Poaceae*, and the group *Glomus* G20 had almost 70% of its sequences distributed between *C. tenuiflorus* and *H. vulgare*, both from the *Asteraceae*. The only member of the *Primulaceae* in this study supported seven exclusive AMF groups (*Glomus* G14, *Glomus* G16, *Glomus* G19, *Glomus* G22, *Glomus* G23, *Glomus* G25, and *Glomus* G26). In contrast, the plants belonging to the family *Poaceae* did not host any specific sequence type; this family seems to not be a source of preferential host plants for the populations of AMF in the ecosystem studied. There is no previous report concerning the AMF preferences or specificities for particular plant

families; unfortunately, the scarce number of plant species considered in our survey does not allow any valid conclusion in this respect.

Within a community of plant species, such as the annual species considered in this study, the interaction between AMF and plant growth may be very complex, and it may be that there were more mycorrhizal-dependent plants than others because of genetic and/or physiological incompatibilities between AMF and host plants (23). However, a higher number of plants cooccurring in the same area (plant diversity) may have increased the AMF diversity (5) in the roots of these annual plant species since AMF diversity is correlated with the diversity and yield of emerging plant communities (50).

In conclusion, the semiarid prairies in Mediterranean conditions can have a high AMF diversity, mostly supported by annual plant species in spite of the fact that their presence is limited to a short period of time related to the late-winter and early-spring rainfall. There were significant differences in the composition of the AMF community among plant species. We cannot conclude the same about differences in the AMF among plant families, although a clear trend is suggested. In order to maintain the biodiversity and productivity of these extreme ecosystems, it seems advisable to protect and even to promote the native annual vegetation.

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