Plant type differently promotes the arbuscular mycorrhizal fungi biodiversity in their rhizospheres after revegetation of a degraded, semiarid land.

M.M. Alguacil[A], **M.P. Torres**[B], E. Torrecillas[A], G. Díaz[B], and A. Roldán[A]

[A] Department of Soil Water Conservation, CEBAS-CSIC, Murcia, Spain, Email mmalguacil@cebas.csic.es
[B] Department of Applied Biology, University Miguel Hernandez of Elche, Elche, Alicante, Spain, Email mp.torres@umh.es

Abstract
The diversity of arbuscular mycorrhizal fungi (AMF) and their association with distinct plants species is crucial information in the early stages of revegetation procedures since the AMF roots colonization plays an important role improving plant establishment and growth. We carried out a study where we analyze the AMF community composition in the roots of *Ephedra fragilis*, *Rhamnus lycioides*, *Pistacia lentiscus* and *Retama sphaerocarpa* fourteen months after revegetation in a Mediterranean semiarid degraded area of southeast Spain in order to verify whether different plant species can variably promote the diversity of AM fungi in their rhizospheres after planted. We amplified a portion of 795 bases pairs of the small-subunit ribosomal DNA by means of nested PCR which was subjected subsequently to cloning, sequencing and phylogenetic analyses. Eight fungal sequence types belonging to *Glomus* group A and B and to the genus *Paraglomus* were identified. The different plant species had different AM fungal community composition. Thus, *R. lycioides* harbored the highest number of fungal types while as *E. fragilis* was colonized only by two fungal types specific for this plant species. *P. lentiscus* and *R. sphaerocarpa* harbored each one three fungal types and two of them were shared. All AMF sequence types were found in the natural soil. These results show that one effective tool to restore degraded lands is an increase in the number of plant species used, which would increase the AMF diversity in the soil and thus the below-ground, positive interactions.

Key Words
Mycorrhizal fungi, rhizosphere, revegetation, functional diversity, semiarid land.

Introduction
Several studies carried out in degraded semiarid Mediterranean areas showed that different shrubs representative of these areas differed in their ability to enhance the development of mycorrhizal propagules in the soil (Azcón-Aguilar *et al*. 2003; Caravaca *et al*. 2005), but whether these differences in the amount of AM fungal propagules correspond with variations in the AMF diversity is still unknown. There is increasing ecological interest in the diversity of AM fungi present in roots in field conditions, particularly with respect to revegetation programmes for degraded ecosystem using autochthonous shrubs, since the most functional species of AM fungi can serve as a source of inoculum for subsequent use in revegetation and/or recovery programmes for degraded soils. In this regard, the selection of plant species able to promote a high AMF biodiversity in their rhizosphere is an important point in the restoration of these difficult sites. In the present study, we used AMF-specific PCR primers, developed recently by Lee *et al*. (2008), to analyse the natural AMF communities which participate in the early colonisation of the roots of four shrub species after revegetation. These plants belong to the natural succession in semiarid Mediterranean ecosystems: *Ephedra fragilis* Desf., *Rhamnus lycioides* L., *Pistacia lentiscus* L. and *Retama sphaerocarpa* (L.) Boiss. The objective of this study was therefore to ascertain whether different plant species promote differently the diversity of AM fungi in their rhizospheres when planted in a semiarid degraded soil.

Methods
Study Site
The experimental area was located in Murcia (Southeastern Spain) (coordinates 38º12' N, 1º13' W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000mm. The mean annual temperature is 19.2 ºC with absence of frost period. The soil in the experimental area is a Typic Torriorthent (SSS, 2006), very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure.

Experimental Design and Sampling
The experiment was conducted using a randomized factorial design with six replication blocks. The

following plant species were selected: *Ephedra fragilis* (Desf.), *Rhamnus lycioides* L., *Pistacia lentiscus* L. and *Retama sphaerocarpa* (L.) Boiss. Once germinated, seedlings were transplanted into the growth substrate, consisting of 1 peat and cocopeat (1:1, v/v) autoclaved (60 min, 120º) in order to avoid any mycorrhizal propagules. They were grown with watering for 8 months under nursery conditions. In early April 2007, an area of 1.200 m^2 was selected to carry out the plantation. 30 seedlings of each plant species (5 per replication block) were planted in individual holes, separated between them at least 1m. The experiment was carried out under strictly natural conditions, without any watering or fertilizer treatments.
Fourteen months after plantation six plants belonging to each of the four selected species (one per replication block) were sampled (a total of 24 plants). Six samples of soil (one per replication block) of 500 g each were also randomly collected at 20-40 cm depths.

Roots and soil DNA extraction and PCR
For each sample, total DNA was extracted from root material (representing approx. 5-8 cm root
21 length) using a DNeasy plant mini Kit. For each of the six soil samples, genomic DNA was extracted from 0.5 g of soil using a FastDNATM 26 Spin kit for soil according to the recommendations of the manufacturer (Q-BIOgene, Heidelberg, Germany). Partial ribosomal small subunit (SSU) DNA fragments were amplified using nested PCR with the universal eukaryotic primers NS1 and NS4 (White *et al*. 1990). Several dilutions were used as template DNA in a second PCR reaction performed using the specific primers AML1 and AML2 (Lee *et al*. 2008).

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

Results
Twenty of the 24 root samples extracted and five of the six soil samples generated PCR products of the expected band of 795 bps, which were used for cloning and creating a clone library. From the 25 clone libraries, a total of 1000 clones were screened by PCR (on average, 40 clones were analysed per library); out of these, a total of 267 clones contained the SSU rRNA gene fragment and subsequently all clones were sequenced. The BLAST search revealed that 176 sequences (65.9%) had a high degree of similarity to sequences from taxa belonging to the phylum *Glomeromycota*, while the remaining sequences (34.1%) were similar to sequences from plant species. After Neighbour-joining (NJ) analyse of the sequences, eight AMF sequence types grouped in the *Glomeraceae* and *Paraglomeraceae* families could be distinguished on the basis of bootstrap values ≥ 77% (Figure 1). Six AMF sequence types belonged to *Glomus* group A, which was the group represented most frequently, one sequence type belonged to *Glomus* group B and one belonged to *Paraglomus*. Only two AMF sequences types identified in this study showed high similarity to sequences of previously-known glomalean species: Glo G1 clustered together with *Glomus lamellosum* and Glo G5 showed high similarity to the species complex *Glomus intraradices*/*Glomus fasciculatum*. Glo G 2b and Glo G4 showed high similarity to previously-described, root-derived sequences in GeneBank belonging to unknown glomalean species. Pa 1, although forming a different clade with a high bootstrap value (100%), showed high homology (95%) with *Paraglomus occultum*. The rest of the sequences (Glo 2a, Glo 2c and Glo G3) received strong support in the phylogenetic analysis but did not seem to be related to any sequences of AM fungi in the database (Figure 1). *R. lycioides* had the highest AMF richness, hosting four of the eight AM fungal types found in this study (Figure 2); also, this shrub showed the highest Shannon-Weaver diversity index (H'=1.15). *R. sphaerocarpa* and *P. lentiscus* harboured three AMF sequence types. The AMF communities of *E. fragilis* had the lowest diversity (H'= 0.56), with the lowest number of AMF sequence types. The sequence type Glo G2b was the most widespread and accounted for 41% of the AMF sequences detected. The eight fungal types were identified in the sequences derived from the soil samples (Figure 2), Glo G5 and Glo G2b being present in the highest proportion.

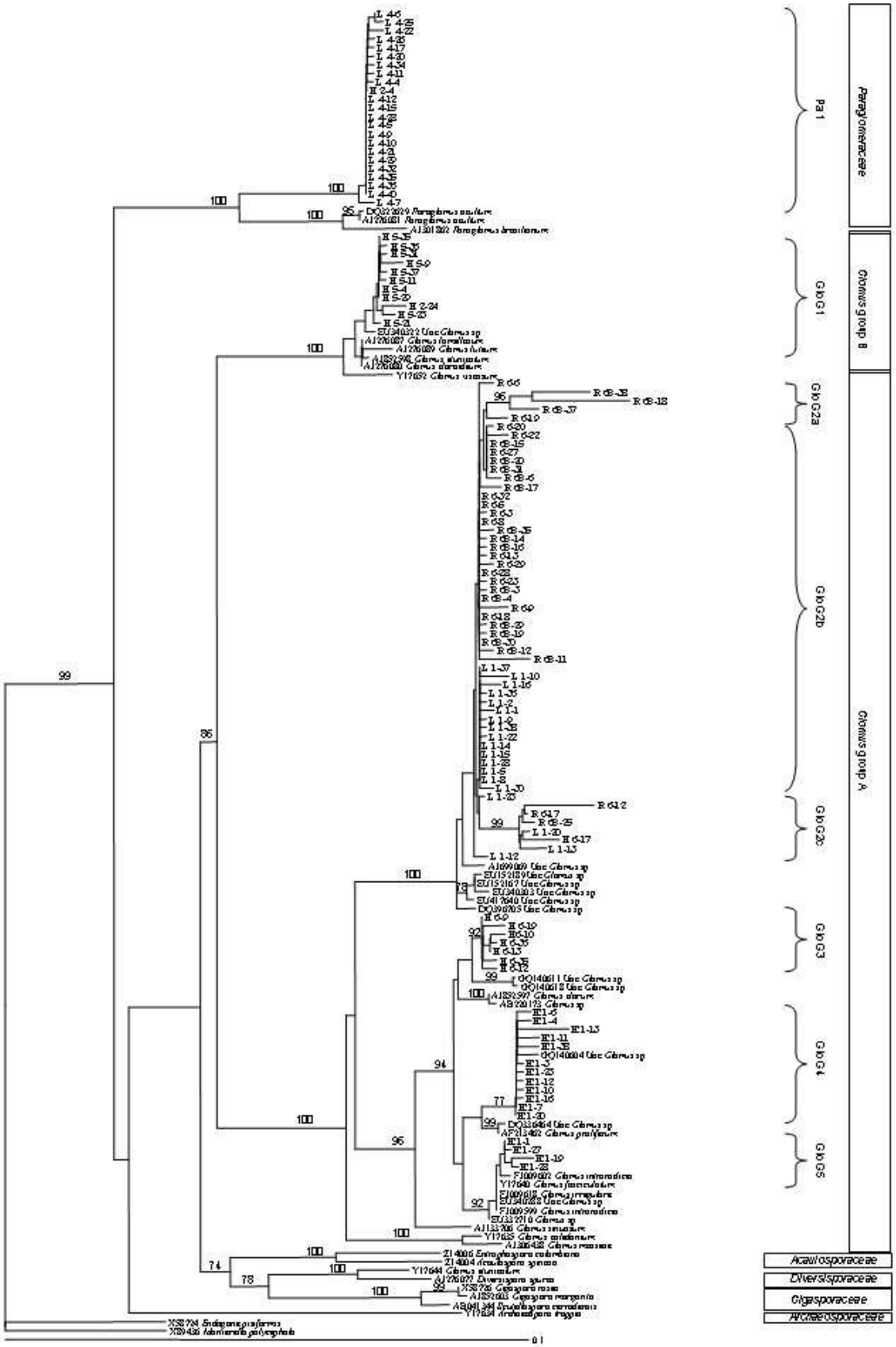

Figure 1. Neighbour-Joining (NJ) phylogenetic tree showing AM fungal sequences isolated from roots of *R. sphaerocarpa*, *R. lycioides*, *P. lentiscus* and *E. fragilis* and reference sequences from GeneBank. All bootstrap values > 75% are shown (100 replicates). Sequences obtained in the present study are shown in bold type. They are labelled with the host plant from which they were obtained (L= *P. lentiscus*, H= *R. lycioides,* R= *R. sphaerocarpa,* and E= *E. fragilis*) and the clone identity number. Group identifiers (for example Glo G1) are AM fungal sequences types found in our study. *Endogone pisiformis* and *Mortierella polycephala* were used as out-groups.

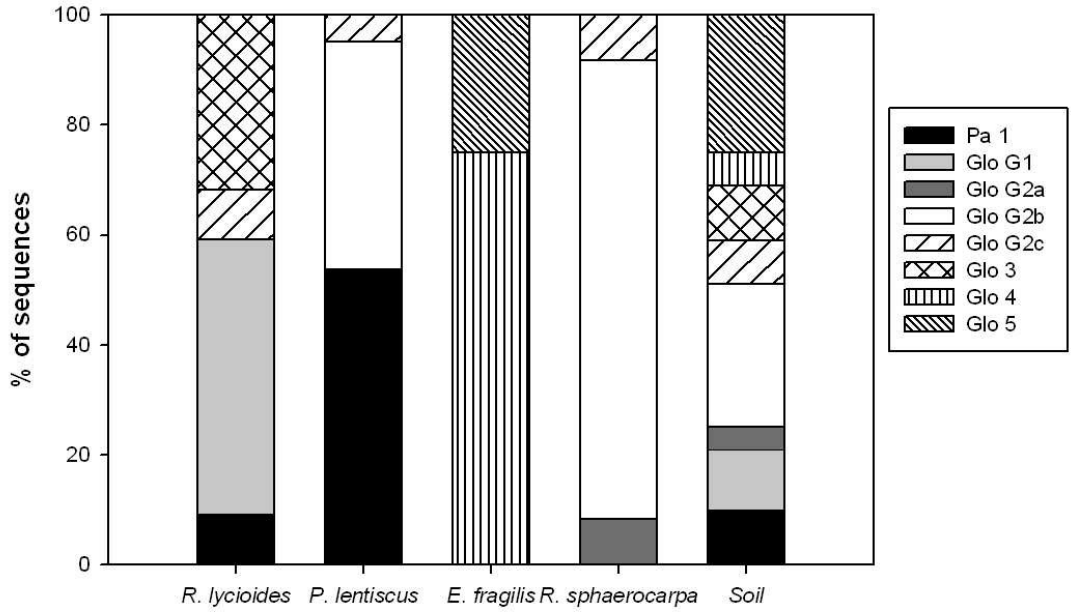

Figure 2. Bar plot showing the relative abundance of the different AM fungal types observed in *R. sphaerocarpa*, *R. lycioides*, *P. lentiscus* and *E. fragilis* roots and soil.

Conclusion

Ecosystems that have been degraded contain fewer AMF isolates. As we have demonstrated, the presence of different plant species results in the formation of mycorrhizae by different AM fungal species which "select" their host. In this way, a new fungal community 1 is created, as in a natural ecosystem where plant communities are associated with communities of AMF (Klironomos *et al*. 2000). In conclusion, this study shows that in revegetation programmes the AMF diversity is different depending on the host plant. Therefore, one effective tool to restore degraded lands is an increase in the number of plant species used, which would increase the AMF diversity in the soil and thus the below-ground, positive interactions.

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