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A molecular approach to ascertain the success of "in situ" AM fungi inoculation in the revegetation of a semiarid, degraded land

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ABSTRACT

The positive effect of arbuscular mycorrhizal fungi inoculation on plant establishment under field conditions has been shown. However, whether this effect is related to the survival of the AMF and how the AMF inoculum affects the colonisation of plant roots by the native AMF remain uncertain.
In this study, we assessed the AMF community composition in *O. europaea* roots inoculated "in situ" with three types of AMF inoculum: *Glomus* sp., *G. intraradices* and a mixture of *Glomus* sp. and *G. intraradices*. All the AMF isolates inoculated showed a good percentage of persistence. Mycorrhizal inoculation with the mixture of *Glomus sp.* and *G. intraradices* was the most effective treatment for increasing the AMF diversity in roots 14 months after plantation. Plant growth was increased significantly by all the inocula tested, although plant biomass was not correlated with variation in the AMF diversity or with the AMF inoculum persistence. Thus, it seems that this positive effect was mediated by the interaction between the AMF inoculum and the natural colonisation. Therefore, the application of native AMF isolates in the planting hole may be considered a good strategy for the revegetation of semiarid, degraded soils, in order to reactivate the indigenous AMF populations and improve the performance of *O. europaea* seedlings, particularly when mixtures of native isolates are used.

1. Introduction

The abandonment of agricultural soils, together with the scarce and irregular rainfall and long, dry and hot summers typical of the Mediterranean, semiarid areas of southeast Spain, has contributed drastically to the acceleration of soil degradation and desertification. Soil degradation implies reduction in the below-ground microbial diversity and/or activity (Kennedy and Smith, 1995). This is due to the influence of the natural plant communities on the microbial processes carried out in the rhizosphere, because of their role in the provision of energy to the soil. The degradation of the soil–plant system affects the microorganism-plant relations, particularly those of arbuscular mycorrhizal fungi (AMF) and their symbioses and propagules. The AMF are key components of natural ecosystems and several studies have shown the important role of these fungi in the early stages of native shrub species establishment and development, after the restoration of degraded Mediterranean ecosystems (Azcón-Aguilar et al., 2003; Barea et al., 1997; Requena et al., 2001; Caravaca et al., 2003, 2005). The AMF increase resistance to environmental stresses, enhance plant nutrient acquisition and improve soil quality (Jeffries and Barea, 2001; Alguacil et al., 2005). However, until now, the success of mycorrhizal plants has been based on plants inoculated with AMF under nursery conditions, while "in situ" AMF inoculation has received little attention.

It has been demonstrated that, in these degraded soils, there is a drastic reduction in the amount, diversity and activity of mycorrhizal propagules capable of colonising the rhizosphere (Requena et al., 2001; Azcón-Aguilar et al., 2003; Heinemeyer and Fitter, 2004); in some cases, they have even disappeared. Therefore, there is increasing interest in the use of arbuscular fungi to reinforce the mycorrhizal soil component (Barea et al., 1997; Azcón-Aguilar et al., 2003), either by the isolation of native AMF from the soil or by inoculation with other, more-efficient and competitive AMF.

The selection of efficient AMF is a key requisite in inoculation programmes and represents the initial step in rehabilitation/restoration approaches with native shrub species (Vallejo et al., 1999; Azcón-Aguilar et al., 2003), since there are different levels of compatibility between host plants and AMF (Roldán et al., 1992; Smith and Read, 2008) and different AMF species have different effects on plant performance (Caravaca et al., 2003). Moreover, some AMF ecotypes show host preference (Alguacil et al., 2011) and also for particular soil and climate conditions (Husband et al., 2002; Öpik et al., 2003), while others are more generalist and widespread (Öpik et al., 2006). Therefore, it is essential to select the AMF ecotypes which show the highest functional and ecological compatibility level for each plant-soil system.

The positive effect of AMF inoculation on plant establishment under field conditions has been shown (Caravaca et al., 2003, 2005,

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Alguacil et al., 2005). However, whether these effects are related to the survival of this AMF and how the AMF inoculum affects the colonisation of plant roots by the native AMF remain unclear. Thanks to the advances in molecular biology techniques in recent years, the identification of these fungi in plant roots is possible allowing biodiversity studies to be achieved. In this regard, different sets of AMF-specific primers, targeting different rDNA regions, have been designed (Helgason et al., 1998; Gollotte et al., 2004; Lee et al., 2008).

In the present study, we used one of the most-representative shrub species from Mediterranean regions, *Olea europaea* L. subsp. *sylvestris*, which is well-adapted to water-stress conditions and belongs to the natural succession of semiarid ecosystems in southeast Spain (Caravaca et al., 2003). In order to ascertain the natural AMF communities developed in *O. europaea* roots after inoculation with different AMF isolates, we used the "AML1-AML2" AMF-specific PCR primers.

The objectives of this study were to determine the persistence and survival of different native AMF inoculated "in situ" and their effects on the establishment of *O. europaea* seedlings and the natural AMF colonisation processes.

2. Materials and methods

2.1. 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, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall less than 270 mm, and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 °C with no frost period. The soil in the experimental area is a Typic Torriorthent (Soil Survey Staff, SSS, 2006), very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone was dominated by *Piptatherum miliaceum* (L.) Cosson., and some shrubs of *Thymus vulgaris L.* and *Rosmarinus officinalis* L. growing in patchy distributions.

2.2. Plants

The plant used, *Olea europaea* subsp. s*ylvestris* is a representative autochthonous shrub species from semiarid shrublands in southeast Spain, well adapted to water stress conditions and, therefore, frequently used in the revegetation of semiarid disturbed lands.

2.3. Isolation and identification of the different AM fungal isolates

AM fungal spores were extracted from soil samples collected in the experimental area by the wet sieving and decanting method, followed by sucrose centrifugation (Sieverding, 1991). Spores were identified at the morphological and molecular level. Criteria for morphological spore identification were based mainly on spore size, colour, wall structure and hyphal attachment (INVAM 1997). AM fungal morphotypes were also analysed by using molecular approaches. Total DNA of the different AM fungal morphotypes was isolated from approximately 50 to 60 spores, which were placed in microcentrifuge tubes containing 40 µl milliQ-water and crushed with a miniature pestle. 10 µl of Chelex® Iso-resin (Biorad) (20% in sterile water) were added to the crushed spores and the mixture was incubated at 95 °C for 5 min followed by 5 min on ice (Wyss and Bonfante, 1993). The resin was removed by centrifugation at 12,000 rpm for 3 min. The supernatant was frozen at −20 °C and used as template for PCR. SSU rRNA gene sequences were PCR amplified from the purified total DNA by using the AM specific primers AML1-AML2 (Lee et al., 2008) and subjected to phylogenetic analysis in the same way as is described below.

2.4. Establishment of the AM fungal cultures

Single species cultures of the different AM fungal morphotypes were established in pots using a mixture of sterile soil/vermiculite (1:1 (v/v)) as growing substrate and *Sorghum bicolor* L. as host plant. About 30 apparently healthy and viable spores of each AM fungal morphotype were used as inoculum by placing them close to the plant root system. Once multiplied and checked for purity, the different AM fungal isolates were used as AM fungal inoculum.

2.5. Mycorrhizal treatments

The different native AMF multiplied in mono-specific cultures were used as mycorrhizal treatments. Thus, *G. intraradices*, *Glomus* sp. and a mixture of both endophytes (half of *G. intraradices* inoculum and half of *Glomus* sp. inoculum) were used.

Arbuscular mycorrhizal fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum bicolor* L.) containing spores, hyphae and mycorrhizal root fragments for each mycorrhizal treatment.

All three types of mycorrhizal inoculum were subjected to a most probable number (MPN) test to determine potential infectivity and to equalize application doses. All three sources of inoculum had a potential infectivity of about 35 infective propagules per gram inoculum.

2.6. Experimental design

The experiment was conducted using a randomized block design with six replication blocks. The factor had four levels: non-inoculation (control = C), inoculation with *G. intraradices* (I), inoculation with *Glomus* sp. (G) and inoculation with the mixture of both endophytes (G + I). Once germinated, the *O. europaea* seedlings were transplanted into the growth substrate, consisting of peat and cocopeat (1:1, v/v) autoclaved (60 min, 120°) in order to avoid any mycorrhizal propagules. They were grown with watering for 8 months under nursery conditions, without any fertilizer. In early April, an area of 2000 m^2 was selected for the plantation. Planting holes (40 cm wide X 40 cm long X 30 cm deep) were dug manually. The corresponding arbuscular mycorrhizal inoculum was applied at a rate of 5% (v/v) in the planting hole closely to pot bound. The seedlings were planted at least 1 m apart, between holes, with 3 m between blocks. At least 24 seedlings per block were planted (6 plants X 4 treatments in each block).

The experiment was carried out under strictly natural conditions, without any watering or fertilizer treatments.

2.7. Sampling

Six, ten and fourteen months after plantation, one plant per replication block and treatment was sampled (a total of 24 plants by sampling). Plants, including root systems, were collected and placed in polyethylene bags for transport to the laboratory, where growth parameters were evaluated. In the case of the last sampling (fourteen months) roots were briefly rinsed, quickly dried on paper and used partly for morphological and partly for molecular analysis.

2.8. Plant analysis

Fresh and dry (105 °C, 5 h) weights of shoots and roots were recorded.

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).

2.9. Roots DNA extraction and PCR

For each sample (total 24), total DNA was extracted from (0.1 g) root material 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 rpm) using a mixer mill (MM 400, Retsch, Haan, Germany). The extracted DNA was resuspended in 20 μl of water. Several dilutions of extracted DNA (1/10, 1/50, 1/100) were prepared and 2 μl were used as template. Partial small subunit (SSU) ribosomal RNA gene 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 (Amershan 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).

Two μl of several dilutions (1/10, 1/20, 1/50 and 1/100) from the first PCR 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 (Amershan 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).

Positive and negative controls using PCR positive products and sterile water respectively were also included in all amplifications. 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.

2.10. 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). Thirty two 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.

Clones 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). 239 representative 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 FR693387-FR693625.

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

2.11. Phylogenetic 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). Neighbour-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-group.

2.12. Statistical analysis

Mycorrhizal treatments effects on measured variables (Shoot and root dry weight and colonization) were tested by a two way analysis of variance, and comparisons among means were made using the Duncan's test calculated at $P<0.05$. Statistical procedures were carried out with the software package SPSS 17.0 for Windows.

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 $H' = -\sum pi(\ln pi)$, where *pi* is the proportion of individuals found in the *i*th species (in a sample, the true value of *pi* is unknown but is estimated as *ni*/*N*, [here and throughout, *ni* is the number of individuals in the *i*th species]).

3. Results

3.1. Identification of the native AMF isolated used

Both native AMF species used showed 99% homology with *Glomus intraradices* and a species from *Glomus* sp. according to sequences from GenBank.

3.2. Plant growth and mycorrhizal colonisation

Regarding the factorial analysis (Table 1), only the inoculation factor was significant for growth parameters. No interaction was found between inoculation and blocks. Six months after planting, the inoculated *O. europaea* plants had significantly-greater shoot biomass than non-inoculated plants (Table 2). Inoculation with *G. intraradices* or the mixture of *G. intraradices* and *Glomus* sp. was significantly more effective than with the *Glomus* sp. alone, with respect to improving the shoot dry weight. The significant increases in shoot biomass mediated by the inoculation treatments were clearly more pronounced during the first stages after plantation (34.8% Control, 127% *Glomus* sp., 221% *G. intraradices* and 213% *Glomus* sp. + *G. intraradices*).

The behaviour of root dry weight was similar to that of shoot dry weight during the 14-months growth period.

The percentage root colonisation did not differ significantly between inoculated and non-inoculated plants, ranging from 24.7% to 33.8% of the root length (Table 2). The plants inoculated with the mixture of *G. intraradices* and *Glomus* sp. showed slightly-higher root colonisation than those receiving the other mycorrhizal treatments.

Table 1
P significance values from the two-way analysis of variance for the growth parameters and root infection of *O. europaea* seedlings as affected by inoculation and block factors.

	Inoculation (I)	Blocks (B)	Interaction (I x B)
Shoot dry biomass	0.003	0.980	0.633
Root dry biomass	0.001	0.895	0.797
Colonization	0.988	0.966	0.984

Table 2
Growth parameters and root infection of *O. europaea* seedlings in response to different mycorrhizal inoculation treatments (n = 6).

Treatments	Shoot (g dry weight)				Root (g dry weight)				Colonization (%) 14 months
	0 month	6 months	10 months	14 months	0 month	6 months	10 months	14 months	
Control	1.64a	2.21a	2.84a	3.27a	0.88a	1.12a	1.56a	2.02a	24.7a
Glomus sp.	1.64a	3.73b	4.62b	5.07b	0.88a	1.96ab	2.12ab	2.66ab	29.7a
G. intraradices	1.64a	5.26c	6.60c	7.77c	0.88a	2.54b	3.02b	3.45b	27.4a
G. intraradices x *Glomus* sp.	1.64a	5.14c	6.85c	7.90c	0.88a	2.36b	2.75b	2.98b	33.8a

Values in columns followed by the same letter do not differ significantly ($P<0.05$) as determined by the LSD test.

3.3. PCR and sequence analysis

Molecular analysis was carried out on six plants for each treatment (C, G, I, G + I) (a total of 24 root samples-one root sample per replication block and for each mycorrhizal treatment) at only the last time point (14 months). The DNA extracted was amplified successfully giving the expected bands of around 795 bps. The PCR products obtained were used for cloning and to construct gene libraries. A total of 24 gene libraries were constructed, from each of which 32 clones were screened by PCR; thus a total of 768 clones were screened by PCR. Subsequently, a total of 239 clones contained a SSU rDNA fragment and all clones were sequenced. No chimeric sequences were detected by BLAST analyses, and the sequences obtained had high degrees of similarity to sequences from taxa belonging to the phylum *Glomeromycota*.

3.4. Phylogenetic analysis of AMF groups

Alignment between the 239 sequences obtained in this study and those corresponding to the closest matches from GenBank were carried out. Thus, a phylogenetic tree was produced which revealed that the sequences belonged to the groups of *Glomerales* and *Paraglomerales*, while nine AMF sequence types could be distinguished on the basis of bootstrap values ≥ 75% (Fig. 1). The sequence similarities within clusters ranged from 98% to 100%. Two sequence types (Glo G1 and Glo G6) clustered with previously identified AMF sequences (*Glomus intraradices* and *Glomus lamellosum*, respectively). Glo G3 and Para 2 did not cluster with any known AMF sequence. The fungal type Para 1, although forming a distinct clade within it with a bootstrap value of 100%, showed high similarity (95%) to *Paraglomus occultum*. The rest of the fungal sequence types showed high similarity to previously described, root-derived sequences in GenBank belonging to unknown glomeralean species.

To determine whether the number of root samples per mycorrhizal treatment was sufficient to represent *Glomeromycota* diversity, sampling effort curves were constructed (Fig. 2). The results show that the number of root samples analysed for each treatment was sufficient to provide coverage of the AMF diversity. In fact, all the curves reached the plateau.

3.5. Persistence of the different types of AMF inoculum in plants

The persistence of the AMF inocula after introduction into the planting hole was checked molecularly, 14 months after inoculation.

Plants inoculated with *Glomus* sp. showed 20% of AMF sequence types belonging to this fungal type (Fig. 3). However, the percentage of sequence types corresponding to *G. intraradices* in plants inoculated with this fungus was almost twice: 48.2%. In the case of inoculation with both types of AMF inoculum together (*G. intraradices* and *Glomus* sp.), the percentage of AMF sequence types corresponding to these AMF was 14.3 % for *G. intraradices* and 39.7% for *Glomus* sp. of the total AMF sequence types detected.

3.6. Distribution of AMF sequence types

The AM fungal community distributions detected in both non-inoculated and inoculated plants are shown in Fig. 3.

The Shannon-Weaver diversity index showed the lowest value in control plants ($H' \approx 1$), and the plants inoculated with the mixture of *G. intraradices* and *Glomus* sp. showed the highest diversity index ($H' \approx 1.5$).

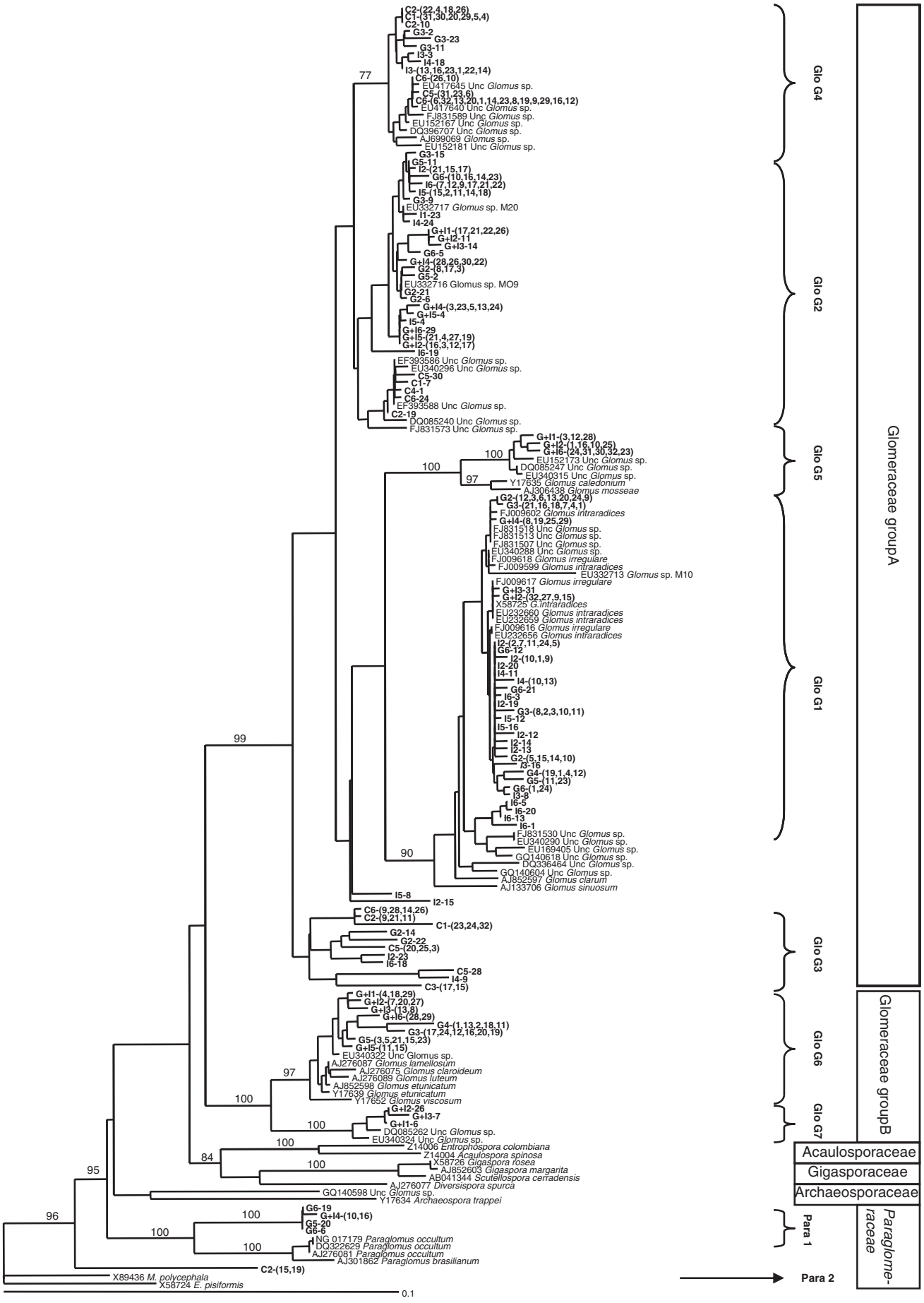
With respect to the AMF sequence types obtained in each inoculation treatment, the control plants hosted four of the nine fungal types found in this study; the sequence type GloG2, corresponding to the *Glomus* sp. inoculum and representing 9.6% of the AMF sequence types detected, was the only fungal type shared by the rest of the mycorrhizal treatments assayed (Fig. 3). The plants inoculated with *G. intraradices* also hosted four fungal types, GloG1 (corresponding to *G. intraradices* inoculum) representing the highest proportion (48.2%) with respect to the other fungal types found with this mycorrhizal treatment. The next-most-frequent sequence type was GloG2 (*Glomus* sp.), which represented 32.1%. The *Glomus* sp. and (G + I) treatments harboured the same and highest number of fungal types (6), although their community compositions differed: only Glo G1, Glo G2, Glo G6 and Para 1 were shared by both. It is of note that Glo G2 represented the highest proportion (39.7%) of AMF sequences types found for the (G + I) mycorrhizal treatment; however, with the *Glomus* sp. treatment, Glo G1 (*G. intraradices*) represented the highest percentage (45.7%).

4. Discussion

This study shows that the inoculation of *O. europaea* seedlings with different, native AM fungi increased the AMF diversity, changing the AMF community composition as well as augmenting the shoot biomass of these plants 14 months after planting.

From the phylogenetic analyses of the AM fungal sequences obtained from roots of *O. europaea* seedlings, nine AMF sequence types or phylotypes belonging to the genus *Glomus* (groups A and B) or *Paraglomus* were identified. Thus, the predominance of *Glomus* and *Paraglomus* may well reflect the real composition of the AMF community in this degraded, semiarid Mediterranean ecosystem.

In the AMF community colonising the roots of *O. europaea* under natural conditions, four AMF sequence types were identified, none of them belonging to known species of AMF in GenBank. Under these conditions, the lowest AM fungal diversity was also shown. When the plants were inoculated with *Glomus* sp. (which showed 99% homology with the sequences grouped in the Glo G2 AMF sequence type), the AMF diversity increased and species composition changed — three new AMF sequence types appearing (Glo G1, Glo G6 and Para 1). Two of them, Glo G1 and Glo G6, belong to known AMF species: *G. intraradices* and *G. lamellosum*, respectively. Although Para 1 formed an independent clade with high support in the bootstrap value (100%), it showed high homology (95%) with *Paraglomus occultum*. It is interesting to note that Glo G2 represented only 20% of the total AMF sequences obtained in plant roots under this mycorrhizal treatment, even though the plants were inoculated with this fungal


C2-(22,4,18,26)
C1-(31,30,20,29,5,4)
C2-10
G3-2
G3-23
G3-11
I3-3
I4-18
I3-(13,16,23,1,22,14)
C6-(26,10)
EU417645 Unc Glomus sp.
C5-(31,23,6)
C6-(6,32,13,20,1,14,23,8,19,9,29,16,12)
EU417640 Unc Glomus sp.
FJ831589 Unc Glomus sp.
EU152167 Unc Glomus sp.
DQ396707 Unc Glomus sp.
AJ699069 Glomus sp.
EU152181 Unc Glomus sp.
77
G3-15
G5-11
I2-(21,15,17)
G6-(10,16,14,23)
I6-(7,12,9,17,21,22)
I5-(15,2,11,14,18)
G3-9
EU332717 Glomus sp. M20
I1-23
I4-24
G+I1-(17,21,22,26)
G+I2-11
G+I3-14
G6-5
G+I4-(28,26,30,22)
G2-(8,17,3)
G5-2
EU332716 Glomus sp. MO9
G2-21
G2-6
G+I4-(3,23,5,13,24)
G+I5-4
I5-4
G+I6-29
G+I5-(21,4,27,19)
G+I2-(16,3,12,17)
I6-19
EF393586 Unc Glomus sp.
EU340296 Unc Glomus sp.
C5-30
C1-7
C4-1
C6-24
EF393588 Unc Glomus sp.
C2-19
DQ085240 Unc Glomus sp.
FJ831573 Unc Glomus sp.
G+I1-(3,12,28)
G+I2-(1,16,10,25)
G+I6-(24,31,30,32,23)
100
EU152173 Unc Glomus sp.
100
DQ085247 Unc Glomus sp.
EU340315 Unc Glomus sp.
97
Y17635 Glomus caledonium
AJ306438 Glomus mosseae
G2-(12,3,6,13,20,24,9)
G3-(21,16,18,7,4,1)
FJ009602 Glomus intraradices
G+I4-(8,19,25,29)
FJ831518 Unc Glomus sp.
FJ831513 Unc Glomus sp.
FJ831507 Unc Glomus sp.
EU340288 Unc Glomus sp.
FJ009618 Glomus irregulare
FJ009599 Glomus intraradices
EU332713 Glomus sp. M10
FJ009617 Glomus irregulare
G+I3-31
G+I2-(32,27,9,15)
X58725 G.intraradices
EU232660 Glomus intraradices
EU232659 Glomus intraradices
FJ009616 Glomus irregulare
EU232656 Glomus intraradices
I2-(2,7,11,24,5)
G6-12
I2-(10,1,9)
I2-20
I4-11
I4-(10,13)
G6-21
I6-3
I2-19
G3-(8,2,3,10,11)
I5-12
I5-16
I2-12
I2-14
I2-13
G2-(5,15,14,10)
I3-16
G4-(19,1,4,12)
G5-(11,23)
G6-(1,24)
I3-8
I6-5
I6-20
I6-13
I6-1
FJ831530 Unc Glomus sp.
EU340290 Unc Glomus sp.
EU169405 Unc Glomus sp.
GQ140618 Unc Glomus sp.
DQ336464 Unc Glomus sp.
GQ140604 Unc Glomus sp.
AJ852597 Glomus clarum
AJ133706 Glomus sinuosum
99
90
I5-8
I2-15
C6-(9,28,14,26)
C2-(9,21,11)
C1-(23,24,32)
G2-14
G2-22
C5-(20,25,3)
I2-23
I6-18
C5-28
I4-9
C3-(17,15)
G+I1-(4,18,29)
G+I2-(7,20,27)
G+I3-(13,8)
G+I6-(28,29)
G4-(1,13,2,18,11)
G3-(17,24,12,16,20,19)
G5-(3,5,21,15,23)
G+I5-(11,15)
EU340322 Unc Glomus sp.
AJ276087 Glomus lamellosum
97
AJ276075 Glomus claroideum
AJ276089 Glomus luteum
AJ852598 Glomus etunicatum
100
Y17639 Glomus etunicatum
Y17652 Glomus viscosum
G+I2-26
G+I3-7
100
G+I1-6
DQ085262 Unc Glomus sp.
EU340324 Unc Glomus sp.
100
Z14006 Entrophospora colombiana
84
Z14004 Acaulospora spinosa
100
X58726 Gigaspora rosea
95
AJ852603 Gigaspora margarita
AB041344 Scutellospora cerradensis
AJ276077 Diversispora spurca
GQ140598 Unc Glomus sp.
Y17634 Archaeospora trappei
96
100
G6-19
G+I4-(10,16)
G5-20
G6-6
100
NG 017179 Paraglomus occultum
DQ322629 Paraglomus occultum
100
AJ276081 Paraglomus occultum
AJ301862 Paraglomus brasilianum
C2-(15,19)
X89436 M. polycephala
X58724 E. pisiformis
0.1
Glo G4
Glo G2
Glo G5
Glo G1
Glo G3
Glo G6
Glo G7
Para 1
Para 2
Glomeraceae groupA
Glomeraceae groupB
Acaulosporaceae
Gigasporaceae
Archaeosporaceae
Paraglome-raceae

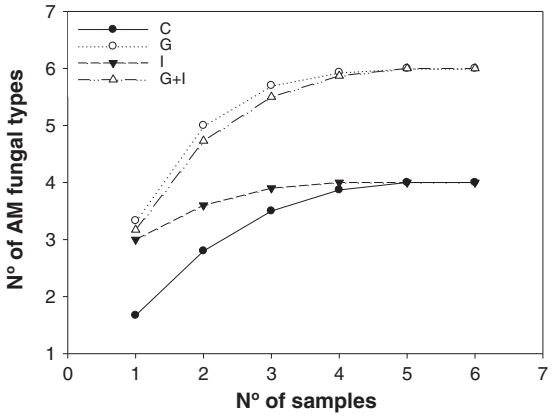


Fig. 2. Sampling effort curves for the AM fungal community in the roots of *O.europaea* L. The sample order was randomized by 100 replications in EstimateS, version 8.0 (Colwell 2005).

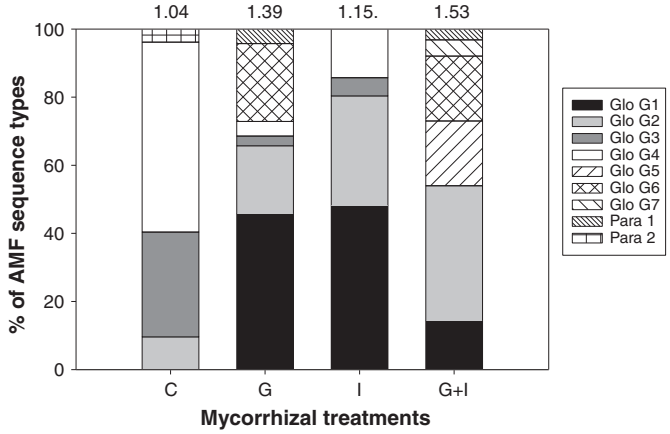


Fig. 3. Bar plot showing the relative abundance of the different AM fungal types observed in *O. europaea* L. roots. The Shannon-Weaver diversity index is indicated on the tops of the respective bars.

isolate. In this regard, *G. intraradices* was the most-frequent AM fungal type, representing 45.7% of the total AMF sequences analysed. Also, this species was dominant in *O. europaea* roots inoculated with *G. intraradices*, representing 48.2% of all the AMF sequences detected. Since *G. intraradices* was a common AM fungus present in plants inoculated with either *Glomus* sp. or *G. intraradices*, it is difficult to determine the success of the inoculation with this species. *G. intraradices* is usually reported as a very-invasive AM fungal species, with a generalistic lifestyle, which has been detected in different locations throughout the world, from both stable and disturbed ecosystems (Öpik et al. 2006), and which produces a large quantity of extra-radical mycelium (Jansa et al. 2003; Öpik et al. 2006) - the main source of AM inoculum in arid and semiarid ecosystems (Requena et al., 1996; Azcón-Aguilar et al., 2003; Ferrol et al., 2004).

The mycorrhizal treatment composed of the mixture of *G. intraradices* and *Glomus* sp. gave the highest Shannon-Weaver diversity index in this study, and although *O. europaea* roots under this treatment had the same number of fungal sequence types as these inoculated with *Glomus* sp. alone, two new fungal sequence types appeared (Glo G5 and Glo G7) - which were exclusive to this mycorrhizal treatment. It is important to emphasize that when both AMF species, *G. intraradices* and *Glomus* sp., were inoculated together, the percentage of *G. intraradices* sequences decreased about 70% with respect to the plants inoculated with *G. intraradices* alone. This could have been due to competition between the introduced AMF and the indigenous populations (Farmer et al., 2007). It has been shown that early colonisation by one AM fungal isolate may prevent further colonisation by another species of AMF, a phenomenon known as autoregulation (Pearson et al., 1993, Vierheilig et al., 2000, Vierheilig, 2004), since the colonisation strategies of different AM fungal isolates differ considerably (Hart and Reader, 2002). Moreover, other factors, such as specificity in the interaction with the host or some form of selective pressure in the soil environment, could influence the dominant presence of some AMF species over others (Cesaro et al., 2008).

With respect to the functional diversity of these isolates, we found that the different AMF treatments showed different levels of effectiveness in improving the performance of *O. europaea*. Different species of AMF have different effects on plant performance (Newsham et al., 1995, van der Heijden et al., 1998, Jeffries and Barea, 2001). In this regard, *G. intraradices* and the mixture of *G. intraradices* and *Glomus* sp. were more effective than *Glomus* sp. regarding increases in shoot biomass, despite the fact that the percentage root colonisation did not differ significantly between inoculated and non-inoculated plants and no correlation between AMF diversity or any AMF sequence type and shoot biomass was found. A higher diversity may not be a prerequisite for greater growth responses in inoculated plants, as has been shown for plants inoculated with *Glomus* sp. Therefore, we suggest that the greater plant growth could have been due to the effects produced by the interaction between different AMF species.

Also, it is important to note that, although we do not know whether plant growth promotion was due to the presence or persistence in the root of the inoculated native species of AMF or to the interaction between the inoculated and indigenous species, our data always revealed a significant improvement in the plant shoot biomass when *G. intraradices* was used as inoculant.

Our experiment demonstrated the important impact of inoculation with native AMF on the growth of shrub species in the first stages of plant development, which are the most critical for the survival of the plants when revegetation programmes are carried out, particularly in degraded, semiarid Mediterranean areas (Caravaca et al., 2003, 2005). The AMF-inoculated plants showed the most-significant increases in growth six months after planting; Caravaca et al. (2003, 2005) reported results similar to ours, although in these previous studies the plants were inoculated and grown for eight months under nursery conditions before being transplanted to the field. In contrast, in our study, the inoculum was added directly to the soil at transplanting. Therefore, the application of an AMF inoculum in the planting hole could be an alternative as effective as the inoculation of plants under nursery conditions.

5. Conclusions

The AMF inocula tested showed good survival percentages. The type of inoculum influenced the AMF diversity found in the *O. europaea* roots. The mycorrhizal inoculation produced significant increases in *O. europaea* growth, although the biomass was correlated neither with the AMF diversity nor with the inoculum persistence.

Fig. 1. Neighbour-Joining (NJ) phylogenetic trees showing AM fungal sequences isolated from roots of *O. europaea* L. and reference sequences from GenBank. All bootstrap values>70% are shown (100 replicates). Sequences obtained in the present study are shown in bold type. They are labelled with the mycorrhizal treatment from which they were obtained (Control = C., *Glomus* sp.= G., *G. intraradices* = I., and *Glomus* sp.+*G. intraradices* = G+I) and the clone identity number. Identical sequences are grouped, followed by the number of clones between brackets having that particular sequence. 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.

Thus, it seems that the positive effect was mediated by the interaction between the AMF inoculum and the natural colonisation. Hence, the application of native AMF isolates in the planting hole may be considered a good strategy for the revegetation of semiarid, degraded soils, in order to reactivate the indigenous AMF populations and improve the performance of *O. europaea* seedlings, particularly when mixtures of native isolates are used.

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