



# Analysing natural diversity of arbuscular mycorrhizal fungi in olive tree (*Olea europaea* L.) plantations and assessment of the effectiveness of native fungal isolates as inoculants for commercial cultivars of olive plantlets

R. Calvente, C. Cano, N. Ferrol, C. Azcón-Aguilar, J. M. Barea\*

*Departamento de Microbiología del Suelo y Sistemas Simbióticos; Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

Received 12 May 2003; received in revised form 21 October 2003; accepted 22 October 2003

## Abstract

The natural diversity of arbuscular mycorrhizal (AM) fungi in the root-associated soil from long-term established olive tree plantations was analysed. Four distinguishable native AM species, namely, *G. intraradices* (BEG 123), *G. mosseae* (BEG 124), *G. clarum* (BEG 125) and *G. viscosum* (BEG 126), were morphologically identified. These strains were also genetically characterised by PCR amplification and sequence analysis of a portion of their SSU rRNA. Phylogenetic analysis of these sequences shows that the new sequences of *G. mosseae*, *G. intraradices* and *G. viscosum* cluster with those of the same species found in the database. However, the new sequence of *G. clarum* fell in a different clade. The effectiveness of these native AM fungi as inoculants for two target varieties of olive (Arbequina and Leccino), currently used in many Mediterranean areas, was assessed. Two AM isolates from our own culture collection, namely *G. intraradices* and *G. mosseae*, were also used as reference inocula. *G. intraradices* and *G. viscosum* isolated from the target agro-system were the most effective fungi to improve the development of both olive varieties. This study supports the need to explore and exploit the natural diversity of AM fungi as a starting point to formulate inoculants to be applied during the commercial nursery production of olive varieties. © 2003 Elsevier B.V. All rights reserved.

**Keywords:** Arbuscular mycorrhizal fungal diversity; Molecular characterization; Olive tree commercial varieties; Micropropagation; Mycorrhizal inoculants

## 1. Introduction

Current interest in applying low-input-based agro-technologies in crop production systems is emphasizing the study and management of microbial interactions in the soil–plant interfaces (Barea et al., 2002). In the particular case of olive tree (*Olea*

*europaea* L.) plantations in Mediterranean agriculture, new technologies for modern olive production include, among other approaches, applying microbial inoculants as bioprotectors, phytostimulators or biofertilizers during the nursery production of quality plantlets (Jiménez-Díaz, 1998). With regard to applying microbial biotechnologies, management of mycorrhizal associations has been proposed because of the role of these symbioses in plant development and health (Azcón-Aguilar and Barea, 1997).

\* Corresponding author. Tel.: +34-958-181600x247; fax: +34-958-129600.  
E-mail address: [josemiguel.barea@eez.csic.es](mailto:josemiguel.barea@eez.csic.es) (J.M. Barea).

Olive plants are known to form arbuscular mycorrhiza (Roldán-Fajardo and Barea, 1985), the most common mycorrhizal type involved in normal cropping systems, being considered as a key component in environmentally friendly agro-biotechnologies (Jeffries and Barea, 2001). Arbuscular mycorrhizal (AM) symbioses are known to play a critical role in plant nutrition, an activity based on the ability of the external fungal mycelium developing around the roots to efficiently explore a bigger volume of soil than non-AM roots, thereby enhancing mineral acquisition by the plant (Smith and Read, 1997).

One critical step for applying AM technology is the appropriate selection of effective fungal isolates to be used as plant inoculants. For this purpose it is recommended to test native ecotypes alongside those considered “highly effective” from established culture collections (Dodd and Thomson, 1994). Therefore, natural diversity of AM fungi in the root-associated soil from established olive plantations in the region must be analyzed first and mono-specific cultures established. The AM fungi, which belong to the order Glomales [to be placed either in the Zygomycetes (Morton and Benny, 1990) or in the new fungal phylum, the Glomeromycota, as recently proposed (Schüßler et al., 2001)], are obligate plant symbionts. Consequently, their multiplication for both characterization and inoculum production requires the establishment of the symbiosis with appropriate host plants. Identification of AM fungi is mainly based on the morphological and developmental characteristics of fungal spores (Morton and Benny, 1990). However, biochemical and molecular approaches are being incorporated to define and relate taxa in the order Glomales (Simon et al., 1992; Graham et al., 1995; Schüßler et al., 2001; Madan et al., 2002), and to study the diversity of their populations in different soil–plant systems (Clapp et al., 1995; Helgason et al., 1998; Kowalchuk et al., 2002; Vandenkoornhuysen et al., 2002). Most of the recent molecular work has focused on the use of PCR techniques to analyze target sequences within the ribosomal gene cluster (van Tuinen et al., 1998; Redecker et al., 2000; Schüßler et al., 2001). These studies have shown that the small subunit of the ribosomal rRNA (SSU rRNA) gene is suitable for analyzing diversity and phylogeny of AM fungi.

Applying AM technology in olive plant production requires consideration of both AM management itself

and the interactions with the plant propagation protocols for the target commercial cultivars. In the case of olive plants micropropagation methods are usually used (Rallo, 1998). In this context, AM inoculation is known to increase survival rate and development of micro-propagated plantlets, improve plant establishment, protect plants against cultural and environmental stresses (particularly increase resistance to root pathogens and tolerance to drought and salinity), and improve soil structure (Azcón-Aguilar and Barea, 1997).

Considering these novel aspects in studying rhizosphere ecology, a series of experiments was carried out aimed at analysing the natural diversity of AM fungi in the rhizosphere of mature olive trees growing under normal cultivation and, then, at determining the efficiency of the AM fungi isolated from target olive plantations, as inoculants for the target olive cultivars.

## 2. Materials and methods

### 2.1. Sampling of olive tree soils

Soil samples from mature field-grown olive trees were collected in a major olive plantation area in the Jaén province, southern Spain. For the mycorrhizal survey 25 individual plants were randomly chosen at 5 plantation sites in the area. Three root-associated soil samples from each individual plant were collected, each sample consisting of five bulked sub-samples (200 cm<sup>3</sup> soil cores) randomly collected at 10 to 20 cm depth.

### 2.2. Isolation of AM fungal spores and establishment of pure cultures

The AM fungal spores were extracted from the rhizosphere soil by wet sieving and decanting followed by sucrose centrifugation (Sieverding, 1991). After centrifugation, the supernatant was poured through a 50 µm mesh and quickly rinsed with tap water. Spores were grouped, under a dissecting microscope, according to their morphological characteristics and used to initiate cultures of the different native AM fungal isolates. For this spore multiplication process, a sterile mixture of soil/sand (v/v) was used as

substrate for the host plant alfalfa (*Medicago sativa*, L.). Alfalfa seeds were surface-sterilized (10 min in a 10% diluted commercial sodium hypochlorite solution followed by several washes with sterile water), pre-germinated and transplanted after emergence into 11 pots and inoculated with about 50 spores belonging to an apparently similar spore morphotype. Alfalfa plants were grown under greenhouse conditions. Soil samples were taken periodically to confirm the AM colonization and the development of apparently mono-specific fungal cultures. The multiplication/isolation cycle was repeated as many times as necessary if mixed spore populations were evidenced until cultures of singly-isolated morphotypes were evident.

### 2.3. Identification of the different AM fungal isolates

#### 2.3.1. Morphological characterization

After confirming under the light microscope the apparent purity of the cultures of the different AM morphotypes, these were identified to genus and, when possible, to species level. Criteria for morphological spore characterization were mainly based on spore size and colour, wall structure and hyphal attachment (Walker, 1983; Morton and Benny, 1990; Schenk and Perez, 1990; Dodd and Rosendahl, 1996; INVAM, 1997) Permanent slides were prepared for each spore morphotype by using both polyvinyl-alcohol and polyvinyl-alcohol plus Melzer's solution (1:1), as described by Walker (1983). The characterized spore morphotypes were registered at the BEG (Banque Europeen de Glomales) culture collection.

#### 2.3.2. Molecular characterization

AM fungal isolates 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  $\mu$ l milliQ-water and crushed with a miniature pestle. 10  $\mu$ l of Chelex<sup>®</sup> 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 primer AM1 (Helgason et al., 1998) and the universal primer NS31 (Simon et al., 1992). The PCR mixture consisted of 1  $\mu$ M of each primer, 23  $\mu$ l of the DNA spore extract and a “ready to go” PCR bead (Amersham Pharmacia Biotech) in a 25  $\mu$ l reaction volume. PCR was performed in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, CA) with an initial denaturation for 10 min at 94 °C, followed by 35 cycles with denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C and extension for 2 min at 72 °C, followed by a 8 min final extension at 72 °C. The PCR products were separated by electrophoresis in 1.6% agarose gels, stained with ethidium bromide, and visualized by UV trans-illumination. The expected bands were purified using a gel extraction kit (Qiagen, Hilden, Germany) and sequenced in both strands using an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) with BLAST programmes (Altschul et al., 1990). Multiple sequence alignments of gene sequences were carried out using the programme CLUSTALW (version 1.5; Thompson et al., 1994). The Kimura two-parameter method was used to estimate distances and the phylogenetic analysis was performed by the Neighbor-joining method by using PHYLIP (Felsenstein, 1993). The relative support of the different groups was determined based upon 1000 bootstrap trees. Phylogenetic trees were drawn using Treeview. The new sequences were deposited in the EMBL database.

### 2.4. Effectiveness of AM fungi isolated from the root-associated soil of olive plantations

AM fungi isolated from the rhizosphere of olive tree plantations in the region, were assayed for their effectiveness as mycorrhizal inoculants, once the mono-specific cultures were established. *G. intraradices* (EEZ 1) and *G. mosseae* (BEG 119), from the mycorrhizal culture collection of the Estación Experimental del Zaidín (EEZ), were also assayed as reference fungi alongside the native AM fungi.

Arbequina and Leccino, two olive commercial varieties currently used to produce high quality olive oil in Mediterranean agro-systems, were chosen. Rooted micro-propagated olive (*O. europaea* L.)

plantlets of these varieties (kindly supplied by COTE-VISA, L'Alcudia, Valencia, Spain), at the beginning of the root-elongation phase, were transplanted into 250 ml pots containing a sterile mixture of soil:sand:vermiculite (1:1:1, v:v:v) and supplied with  $1 \text{ g l}^{-1}$  of a slow release fertilizer (mini-osmocote). The soil was a Cambisol with pH ( $\text{H}_2\text{O}$ ) of 7.6, available ( $\text{NaHCO}_3$ -extractable) P of  $9 \text{ mg l}^{-1}$ , total N of  $1300 \text{ mg l}^{-1}$ , 2.1% organic matter, and a texture of 38% sand, 43% silt, and 19% clay (Lachica et al., 1973). This soil was taken from an agricultural region where olives are usually cropped. Plantlets were inoculated with appropriate AM fungal inocula which consisted of rhizospheric soil containing spores, hyphae and mycorrhizal root fragments and were obtained on a defined substrate [vermiculite/sepiolite mixture (1:1, vol:vol)] and clover as host plant (Azcón-Aguilar et al., 2000). All the AM inocula supply around 375 AM propagules per plant, following previous own experience (unpublished results). The mycorrhizal potential in this inoculum was determined by following a dilution technique (Sieverding, 1991) and further calculation of the most probable number (MPN) of "infective" (able to develop colonization units in the root of a test plant) mycorrhizal propagules. Inoculated and non-inoculated control plantlets were grown in a greenhouse. Environmental conditions were 16/8 h light/dark photoperiod, 25/18 °C, day/night temperature and 60/80% day/night relative humidity. Plants were watered every 2 days with tap water and fed once a week with a low-P-content (25%) of Long Ashton nutrient solution (Hewitt, 1966). Seven replicates per treatment were prepared.

### 2.5. Harvest, measurements and statistical analyses

Plants were harvested 19 weeks after inoculation. The response variables studied were the height of the plant (shoots), shoot dry and fresh weights, root fresh weight, inoculum efficiency and mycorrhizal colonization. The efficiency of the mycorrhizal inoculum was calculated as the percentage increase in shoot weight produced by each AM fungus over the un-inoculated control. An aliquot of the root system was separated to determine AM colonization. Microscopic estimations were carried out in stained root samples processed as described by Phillips and Hayman (1970). More than

50 root segments (of about 1 cm) per plant were examined. These data were arc-sin square root transformed before statistical analysis.

Data were processed by the analysis of variance (ANOVA) using the procedures of the SAS Institute, SAS/STAT version 6 (1990). When appropriate, statistical differences were determined by using the Fisher's Protected Least Significant Difference Test at  $P < 0.05$ .

## 3. Results

### 3.1. Characterization of AM fungi isolated from olive plantations

The root fragments present in the root-associated soil samples taken from the long-term established olive tree plantations consistently showed AM fungal structures. This corroborated the expected mycotrophic habit of this crop. Four distinguishable morphotypes of *Glomus* spp. were identified in these soil samples. The assigned species and the corresponding BEG registration number is: *G. intraradices* (BEG 123), *G. mosseae* (BEG 124), *G. clarum* (BEG 125) and *G. viscosum* (BEG 126). These morphotypes were consistently present in all the rhizospheric soil samples.

The four AM fungi morphotypes were genetically characterised by PCR amplification and sequence analysis of a portion of their SSU rRNA to verify their morphological identification. The accession number for the new sequences, as deposited in the EMBL database are as follows: *G. intraradices* (AJ505617), *G. mosseae* (AJ505618), *G. clarum* (AJ505619) and *G. viscosum* (AJ505620). Comparisons of the obtained sequences with those present in the database revealed that they show more than 95% identity to the SSU rRNA gene of different AM fungal isolates. Phylogenetic analysis of the sequences obtained in this study and others from selected glomalean SSU rRNA genes from the database shows that the new sequences of *G. mosseae*, *G. intraradices* and *G. viscosum* cluster with the sequences obtained from these species in other laboratories (Fig. 1). However, the sequence of *G. clarum* (BEG 125) is grouped in a different clade than other *G. clarum* sequences recorded in the database.

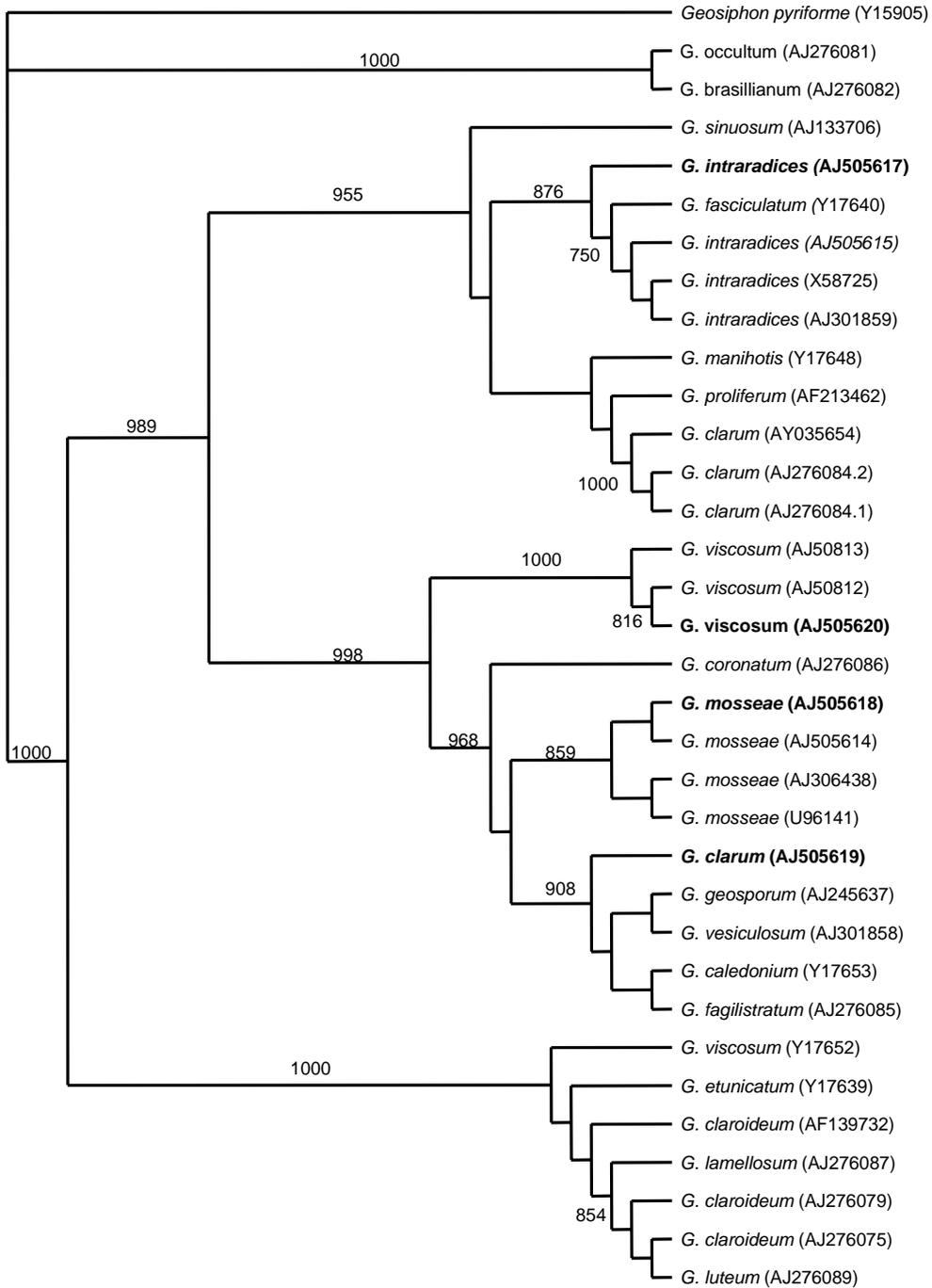


Fig. 1. Phylogenetic analysis of the NS31-AM1 region of the SSU rRNA from different AM fungi, including those isolated from olive plantations in the present study (in bold). Bootstrap values of the neighbour-joining analysis over 750 from 1000 replications are shown on the branches.

Table 1

Effectiveness of different AM fungi isolated from olive rhizospheres, in comparison with two reference strains, *Glomus mosseae* (BEG 119) and *Glomus intraradices* (EEZ 1), as inoculants for commercial varieties of olive plants

Experimental variables		Response variables <sup>a</sup>				
Plant cultivar	AM fungal inoculum <sup>b</sup>	Plant height (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Inoculum efficiency <sup>c</sup>	AM colonization (%)
Arbequina	Control	8.6 ± 0.29 a	1.03 ± 0.05 a	1.40 ± 0.13 bc	–	0 a
	<i>G.intraradices</i> (BEG 123)	21.1 ± 0.91 d	2.53 ± 0.17 e	2.10 ± 0.07 de	146	72 ± 2.40 c
	<i>G.mosseae</i> (BEG 124)	15.2 ± 0.68 bc	1.73 ± 0.16 bc	1.12 ± 0.20 ab	68	53 ± 6.59 b
	<i>G.clarum</i> (BEG 125)	12.4 ± 0.94 b	1.51 ± 0.10 b	1.54 ± 0.19 c	47	55 ± 4.12 b
	<i>G.viscosum</i> (BEG 126)	21.5 ± 0.65 d	2.55 ± 0.14 e	2.16 ± 0.20 e	148	57 ± 2.52 b
	<i>G.mosseae</i> (BEG 119)	15.5 ± 0.74 bc	1.89 ± 0.04 cd	1.19 ± 0.09 ab	84	77 ± 0.93 c
	<i>G.intraradices</i> (EEZ1)	16.3 ± 0.73 d	2.16 ± 0.04 d	1.75 ± 0.12 cd	110	52 ± 2.89 b
Leccino	Control	22.7 ± 1.04 a	3.08 ± 0.11 ab	2.08 ± 0.12 ab	–	0 a
	<i>G.intraradices</i> (BEG 123)	31.0 ± 0.72 d	4.11 ± 0.10 d	2.74 ± 0.14 c	33	21 ± 4.08 b
	<i>G.mosseae</i> (BEG 124)	23.7 ± 1.75 ab	3.16 ± 0.17 b	2.55 ± 0.29 c	3	21 ± 6.00 b
	<i>G.clarum</i> (BEG 125)	21.9 ± 0.98 a	2.75 ± 0.08 a	1.93 ± 0.07 a	–11	3 ± 0.76 a
	<i>G.viscosum</i> (BEG 126)	30.1 ± 0.48 d	3.63 ± 0.12 c	2.34 ± 0.16 bcd	18	38 ± 3.62 cd
	<i>G.mosseae</i> (BEG 119)	26.3 ± 0.90 bc	3.15 ± 0.15 b	2.08 ± 0.15 ab	2	31 ± 5.74 c
	<i>G.intraradices</i> (EEZ1)	29.3 ± 1.59 cd	3.66 ± 0.14 c	2.36 ± 0.14 bc	19	41 ± 1.92 d

<sup>a</sup> Means ± S.E.,  $n = 7$ . For each olive cultivar, data not sharing a letter in common differ significantly ( $P < 0.05$ ) by the Fisher's Protected Least Significant Difference Test. Data were rounded after statistical analysis.

<sup>b</sup> Registration number.

<sup>c</sup> Calculated as the percentage increase in shoot fresh weight produced by each AM fungus over the un-inoculated control.

### 3.2. Evaluation of the effectiveness of the AM fungi isolated from olive plantations as inoculant for olive plantlets

Data summarized in Table 1 confirm that both mycorrhizal dependency and root colonization were higher in Arbequina than in Leccino plants. *G. intraradices* (BEG 123) and *G. viscosum* (BEG 126) were the most effective fungi to improve the development of micro-propagated plants of the two target olive varieties (Table 1). It is noteworthy that the *G. intraradices* (BEG 123) strain, isolated from olive rhizosphere, was more effective than the *G. intraradices* EEZ1 from culture collection. In spite of the low mycotrophy level of the Leccino plants, inoculation with *G. intraradices* (BEG 123) produced a considerable growth response (33% of inoculum efficiency) in this cultivar. The percentage of root colonization was not directly related to the size of the growth response.

## 4. Discussion

Both morphology-based and novel molecular approaches were applied to characterize the isolated AM

fungi from root-associated soil in long-time established olive plantations. This is the first step to analyse the diversity of AM fungi in the target agrosystems. By following these approaches, only four distinguishable spore morpho-types, further corroborated by molecular techniques, were found. It is evident that mono-cultured olive trees select for a low number of AM fungal species. This supports previous statements indicating that AM fungal diversity is low in agricultural field (Helgason et al., 1998), particularly in the case of monoculture (Menéndez et al., 2001), as is the case with olive plantations. However, as previous studies have demonstrated (Stutz and Morton, 1996; Ferrol et al., 2003) field spores often represent just a fraction of the functional AM community. The low species richness found in the olive rhizosphere might reflect limitations in the sporulation patterns under field conditions. Establishment of long-term trap cultures might reveal a higher level of AM fungal diversity in the target soil.

The molecular characterization of AM fungi has been a complicated task in the past because of the considerable degree of variation detected among the copies of ribosomal DNA within single spores, which brought into question the validity of these methods for

phylogenetic investigations into the Glomales (Hijri et al., 1999; Sanders, 1999). However, recent reports indicate that SSU rRNA sequence analysis is a suitable tool to infer phylogenetic relationships among AM fungi since the expected sequence difference could be more easily linked to a taxonomic entity than the possibly multi-allelic ITS sequences (Schwarzott and Schüßler, 2001). For this reason, the different morphotypes isolated in this study were genetically characterised by analysis of their SSU rRNA sequences. The fact that the phylogenetic tree groups the sequences of our isolates *G. intraradices* (BEG 123), *G. mosseae* (BEG 124) and *G. viscosum* (BEG 126) with different isolates of these species from other laboratories, indicates that strains of the same species present very close phylogenetic relationships (Schwarzott and Schüßler, 2001). However, the sequence of *G. clarum* (BEG 125) was surprisingly located in a different clade than other *G. clarum* sequences recorded in the database. In a previous study, Schwarzott and Schüßler (2001) also observed that different isolates of this species were placed in different branches when a different region of the SSU rRNA (NS1-NS2 fragment) was analysed. Although the reasons for this divergence are not known, it has been proposed that it could be due to difficulties in distinguishing *G. clarum* from *G. manihotis*. Further work is in progress to elucidate why different isolates of *G. clarum* do not present close phylogenetic relationships. The increasing amount of sequence data currently becoming available will soon improve classification of AM fungi.

In testing the effectiveness of the isolated native AM fungi it must be considered that the use of AM fungi native to the target agro-system does not necessarily mean high effectiveness as inoculants (Enkhtuya et al., 2000). However, in this study the native *G. intraradices* (BEG 123) and *G. viscosum* (BEG 126) were the most effective fungi for improving growth of both olive varieties, among the AM isolates tested. As described for other plant species (Camprubi and Calvet, 1996; Linderman and Davis, 2001), results here described for olive plants, show that the degree of responsiveness to AM inoculation not only varies depending on olive genotype but also on the AM fungi inoculated. It is noteworthy that the *G. intraradices* (BEG 123) isolated from root-associated soil in the target olive plantation was more effective than the *G. intraradices* (EEZ 1) belonging to our culture collec-

tion. This indicates a certain level of physiological and ecological adaptations of the AM fungi to the target environment (Requena et al., 1997; Jeffries and Barea, 2001) and supports the importance of appropriate selection processes aimed at choosing the most effective inoculants for the target plant species and/or variety.

Inoculation of micro-propagated plantlets with selected AM fungi to improve the quality of nursery-produced plants has been tested for many cash crops (Varma and Schuepp, 1995; Lovato et al., 1996; Azcón-Aguilar and Barea, 1997). However, no information is available with regard to olive plantlets. The reported experiments support the potentiality of AM inoculation for two olive cultivars (Arbequina and Leccino) of current agronomic interest.

In conclusion, this study emphasizes the importance of exploring and exploiting the natural diversity of AM fungi as a starting point to formulate inoculants to be applied for the production of appropriate plant varieties with optimised quality.

## Acknowledgements

The authors gratefully acknowledge the Spanish Research Programmes CICYT project FEDER-UE 1FD97 0763 and INIA project CA 99 010, that supported the reported studies.

## References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipton, D.J., 1990. Basic local alignment search tool. *J. Moll. Biol.* 215, 403–410.
- Azcón-Aguilar, C., Barea, J.M., 1997. Applying mycorrhiza biotechnology to horticulture: significance and potentials. *SCI. Aortic.* 68, 1–24.
- Azcón-Aguilar, C., Palenzuela, J., Barea, J.M., 2000. Sustrato para la producción de inóculos de hongos formadores de micorrizas. Patent number 9901814, Spain.
- Barea, J.M., Azcón, R., Azcón-Aguilar, C., 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. *Anton. Leeuw.* 81, 343–351.
- Camprubi, A., Calvet, C., 1996. Isolation and screening of mycorrhizal fungi from citrus nurseries and orchards and inoculation studies. *Hortscience* 31, 366–369.
- Clapp, J.P., Young, J.P.W., Merryweather, J.W., Fitter, A.H., 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytol.* 130, 259–265.

- Dodd, J.C., Rosendahl, S., 1996. The BEG Expert System—a multimedia identification system for arbuscular mycorrhizal fungi. *Mycorrhiza* 6, 275–278.
- Dodd, J.C., Thomson, B.D., 1994. The screening and selection of inoculant arbuscular-mycorrhizal and ectomycorrhizal fungi. *Plant Soil* 159, 148–149.
- Enkhtuya, B., Rydlová, J., Vosátka, M., 2000. Effectiveness of indigenous and non-indigenous isolates of arbuscular mycorrhizal fungi in soils from degraded ecosystems and man-made habitats. *Appl. Soil Ecol.* 14, 201–211.
- Felstein, J., 1993. PHYLIP, Version 3.5, Department of Genetics, University of Washington, USA.
- Ferrol, N., Calvente, R., Cano, C., Barea, J.M., Azcón-Aguilar, C., 2003. Analysing arbuscular mycorrhizal fungal diversity in shrub-associated resource-islands from a desertification-threatened semiarid mediterranean ecosystem. *Appl. Soil Ecol.*, in press.
- Graham, J.H., Hodge, N.C., Morton, J.B., 1995. Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. *Appl. Environ. Microbiol.* 61, 58–64.
- Helgason, T., Daniell, T.J., Husband, R., Fitter, A., Young, P., 1998. Ploughing up the wood-wide web? *Nature* 394, 431.
- Hewitt, E.J., 1966. Sand and water culture methods used in the studies of plant nutrition. In: *Tech Comm 22*. London, Comm. Agri. Bur., pp. 430–434.
- Hijri, M., Hosny, M., van Tuinen, D., Dulieu, H., 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. *Fungal Genet. Biol.* 26, 141–151.
- INVAM 1997, <http://www.invam.caf.wvu.edu/>.
- Jeffries, P., Barea, J.M., 2001. Arbuscular mycorrhiza—a key component of sustainable plant-soil ecosystems. In: Hock, B. (Ed.), *The Mycota IX Fungal Associations*. Springer, Berlin, pp. 95–113.
- Jiménez-Díaz, R., 1998. Control de enfermedades. In: Jiménez-Díaz, R., Lamo de Espinosa, J. (Eds.), *Agricultura Sostenible*. Mundi-Prensa, Madrid, pp. 345–375.
- Kowalchuk, G.A., De Souza, F.A., Veen, J.A.V., 2002. Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. *Mol. Ecol.* 11, 571–581.
- Lachica, M., Aguilar, A., Yañez, J., 1973. Análisis foliar, métodos utilizados en la Estación Experimental del Zaidín. *Anal. Edafol. Agrobiol.* 32, 1033–1047.
- Linderman, R.G., Davis, E.A., 2001. Comparative response of selected grapevine rootstocks and cultivars to inoculation with different mycorrhizal fungi. *Am. J. Enol. Vitic.* 52, 8–11.
- Lovato, P.E., Gianinazzi-Pearson, V., Trouvelot, A., Gianinazzi, S., 1996. The state of art of mycorrhizas and micropropagation. *Adv. Hortic. Sci.* 10, 46–52.
- Madan, R., Pankhurst, C., Hawke, B., Smith, S., 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. *Soil Biol. Biochem.* 34, 125–128.
- Menéndez, A.B., Scervino, J.M., Godeas, A.M., 2001. Arbuscular mycorrhizal populations associated with natural and cultivated vegetation on a site of Buenos Aires province, Argentina. *Biol. Fertil. Soils* 33, 373–381.
- Morton, J.B., Benny, G.L., 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glominae and Gigasporinae, and two new families, Acaulosporaceae, with an emendation of glomaceae. *Mycotaxon* 37, 471–491.
- Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55, 159–161.
- Rallo, L., 1998. Sistemas frutícolas de secano: el olivar. In: Jiménez-Díaz, R., Lamo de Espinosa, J. (Eds.), *Agricultura Sostenible*. Mundi-Prensa, Madrid, pp. 471–487.
- Redecker, D., Morton, J.B., Bruns, T.D., 2000. Molecular phylogeny of the arbuscular mycorrhizal fungi *Glomus sinuosum* and *Sclerocystis coremioides*. *Mycologia* 92, 282–285.
- Requena, N., Jimenez, I., Toro, N., Barea, J.M., 1997. Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. *New Phytol.* 136, 667–677.
- Roldán-Fajardo, B.E., Barea, J.M., 1985. Mycorrhizal dependency in the olive tree (*Olea europaea* L.). In: Gianinazzi-Pearson, V., Gianinazzi, S. (Eds.), *Physiological and Genetical Aspects of Mycorrhizal*. INRA, Paris, pp. 323–326.
- Sanders, I.R., 1999. No sex please, we're fungi. *Nature* 399, 737–738.
- SAS Institut (1990) SAS/STAT Users Guide: Statistic, Version 6, fourth ed. SAS Institut, Cary, NC.
- Schenk, N.C., Perez, Y., 1990. Manual for Identification of VA Mycorrhizal Fungi. Synergistic Publications, Gainesville, Florida, 250 pp.
- Schwarzott, D., Schüßler, A., 2001. A simple and reliable method for SSU rRNA gene DNA extraction, amplification and cloning from single AM fungal spores. *Mycorrhiza* 10, 203–207.
- Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol. Res.* 105, 1413–1421.
- Sieverding, E., 1991. Vesicular-Arbuscular Mycorrhiza Management in Tropical Agrosystems. Deutsche GTZ. GmbH Eschborn, 371 pp.
- Simon, L., Lalonde, M., Bruns, T.D., 1992. Specific amplification of 18S fungal ribosomal genes from VA endomycorrhizal fungi colonising roots. *Appl. Environ. Microbiol.* 58, 291–295.
- Smith, S.E., Read, D.J., 1997. *Mycorrhizal Symbiosis*. Academic Press, London, 605 pp.
- Stutz, J.C., Morton, J.B., 1996. Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. *Can. J. Bot.* 74, 1883–1889.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22, 4673–4680.

- Vandenkoornhuyse, P., Husband, R., Daniell, T.J., Watson, I.J., Duck, J.M., Fitter, A.H., Young, J.P., 2002. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Mol. Ecol.* 11, 1555–1564.
- van Tuinen, D., Jacqout, E., Zhao, B., Gollotte, A., Gianinazzi-Pearson, V., 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Mol. Ecol.* 7, 879–887.
- Walker, C., 1983. Taxonomic concepts in the Endogonaceae: spore wall characteristics in species descriptions. *Mycotaxon* 18, 443–455.
- Varma, A., Schuepp, H., 1995. Mycorrhization of the commercially important micropropagated plants. *Crit. Rev. Biotech.* 15, 313–328.
- Wyss, S., Bonfante, P., 1993. Amplification of genomic DNA of arbuscular mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycol. Res.* 97, 1351–1357.