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# Changes in the composition and diversity of AMF communities mediated by management practices in a Mediterranean soil are related with increases in soil biological activity

M.M. Alguacil [a,*], E. Torrecillas [a], F. García-Orenes [b], A. Roldán [a]

[a] CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, P.O. Box 164, Campus de Espinardo, 30100 Murcia, Spain

[b] GEA – Grupo de Edafología Ambiental, Departamento de Agroquímica y Medio Ambiente, Universidad Miguel Hernández, Avda. de la Universidad s/n, E-03202 Elche, Alicante, Spain

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ABSTRACT

A field experiment was performed in a semi-arid Mediterranean agroecosystem to assess the influence of different management practices on the composition and diversity of the arbuscular mycorrhizal fungi (AMF) present in the soil. The management practices included residual herbicide use (RH), plowing (P), plowing + oats (OP), addition of oats straw mulch (OS), and a control (land abandonment) (C). An adjacent, non-cultivated area under natural vegetation was used as a standard for local, high-quality soil, and as a reference for natural plant cover (NC). After eight years of management, soil was sampled, DNA was extracted, and the AM fungal small-subunit (SSU) rRNA genes were subjected to PCR, cloning, sequencing, and phylogenetic analyses. Thirty-five different phylotypes were identified, which were grouped in five families: the Glomeraceae, Paraglomeraceae, Diversisporaceae, Ambisporaceae, and Claroideoglomeraceae. The different agricultural management practices assayed affected both the community composition and the phylotypes richness of the AMF recovered from the soil, there being four clearly-different AMF communities (C together with OS, P together with OP, RH, and NC). The diversity of the AMF was positively correlated with soil parameters related with biological activity. The treatments involving plowing greatly altered the composition of the AMF communities but did not affect significantly the diversity and richness of the AMF with respect to treatment NC; however, the addition of herbicide gave the lowest AMF diversity found in this study. The treatment based on the addition of oats straw appears to be the most-suitable management strategy with respect to promotion of the AMF diversity and biological activity in this soil.



## 1. Introduction

Arbuscular mycorrhizal fungi (AMF) are the main component of the soil microbiota in most ecosystems and may be used as sensitive indicators of soil ecological quality, if they respond to environmental variation (Verbruggen et al., 2012). They are generalists and represent one of the most-abundant underground symbioses, since they form symbiotic relationships with the majority of land plants (Smith and Read, 2008). The AMF inhabit both plant roots and the surrounding soils, where they facilitate mineral nutrient uptake from the soil in exchange for plant carbohydrates. Moreover, AMF can protect their hosts from pathogens (Sikes et al., 2009) and enhance the sustainability of ecosystems by improving the soil structure (Caravaca et al., 2006; Wilson et al., 2009). These observations imply that AMF play an important role in the maintenance of agroecosystems stability and sustainable agricultural development.

Unsuitable land management practices together with the climatic conditions typical of the semi-arid areas of southeastern Spain (scarce, irregular rainfall and frequent drought periods) can contribute to increases in the erosion rates and degradation processes of Mediterranean agricultural land. Under these climatic conditions, a loss of soil fertility is produced, as well as a reduction in the abundance and diversity of the AMF present in the soil (Requena et al., 2001; Azcón-Aguilar et al., 2003). Most of the biodiversity of agroecosystems lies in the soil (Young and Crawford,

* Corresponding author. Tel.: +34 968 396337; fax: +34 968 396213.
*E-mail address:* mmalguacil@cebas.csic.es (M.M. Alguacil).

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0038-0717/

2004), and the functions performed by soil biota have great direct and indirect effects on crop growth and quality, the quality of nutrient cycling, the sustainability of soil productivity (Roger-Estrade et al., 2010), and thus, the ecosystem multifunctionality (Wagg et al., 2014). Therefore, in order to improve or maintain the soil quality and biodiversity, the development and implementation of new, sustainable agricultural practices are necessary. Currently, several strategies are being applied on experimental farms on rain-fed agricultural land in southeastern Spain, in order to reduce the high erosion rates. These include catch crops, no-tillage or reduced tillage, pruned and chipped branches, straw mulches, and weed control by herbicides (García-Orenes et al., 2009). Previous studies (carried out at the site that is also the subject of the current work) showed that some of these strategies contribute to the reduction of soil erosion (García-Orenes et al., 2010), the enhancement of soil productivity and aggregate stability (García-Orenes et al., 2012), and the maintenance of the microbial community structure (García-Orenes et al., 2013). Soil management practices affect not only the erosion processes and soil quality but also the composition and species diversity of the AMF communities present in the soil (Mathimaran et al., 2005; Alguacil et al., 2009b; Lumini et al., 2010; Avio et al., 2013; Köhl et al., 2014) it is very important to know the impact that these agriculture management practices have on the soil properties and, as a consequence, on the species diversity and community structure of AMF. As mentioned above, the AMF play a vital role in ecosystem services such as plant growth and productivity, since they provide nutrients to plants and improve the soil structure, thus helping to enhance the sustainability of ecosystems (Smith and Read, 2008).

Until now, most studies on AMF communities in agricultural fields have been restricted to conventional (tillage) management or conventional agricultural practices (Jansa et al., 2003; Alguacil et al., 2008; Schnoor et al., 2011; Borriello et al., 2012; Brito et al., 2012), and very few have been conducted in Mediterranean conditions (Brito et al., 2012; Avio et al., 2013; Bedini et al., 2013). In general, these studies indicated that the abundance and diversity of AMF were related negatively to the management intensity (Verbruggen et al., 2012). It has been shown that the dispersal of AMF taxa is strongly influenced by chance; thus, shifts in the distributions of AMF species are caused by the habitat preferences of taxa due to factors such as nutrient availability (Palacio et al., 2007; Alguacil et al., 2010; Antunes et al., 2012), soil type (Lekberg et al., 2007; Helgason and Fitter, 2009; Balestrini et al., 2010; Oehl et al., 2010), soil disturbance (Schnoor et al., 2011; Brito et al., 2012), or toxic compounds (Hassan et al., 2011; Ipsilantis et al., 2012). Therefore, because the AMF taxa are functionally distinct, it is necessary to identify the major factors driving the AM fungal community assembly in soils. Knowledge of these factors will help in the conservation of the biodiversity and functions of semi-arid ecosystems.

In the present study, we examined the variations in composition and richness of AMF communities after application of different management practices in an experimental semi-arid, rain-fed orchard in eastern Spain. We hypothesized that variations in AMF populations can be related to alterations in soil biological activity. Our aim was 1) to ascertain the factors affecting the distribution of AMF communities; and 2) to assess which management practices were the most effective at promoting the richness and diversity of AMF in the soil, in order to implement agricultural management systems more suitable with regard to improving the biological fertility in Mediterranean soils.

## 2. Materials and methods

### 2.1. Study area

The experiment was established in a homogeneous field with a 5% slope, located at the El Teularet Experimental Station (García-Orenes et al., 2009, 2010) in the Enguera Range (Valencia, Spain 38° 50′ N; 0° 42′ W). The soil was classified as a Typic Xerorthent (SSS, 2010) developed from Cretaceous marls. The climate is typically Mediterranean, with three to five months of summer drought, usually from June to September. The mean annual rainfall in the study area is 479 mm, with an average annual temperature of 14.2 °C over the last 10 years.

### 2.2. Experimental design and layout

An experimental randomized-plot layout was established in a rain-fed olive orchard in the autumn of 2003, and the area was plowed to create uniform surface soil conditions before the start of the experiment. Before the experiment began, the soil was sampled at different points across the field, and no significant differences were found in the soil properties before the different treatments were established. Over centuries, plowing has contributed to a reduction in the spatial variability of the soil properties, as shown in Table 1. The experimental area was then divided, and five different treatments were applied in February of 2004, with three replicate plots per treatment (6 × 10 $m^2$). An adjacent area with the same type of soil, under natural forest vegetation, was used as a standard for local, high-quality soil. Here, three plots (6 × 10 $m^2$), situated 20 m apart, were established. We used soil under the wild, native vegetation, as close as possible to the potential vegetation, which had not been recently altered by human action (>60 years). The idea of using undisturbed soils under wild, native vegetation was suggested by Fedoroff (1987) and is based on the fact that soils that develop freely reach an equilibrium amongst their properties that leads to long-term stability, being considered as high-quality soils. The treatments were selected based on the practices commonly used by farmers at the study site, where rain-fed

**Table 1**
Analytical characteristics of the soil used in the experiment ($n = 20$).

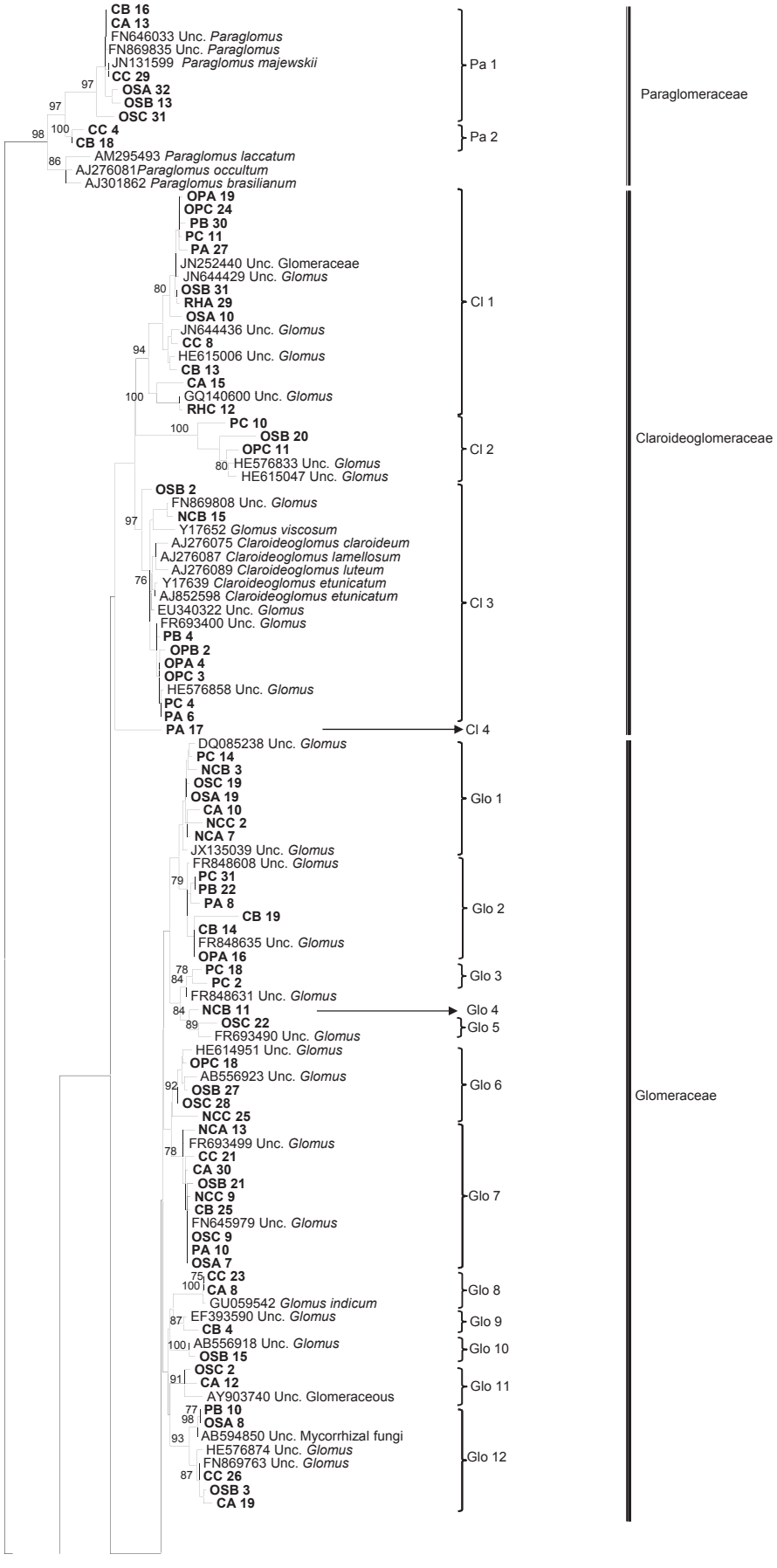
| | |
|---|---|
| Texture (%)[a] | 39, 38, 23 |
| pH (1:5, $H_2O$) | 8.30 ± 0.02 |
| Electrical conductivity EC (1:5, μS $cm^{-1}$) | 185 ± 4 |
| $CaCO_3$ (%) | 60 ± 3 |
| Total organic carbon (g $kg^{-1}$) | 12.5 ± 0.1 |
| Soluble C (μg $g^{-1}$) | 74 ± 1 |
| Microbial carbon biomass ($C_{mic}$) (μg $g^{-1}$) | 270 ± 2 |
| Total N (g $kg^{-1}$) | 0.78 ± 0.03 |
| Available P (mg $kg^{-1}$) | 2 ± 0 |
| Extractable K (mg $kg^{-1}$) | 303 ± 12 |
| Basal respiration rate (μg $CO^2$ $g^{-1}$ $h^{-1}$) | 5.7 ± 0.3 |

Mean ± standard deviation.
[a] Sand: 2–0.02 mm, Silt: 0.02–0.002 mm, Clay: <0.002 mm.

**Table 2**
Properties of soil in response to different agricultural management practices after 8 years establishment ($n = 3$).

| Treatments | C | NC | OS | P | OP | RH |
|---|---|---|---|---|---|---|
| OM (%) | 3.40c | 4.05c | 3.32c | 2.54b | 2.45b | 2.05a |
| MBC (mg $kg^{-1}$) | 335c | 310c | 419d | 240b | 322c | 201a |
| SCH (mg $kg^{-1}$) | 73.1c | 98.8d | 95.2d | 37.4b | 40.1b | 10.2a |

OM: soil organic matter content; MBC: microbial biomass C; SCH: soluble carbohydrate content. Values in row followed by the same letter do not differ significantly ($P < 0.05$) as determined by the Duncan's test. C = control; NC = natural cover; OS = oats straw; P = plowing; OP= oats + plowing; RH = residual herbicide.


CB 16
CA 13
FN646033 Unc. Paraglomus
FN869835 Unc. Paraglomus
JN131599 Paraglomus majewskii
CC 29
OSA 32
OSB 13
OSC 31
CC 4
CB 18
AM295493 Paraglomus laccatum
AJ276081Paraglomus occultum
AJ301862 Paraglomus brasilianum
Pa 1
Pa 2
Paraglomeraceae
OPA 19
OPC 24
PB 30
PC 11
PA 27
JN252440 Unc. Glomeraceae
JN644429 Unc. Glomus
OSB 31
RHA 29
OSA 10
JN644436 Unc. Glomus
CC 8
HE615006 Unc. Glomus
CB 13
CA 15
GQ140600 Unc. Glomus
RHC 12
Cl 1
PC 10
OSB 20
OPC 11
HE576833 Unc. Glomus
HE615047 Unc. Glomus
Cl 2
OSB 2
FN869808 Unc. Glomus
NCB 15
Y17652 Glomus viscosum
AJ276075 Claroideoglomus claroideum
AJ276087 Claroideoglomus lamellosum
AJ276089 Claroideoglomus luteum
Y17639 Claroideoglomus etunicatum
AJ852598 Claroideoglomus etunicatum
EU340322 Unc. Glomus
FR693400 Unc. Glomus
PB 4
OPB 2
OPA 4
OPC 3
HE576858 Unc. Glomus
PC 4
PA 6
Cl 3
PA 17
Cl 4
Claroideoglomeraceae
DQ085238 Unc. Glomus
PC 14
NCB 3
OSC 19
OSA 19
CA 10
NCC 2
NCA 7
Glo 1
JX135039 Unc. Glomus
FR848608 Unc. Glomus
PC 31
PB 22
PA 8
CB 19
CB 14
FR848635 Unc. Glomus
OPA 16
Glo 2
PC 18
PC 2
Glo 3
FR848631 Unc. Glomus
NCB 11
Glo 4
OSC 22
FR693490 Unc. Glomus
Glo 5
HE614951 Unc. Glomus
OPC 18
AB556923 Unc. Glomus
OSB 27
OSC 28
NCC 25
Glo 6
NCA 13
FR693499 Unc. Glomus
CC 21
CA 30
OSB 21
NCC 9
CB 25
FN645979 Unc. Glomus
OSC 9
PA 10
OSA 7
Glo 7
CC 23
CA 8
GU059542 Glomus indicum
Glo 8
EF393590 Unc. Glomus
CB 4
Glo 9
AB556918 Unc. Glomus
OSB 15
Glo 10
OSC 2
CA 12
AY903740 Unc. Glomeraceous
Glo 11
PB 10
OSA 8
AB594850 Unc. Mycorrhizal fungi
HE576874 Unc. Glomus
FN869763 Unc. Glomus
CC 26
OSB 3
CA 19
Glo 12
Glomeraceae

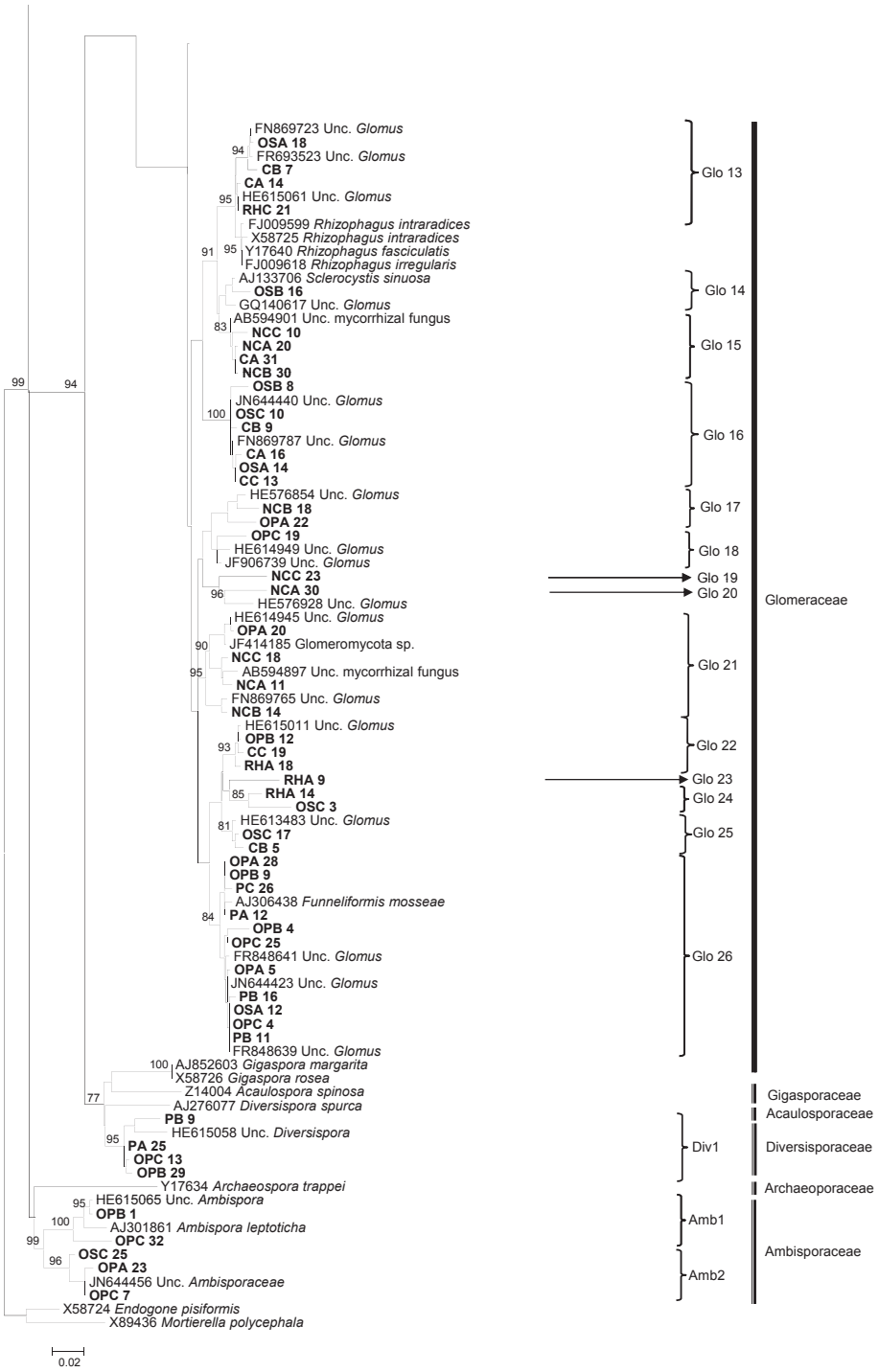



**Fig. 1.** (*continued*).

agriculture is the most-common farming activity (olive, almond, and cereal crops). The treatments, applied every year along the 8-year experimental period, were as follows:

RH, residual herbicide (3 applications $yr^{-1}$ (240 g $l^{-1}$ Oxyfluorfen); 1.5 kg $ha^{-1}$);

P, plowing (4 times $yr^{-1}$, to a depth of 20 cm);

OP, oats + plowing (plowing 4 times $yr^{-1}$ to a depth of 20 cm and sown 100% oats added to the soil in spring);

OS, oats straw (0.25 kg $m^{-2}$ $yr^{-1}$, straw mulch chopped and left on the surface of the soil);

C, a control was established with no management – simulating an abandoned field. These plots were recolonized spontaneously by

**Fig. 1.** Neighbor-Joining (NJ) phylogenetic tree showing AM fungal sequences isolated from soils under the different treatments (C (control), NC (natural cover), OS (oats straw), P (plowing), OP (oats plowing), RH (residual herbicide)), and reference sequences from GenBank. All bootstrap values >75% are shown (1000 replicates). Numbers above branches indicate the bootstrap values of the maximum likelihood analysis. Sequences obtained in the present study are shown in bold type. They are labeled with the name of treatment and repetition (A: repetition 1, B: repetition 2, C: repetition 3) from which they were obtained and the clone identity number. Group identifiers (for example Glo1) are phylotypes found in our study. *Endogone pisiformis* and *Mortierella polycephala* were used as out-groups.

native plant species (*Brachypodium retusum* (Pers.) Beauv., *Cistus albidus* L., etc.);

NC, natural cover (adjacent, non-cultivated area with *Quercus coccifera* L., *Pistacia lentiscus* L., *Juniperus oxycedrus* L., and *Brachypodium retusum (Pers.) Beauv.)*. This area, with the same type of soil but under natural vegetation, was used as a standard for local, high-quality soil and as a reference for natural cover.

No further fertilization or pest management was applied over the experimental period.

### 2.3. Soil sampling

After applying the treatments for eight years, samples were collected in July 2012 – immediately after the rainy season, when the microbial activity was expected to be higher. Each sample consisted of six bulked sub-samples per replicate plot (100-$cm^3$ cores), randomly collected at 0–5 cm depth. The 18 soil samples (6 treatments × 3 replicates) were sieved at 2 mm, frozen, and stored until analysis.

### 2.4. Soil analyses

The microbial biomass carbon ($C_{mic}$) was determined by the fumigation-extraction method (Vance et al., 1987). Water-soluble carbohydrates (SCH) extracted with water was determined using the method of Brink et al. (1960) and was measured spectrophotometrically at 630 nm. Soil organic carbon was determined using the potassium dichromate oxidation method (Nelson and Sommers, 1982), and the organic matter (OM) values were calculated using the Wasksman coefficient (1.72).

### 2.5. DNA extractions from soils and PCR

Two independent DNA extractions (0.5 g of soil fresh weight) from the 18 soil samples were carried out using a FastDNA™ Spin kit for soil according to the recommendations of the manufacturer (Q-BIOgene, Heidelberg, Germany). DNA extracts were stored at −20 °C.

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 PuReTaq Ready-To-Go PCR beads (Amersham 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 PuReTaq Ready-To-Go PCR beads (Amersham 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 samples 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 *GelRed*™ (Biotium).

### 2.6. Cloning and sequencing

The PCR products were purified using a Gel extraction Kit (Qiagen) cloned into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* (Xl1 blue). Putative positive transformants were screened in each resulting SSU rRNA gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and the supplied reaction buffer to a final volume of 25 μl and a re-amplification with AML1 and AML2 primers with the same cycling parameters 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 using the universal primers SP6 and T7 by Laboratory of Sistemas Genómicos (Valencia, Spain).

123 unique sequences of the clones generated in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under the following accession numbers HF913444–HF913566.

### 2.7. Phylogenetical analysis

Sequence editing was done using the program FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). A search for similar sequences to the ones from this study was conducted with the BLAST tool (Zhang et al., 2000) provided by GenBank. 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 and Walker (2010). All the sequences were aligned, using the multiple sequence comparison program, MAFFT, version 7.0 (available at http://align.bmr.kyushu-u.ac.jp/mafft/software) and the alignment was adjusted manually in BioEdit software version 7.0.4.1 (Hall, 1999).

Maximum likelihood (ML) phylogenetic analyses were performed with MEGA v.5.05 software (Tamura et al., 2011). Nucleotide data files were first tested to find the best DNA evolution model. The General Time Reversible model with a discrete Gamma distribution showed the lowest BIC scores (Bayesian Information Criterion) and was deemed to best describe the nucleotide substitution pattern. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The robustness of all trees obtained was evaluated by 1000 bootstrap replications. *Endogone pisiformis* Link and *Mortierella polycephala* Coem, were used as the out-groups.

Different AMF 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 85%) and sequence similarity ≥ 97%. The analysis for clustering sequences was carried out using the CD-HIT program considering a sequence identity cut-off of 97% (Fu et al., 2012).

### 2.8. Diversity of AM fungal community

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 $H' = -\sum p_i(\ln p_i)$, where $p_i$ is the proportion of individuals found in the $_i$th 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 $_i$th species]).

The Jaccard index ($J$) measures similarity between sample sets. It is calculated from the equation $J = C/S_1 + S_2 - C$, where $C$ is the number of phylotypes shared between treatments 1 and 2, $S_1$ the number of phylotypes in treatment 1, $S_2$ the number of phylotypes in treatment 2.

Rarefaction analyses were applied in order to determine if the number of tested clones sufficiently represented the AMF diversity in the soil samples (Raup, 1975). The rarefaction curves were produced by plotting the number of phylotypes observed against the number of sequences obtained using the freely available Analytic Rarefation v. 1.3 software (http://www.uga.edu/~strata/software/anRareReadme.html).

### 2.9. Statistical analysis

The effects of the treatments on soil parameters were tested using a one-way analysis of variance, and comparisons between the means were made using the Duncan's test calculated at $p < 0.05$. The data were tested for normality using the Kolmogorov–Smirnov test. Correlation analysis between all the soil parameters measured, and the AMF diversity and richness was carried out using Pearson's rank correlation coefficients.

The Shannon diversity index and the AMF phylotypes richness were subjected to ANOVA to test for significant differences between different management practices. For the comparisons among means the Duncan's test at $P < 0.05$ was used. All the statistical procedures were carried out with the software package IBM SPSS Statistic 19.0 for Windows.

To elucidate the relationships between the AM fungal community composition, soil properties and management treatments, ordination analyses were conducted in CANOCO for Windows v. 4.5 (ter Braak and Smilauer, 2004) using the relative abundance data for each AMF phylotype. Initial detrended correspondence analysis suggested a unimodal character of the data response to the sample origin (the lengths of gradients were >4); therefore, the canonical-correspondence analysis (CCA) was used. Monte Carlo permutation tests were conducted using 499 random permutations. The subsequent forward-selection procedure ranked the environmental variable according to their importance and significance for the distribution of the phylotypes.

## 3. Results

### 3.1. Soil properties

The application of oats straw to the soil yielded significantly-higher levels of MBC than the other treatments assayed (natural vegetation (NC), control (C), plowing (P), oats + plowing (OP), and residual herbicide (RH)) (Table 2). For all soil properties measured, the lowest values – significantly so – were found in the RH treatment. The OM and SCH contents did not differ significantly between the soils under the plowing and oats + plowing treatments; however, the MBC content was significantly higher under the OP treatment. The uncultivated plot under natural vegetation (NC) showed significantly-higher SCH contents than the control, but no significant differences between the C and NC plots were found with respect to the OM and MBC contents (Table 2).

### 3.2. Molecular analysis of AMF

We successfully amplified AM fungal DNA from all soil samples, with the primer combination AML1/AML2. Eighteen clone libraries, coming from six treatments and three replicates per treatment, were created. Approximately 100 clones per treatment were screened, sequenced, and analyzed phylogenetically. The BLAST searches revealed that 447 sequences (83% of the total sequences) showed high similarity (98–100% identity) to sequences from AMF taxa and belonged to members of the phylum Glomeromycota. Unique sequences of each phylotype were submitted to the EMBL database and are included in the phylogenetic tree shown in Fig. 1.

Thirty-five AM fungal phylotypes were detected in this study. By a large margin, the most-abundant and diverse group in the soil samples was the Glomeraceae, with 308 sequences grouped in 26 phylotypes, followed by the Claroideoglomeraceae group, represented by four phylotypes with 103 sequences. The Paraglomeraceae and Ambisporaceae were represented by two phylotypes each and the Diversisporaceae by only one phylotype (Fig. 1). Only seven phylotypes nested within a clade of sequences of AMF in culture and could be assigned to named species. These included Pa 1 (*Paraglomus majewskii*), Cl 3 (*Claroideoglomus lamellosum/etunicatum/claroideum* group) – the second-most-frequent phylotype found in this study, Glo 8 (*Glomus indicum*), Glo 13 (*Rhizophagus intraradices/irregularis* group), Glo 14 (*Sclerocystis sinuosa*), Glo 26 (*Funneliformis mosseae*) – the third-most-frequent phylotype detected in these soil samples, and Amb 1 (*Ambispora leptoticha*). Six phylotypes had no closely-related reference sequences in the database and the remaining phylotypes were related to uncultured Glomeromycota species sequences available in GenBank.

We explored whether the number of clones sequenced was sufficient to represent the diversity of AMF communities in the soil, by constructing rarefaction curves (Fig. 2). For the C and RH treatments, there was a well-defined leveling-off of the curves and it is highly unlikely that the sequencing of more clones would have revealed more phylotypes. For the OS, P, and OP treatments and the plot under natural vegetation, the number of phylotypes also approached saturation and the curve slope indicates that in order to find one more phylotype the number of sequences would have to be approximately doubled. Therefore, these data show that the clones sequenced in the soil samples of each treatment were sufficient to allow the detection of the vast majority of phylotypes.

### 3.3. Analysis of the AM fungal community composition in the soils with the different management practices

We did not find any phylotype common to all six treatments (Table 3). The only phylotype detected in the samples of the soils under all five different agricultural management practices was Cl 1. The oats straw (OS) addition to the soil produced the most-diverse AM fungal community, the diversity being significantly higher than in the rest of the assayed treatments (Fig. 3A). The OS treatment hosted 19 of the 35 phylotypes found in this study, being specific to this treatment Glo 5, Glo 10, and Glo 14 (related to *S. sinuosa*). The Shannon diversity index showed a clear difference between the OS treatment and the NC, P, OP, and RH treatments (Fig. 3B). The C (control) plot had the second-highest richness (15 phylotypes) and diversity ($H' = 2.24$). The C and OS plots shared the highest number of phylotypes (10), being exclusive to these two treatments Pa 1, Pa 2, Glo 11, and Glo 16. The phylotypes Glo 7 and Glo 12, although detected also in other soil management treatments, exhibited their highest appearance frequency (evenness) in these plots (C and OS) (Table 3).

The treatments involving plowing (P and OP) showed similar Shannon index values, which were close to that of the uncultivated plot under natural vegetation ($H' \approx 2.00$) (Fig. 3B). The numbers of phylotypes found under P and OP were also similar (12 and 14, respectively), being specific to these two management treatments the phylotype Div 1 (Table 3). Moreover, 92% of the clones belonging to Cl 3 and 96% of those belonging to Glo 26 (both are phylotypes related to known AMF species: the *C. lamellosum/etunicatum/claroideum* group and *F. mosseae*, respectively) were found under both soil management regimes which involved plowing. On the other hand, Cl 4 and Glo 3 were found exclusively in treatment P plots, and Glo 18 and Amb 1 only in OP plots (Table 3).

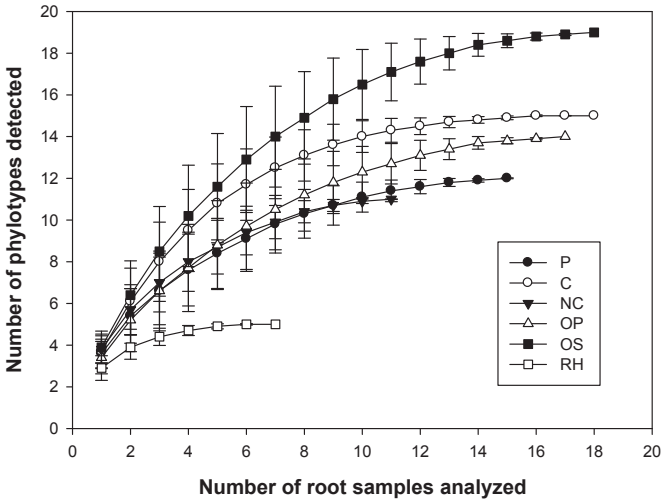



**Fig. 2.** Rarefaction curves for SSU rDNA libraries from soil under different agricultural management practices. C, control; NC, natural cover; OS, oats straw; P, plowing; OP, oats plowing; RH, residual herbicide. Curves were obtained using the Analytical Rarefaction Program version 1.3 (http://www.uga.edu/~strata/software/anRareReadme.html).

The application of herbicide to the soil gave rise to the lowest (significantly so) AMF richness and Shannon diversity index, with only five phylotypes retrieved and an $H'$ value of 1.32 (Fig. 3A,B).

**Table 3**
Number of clones detected for each AMF phylotype in the soil samples under different agricultural management practices ($n = 3$).

| Treatments | C | NC | OS | P | OP | RH |
|---|---|---|---|---|---|---|
| Pa1 | 5 | – | 7 | – | – | – |
| Pa2 | 3 | – | 2 | – | – | – |
| Cl1 | 6 | – | 4 | 13 | 3 | 9 |
| Cl2 | – | – | 2 | 2 | 2 | – |
| Cl3 | – | 2 | 3 | 23 | 32 | – |
| Cl4 | – | – | – | 2 | – | – |
| Glo1 | 3 | 16 | 3 | 3 | – | – |
| Glo2 | 3 | – | – | 4 | 2 | – |
| Glo3 | – | – | – | 2 | – | – |
| Glo4 | – | 2 | – | – | – | – |
| Glo5 | – | – | 2 | – | – | – |
| Glo6 | – | 2 | 4 | – | 2 | – |
| Glo7 | 36 | 7 | 32 | 2 | – | – |
| Glo8 | 3 | – | – | – | – | – |
| Glo9 | 4 | 2 | – | 2 | – | – |
| Glo10 | – | – | 2 | – | – | – |
| Glo11 | 6 | – | 2 | – | – | – |
| Glo12 | 5 | – | 7 | 2 | – | – |
| Glo13 | 3 | – | 2 | – | – | 4 |
| Glo14 | – | – | 2 | – | – | – |
| Glo15 | 2 | 10 | – | – | – | – |
| Glo16 | 6 | – | 10 | – | – | – |
| Glo17 | – | 2 | – | – | 2 | – |
| Glo18 | – | – | – | – | 2 | – |
| Glo19 | – | 2 | – | – | – | – |
| Glo20 | – | 2 | – | – | – | – |
| Glo21 | – | 11 | – | – | 2 | – |
| Glo22 | 2 | – | – | – | 2 | 19 |
| Glo23 | – | – | – | – | – | 2 |
| Glo24 | – | – | 2 | – | – | 4 |
| Glo25 | 5 | – | 2 | – | 2 | – |
| Glo26 | – | – | 2 | 21 | 23 | – |
| Div1 | – | – | – | 4 | 7 | – |
| Amb1 | – | – | – | – | 2 | – |
| Amb2 | – | – | 2 | – | 4 | – |
| Total | 92 | 58 | 92 | 80 | 87 | 38 |

The CCA analysis showed that the different agricultural management practices altered the AMF communities ($p = 0.002$, $F = 2.79$), as revealed by Monte Carlo permutation tests (Fig. 4). Four AMF communities were clearly differentiated. There were no significant differences between the AMF communities under the P and OP treatments; also, the C and OS plots shared a similar AMF community. In fact, in the diagram, the centroids representing these treatments are close to each other, indicating that these treatments gave rise to similar AMF communities. These results are supported also by the Jaccard index, which is closer to 1 between the AMF communities of the P and OP plots and between those of the C and OS plots than for the rest of the plots (Table 4).

The forward selection procedure revealed the scoring of the environmental variables with respect to their importance for determination of the phylotype distribution, which corresponds to the variance explained by each environmental variable at the time it was included in the model. Only the SCH content ($P = 0.002$), RH treatment ($P = 0.004$), and NC treatment ($P = 0.002$) had a significant influence on the variance at that time.

In a parallel analysis excluding the soil parameters, the forward selection procedure indicated that the AMF communities in the RH, NC, OP, and P treatments were significantly different from those of

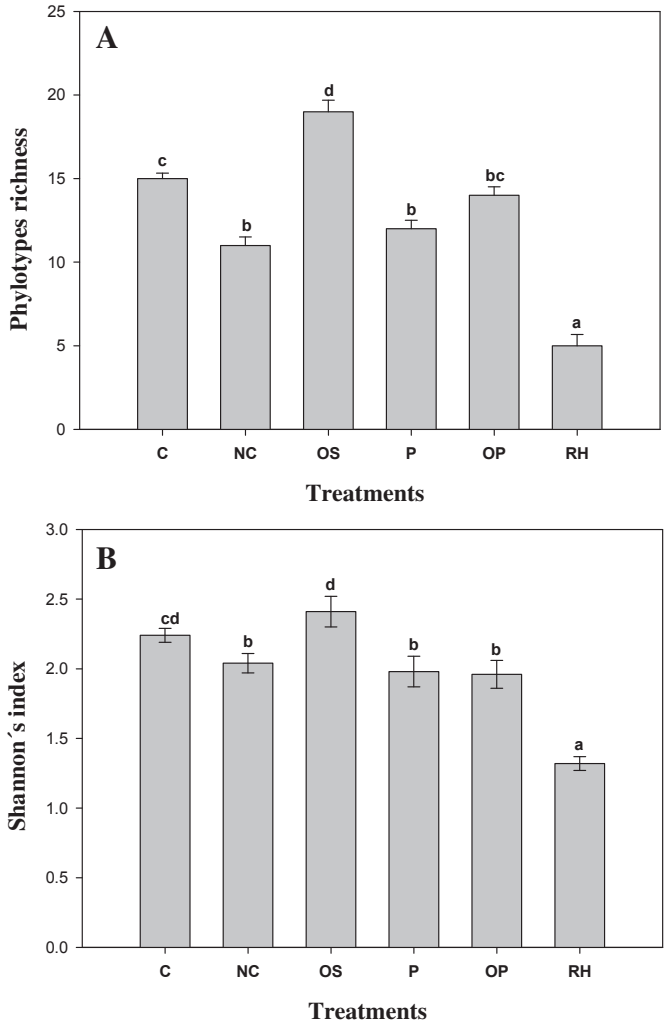



**Fig. 3.** Graphical representation of the (A) Phylotypes richness and (B) Shannon's index in the soil under different agricultural management practices. Abbreviations are: C, control; NC, natural cover; OS, oats straw; P, plowing; OP, oats plowing; RH, residual herbicide ($n = 3$, ±SE).

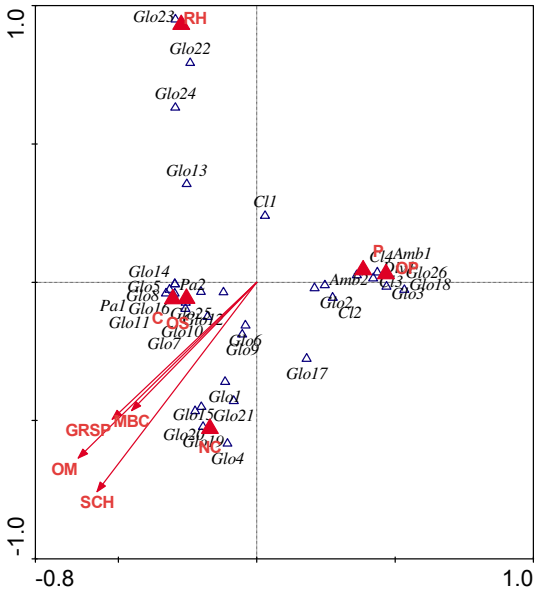



**Fig. 4.** Canonical Correspondence analysis (CCA) of the AM fungal community composition recovered in the soil under the different treatments and soil properties. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as follows: CCA1: 0.68 and CCA2: 0.65. The model explained 52.9% of the whole variance. Abbreviations: C (control), NC (natural cover), OS (oats straw), P (plowing), OP (oats plowing), RH (residual herbicide), OM (soil organic matter content), MBC (microbial biomass C), GRSP (glomalin related soil protein), SCH (soluble carbohydrate content).

treatments C and OS (RH: $P = 0.008$; NC: $P = 0.004$; OP: $P = 0.024$; P: $P = 0.004$).

### 3.4. The relationship between the diversity of the AMF and the soil properties

Positive correlations between all the soil properties related with biological activity measured and the Shannon diversity index were found ($P < 0.05$) (Table 5). Moreover, the richness of phylotypes was related positively also with the microbial biomass C content ($r = 0.921$; $P < 0.01$).

## 4. Discussion

In this work, the diversity of AMF occurring in plots subjected to different management practices under semi-arid conditions was investigated. To the best of our knowledge, this study shows the highest number of phylotypes detected so far in agricultural fields, using the same molecular approach as ours (32 in the treated soils plus three exclusive to the natural cover soil). It should be mentioned that the majority of papers on this topic used fungal DNA extracted from mycorrhizal roots (Helgason et al., 1998; Daniell et al., 2001; Hijri et al., 2006; Alguacil et al., 2008; Verbruggen et al., 2010; Sasvári et al., 2011). Therefore, it is quite probable that the higher AMF richness in our survey is due to the fact that the AMF diversity in the soil is higher than that within the roots, as observed by other authors in a grassland ecosystem (Hempel et al., 2007) and in agricultural soils (Cesaro et al., 2008; Balestrini et al., 2010; Borriello et al., 2012). The soil contains both spores and extra-radicular mycelium; however, in the root tissue only the symbiotically-active AMF community is present at a given time.

In our study, the most-common sequence group was the Glo 7 phylotype, which showed 99% homology with sequences from unidentified *Glomus* from a Mediterranean degraded, semi-arid area (Alguacil et al., 2011a, 2011b). This phylotype was present in all the treatments assayed, except OP and RH. The second-most-common sequence group was the Cl 3 phylotype, affiliated with the *C. lamellosum*/*etunicatum*/*claroideum* group. This group was retrieved mainly from the soils subjected to plowing (P and OP). Jansa et al. (2003) also found that *C. claroideum* increased significantly with tillage intensity, in a long-term field experiment carried out in Switzerland. Thus, these AMF taxa may have a certain degree of resistance to plowing.

In agreement with the majority of surveys carried out in agricultural fields, we also found the phylotype assigned to *F. mosseae* to be present in our analyses. In fact, this AM fungus was found principally in the soils subjected to plowing (treatments P and OP). It has been reported that *F. mosseae* is common and typical in arable fields (Oehl et al., 2003; Hijri et al., 2006; Rosendahl et al., 2009; Borriello et al., 2012; Avio et al., 2013).

Surprisingly, *R. intraradices*, the most-abundant and widespread AMF taxon in the majority of studies carried out until now, in different locations and in both natural and managed ecosystems around the world (Öpik et al., 2006), was found only occasionally in our experiment. This phylotype only appeared in 2% of the total sequences, distributed in three treatments (C, OS, and RH) (Table 3). This finding was rather unexpected and it is difficult to explain the low presence of this AMF phylotype in our study since *R. intraradices* is considered a very-invasive AM fungal species, with a generalistic lifestyle, that produces a large quantity of extra-radicular mycelium (Jansa et al., 2003; Öpik et al., 2006).

Another finding of note in our study was the lack of detection of Archaeosporaceae and Paraglomeraceae in plots under plowing (P and OP), which is in agreement with the results of other studies that indicated that these AMF species do not appear to be dominant or detectable in arable soils (Oehl et al., 2003, 2005; Hijri et al., 2006; Alguacil et al., 2008; Borriello et al., 2012). Taxa of AMF belonging to the families Ambisporaceae and Diversisporaceae were recovered under both plowing treatments. In the studies carried out to date, these families were generally rare components of the AMF communities found in agricultural systems (Daniell et al., 2001; Hijri et al., 2006; Alguacil et al., 2008; Toljander et al., 2008), although none of these studies were performed in Mediterranean semi-arid conditions – where these AMF have been recovered widely (Alguacil et al., 2012; Torrecillas et al., 2012a, 2012b).

**Table 4**
Jaccard similarity index.

| | C | NC | OS | P | OP | RH |
|---|---|---|---|---|---|---|
| C | 1 | 0.18 | 0.42 | 0.29 | 0.16 | 0.18 |
| NC | | 1 | 0.15 | 0.21 | 0.19 | 0 |
| OS | | | 1 | 0.29 | 0.27 | 0.14 |
| P | | | | 1 | 0.30 | 0.06 |
| OP | | | | | 1 | 0.12 |
| RH | | | | | | 1 |

C = control; NC = natural cover; OS = oats straw; P = plowing; OP = oats + plowing; RH = residual herbicide.

**Table 5**
Pearson's coefficients of correlation and significance level between the soil parameters measured, the richness of phylotypes and the AMF diversity ($n = 3$).

| Parameters | Shannon diversity index | Richness |
|---|---|---|
| Soil organic matter content | 0.790 (0.050) | 0.663 (0.151) |
| Soluble carbohydrate content | 0.821 (0.045) | 0.674 (0.142) |
| Microbial biomass C | 0.882 (0.020) | 0.921 (0.009) |

In the present study, we observed that the different management practices had a significant effect on the AMF community composition ($P < 0.001$). It is of note that a relatively-short period of time (8 years) has yielded dramatic changes in the composition and diversity of the AMF communities, attributable to the practices assayed. The highest number of AMF phylotypes (19) was detected with oats straw addition. This OS treatment increased significantly the values of all the soil parameters measured (Table 2), which are commonly associated with the soil biological activity (Caravaca et al., 2006). Thus, the increased AMF diversity under this treatment might have been a consequence of an improvement in soil biological quality. Quite often, soil properties have been reported to play an important role in the structure and composition of AMF communities (Helgason and Fitter, 2009; Schreiner and Mihara, 2009; Alguacil et al., 2009a, 2010; Jansa et al., 2014). In fact, in our study, there was a significant, positive correlation between the AMF diversity and all the soil parameters measured. It has been suggested that the sporulation of some AMF can be stimulated by an increase in the organic matter content (Gryndler et al., 2005; Oehl et al., 2009) and in our work the highest organic matter content also occurred under the OS treatment, which gave the highest AMF richness (Table 3, Fig. 2). Oats straw contains organic compounds which are easily available to soil microorganisms, such as celluloses. These substrates may have promoted the microbial activity, as shown by the increased content of microbial biomass C in the OS plot (Table 2); this has been considered a sensitive indicator of soil microbial activity (Garcia et al., 1997; Caravaca et al., 2003). The community composition of the AMF for the control (C) treatment was very similar to that of the OS treatment (Fig. 4) and had a higher AMF richness than the plots used as a reference (NC). These results might be due to the changes in the composition of the (natural) weed plant community in response to the management practices (O'Donovan and McAndrew, 2000; Streit et al., 2000; Jansa et al., 2003) and to differences in the strategies of colonization among species. In fact, this control soil was recolonized by native plant species which were different from the plants present in the natural, non-managed soil. In accordance with this, some authors have reported that the plant diversity influences the AMF community structure and increases AMF abundance (Burrows and Pfleger, 2002; König et al., 2010; De Deyn et al., 2011). It seems clear that, after cultivation, the soil AMF composition changes greatly (Oehl et al., 2003); in our case, both the abandonment of cultivation (control) and the OS treatment yielded higher phylotypes richness than the natural cover soil, in spite of the fact that in this never-treated soil the highest values of the soil C fractions and biological activity were recorded. Although the principal reason might be a change in the plant species composition, as has been discussed above, some authors have suggested that in non-cultivated areas hardening of the soil surface can occur – which can produce a decrease in soil porosity, leading to a limited $O_2$ supply for soil microorganisms (Álvaro-Fuentes et al., 2008). These conditions are unfavorable to the distribution of AMF propagules and may decrease their activity and, as a result, their abundance (Yang et al., 2012), even though the accumulation of soil organic C is high (Curaqueo et al., 2011), in accordance with our results.

It is of note that the addition of oats straw together with plowing produced an AMF community with a composition very similar to that resulting from plowing alone (Fig. 4). Also, the soil properties, AMF richness, and AMF diversity of the P and OP treatments were similar. This suggests that the plowing effect was predominant over the oats addition effect and only the AMF resistant to plowing prevailed. In agreement with previous studies (Jansa et al., 2003; Oehl et al., 2003, 2005; Hijri et al., 2006; Toljander et al., 2008; Sasvári et al., 2011), a relatively-high AMF richness was found in the arable soils P and OP, which showed 12 and 14 phylotypes, respectively. Also, the AMF community composition under both plowing treatments was different from the other treatments assayed. Therefore, a shift in the AMF community composition was caused by plowing (Jansa et al., 2003; Schnoor et al., 2011; Brito et al., 2012); in fact, few phylotypes were shared between the natural and plowed soils, as was shown clearly in the CCA analysis also (Fig. 4). According to Oehl et al. (2005), in the deeper soil layers reside many AMF species that remain "inactive"; therefore, these layers may serve as an important source of inoculum. Thus, with the plowing these layers may have been moved and new AMF species "activated" or "recovered", in addition to the disturbance-tolerant phylotypes that proliferate after plowing. On the other hand, Boddington and Dodd (2000) suggested that an increase in the number of AMF species in plowed soils could be due to a stimulatory effect of tillage on hyphal growth.

The residual herbicide induced a very-significant decrease in the richness and diversity of the AMF community in our experiment (Table 3, Figs. 2 and 4). This treatment produced the lowest AMF diversity of this study, with only five phylotypes recovered. It seems obvious that an absence of weeds could mediate a decrease in those AMF phylotypes which show host preferences; what is of note is that most of the AMF community in the soil disappeared in a relatively-short period of time (8 years). Thus, the five AMF phylotypes found in the RH treatment have some traits that allow them to adapt to this stressful situation or might remain as very-resistant spores in the soil. Some studies have demonstrated negative effects of oxyfluorfen on the mycorrhizal colonization of cassava (Sieverding and Leihner, 1984) and grapevine roots (Baumgartner et al., 2005).

In conclusion, the different practices assayed affected both the community composition and the phylotypes richness of the AMF recovered in the soil, with four clearly-different AMF communities being identified (C together with OS, P together with OP, RH, and NC) in a relatively-short period of time (8 years). The diversity of the AMF was significantly correlated with improvements in soil biological properties, while disturbance mediated by plowing altered greatly the AMF composition but did not affect significantly the AMF diversity and richness, with respect to the reference treatment (NC). Since one of the main targets in sustainable land use is the protection of biodiversity (Kleijn et al., 2001; Billeter et al., 2008), the treatments based on the addition of oats straw appear to be the most-suitable strategy with respect to promotion of the AMF diversity and biological activity in soil.

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