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ARTICLE

Loss of arbuscular mycorrhizal fungal diversity in trap cultures during long-term subculturing

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Abstract: Long-term successional dynamics of an inoculum of arbuscular mycorrhizal fungi (AMF) associated with the maize rhizosphere (from traditionally managed agroecosystems in Los Tuxtlas, Veracruz, Mexico), was followed in *Bracchiaria comata* trap cultures for almost eight years. The results indicate that AMF diversity is lost following long-term subculturing of a single plant host species. Only the dominant species, *Claroideoglomus etunicatum,* persisted in pot cultures after 13 cycles. The absence of other morphotypes was demonstrated by an 18S rDNA survey, which confirmed that the sequences present solely belonged to *C. etunicatum*. Members of *Diversisporales* were the first to decrease in diversity, and the most persistent species belonged to *Glomerales*.

Key words:
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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) belonging to the phylum *Glomeromycota* are important soil organisms that form mutualistic associations with plants, and which are involved in the uptake and transport of mineral nutrients to plant roots (Barea *et al*. 2002). Up to 90 % of analysed plant species are able to form this symbiosis (Smith & Read 1997). Soil structure is improved by the presence of AMF, which likely represents a large component of the microbial biomass (Sieverding & Oehl 2005).

Several studies have suggested that there has been very little selective pressure for either symbiotic partner to develop a high degree of specificity (Harley & Smith 1983, Law & Lewis 1983, Clapp *et al*. 1995). However, Chanway *et al*. (1991) noted several factors that may result in strong selection for plant host specificity, such as geographical distance, environmental heterogeneity, host identity, and habitat, which might also affect distribution of AMF. While some authors have noted a lack of host specificity (Merckx *et al*. 2012), others report that certain hosts show preferences for certain AMF (Yang *et al*. 2012).

Edaphic conditions and cultural management in different agroecosystems are factors that may influence soil AMF (Oehl *et al*. 2003, 2004, 2005). Very few studies describe the selective pressure on AMF species when they are taken from their natural environments to trap cultures (Antunes 2012, Oliveira 2010). Cuenca *et al*. (2003) reported that AMF propagation in trap cultures exhibits difficulties because exact natural conditions cannot be reproduced; this causes a bias towards proliferation of species that are able to tolerate greenhouse conditions, and that associate better with the plant host under the specific trap culture conditions. Leal *et al*. (2009) have also reported that the trap-plant technique does not necessarily facilitate AMF identification from a specific ecosystem, since diverse factors in the trap cultures might prevent the propagation of all the species present in the initial inoculum. Factors such as the soil or substrate type used and handling, can also include the broad variety of plant species used for propagating AMF in trap cultures (Yao *et al*. 2010). These results do not necessarily support or contradict the observations that either multiple infections or AMF selectivity occur between certain host-fungus combinations in co-existing plants of a natural community. They do, however, indicate that mycorrhizal fungi respond differentially according to the host species; these differences could thus result in a selective pressure that favours certain host-fungus combinations (Bever 2002, Sanders & Fitter 1992).

This study aims to present a long-term successional analysis of an AMF maize rhizosphere community from traditionally managed sites in Los Tuxtlas, Veracruz, maintained in trap cultures of *Bracchiaria comata* in continuous subculturing for almost eight years.

MATERIALS AND METHODS

Site description

Sampling was carried out in the Los Tuxtlas, Biosphere Reserve area (UNESCO – MAB Biosphere Directory), in the state of Veracruz, Mexico (18° 24´ 56" N – 18°26´33" N; 94° 56´ 53" W – 94° 58´ 18" W), situated at an altitude of 238.39 ± 37.45 m (García *et al*. 2009). The annual precipitation is 2 000–2 500 mm; the weather is characterised as Am (f), i.e. tropical warm and humid, and the mean annual temperature ranges from 22 to 26 °C with abundant rain throughout the year (García 1998). The site was traditionally managed, and referred to as "milpa" by the indigenous inhabitants. It is a diverse agroecosystem (polycultures) where the main crop is maize. Milpas normally contain native maize types (criollos), always accompanied by other crop plants such as bean (*Phaseolus* spp.) and pumpkin (*Cucurbita* spp.); these products constitute the traditional pre-Hispanic agriculture "food triad" system. Other common crops associated with this polyculture system are jicama (*Pachyrhizus erosus*) and cassava (*Manihot esculenta*) (López-Cano & Castillo-Campos 2009, Negrete-Yankelevich *et al*. 2013). Soil cores were taken at the end of the rainy season during December 2003 and January 2004. The soil is an umbric Andisol, composed of 66 % sand, 11.5 % clay, and 22.5 % lime. Other soil characteristics include 11.51 % organic matter, 9–19 mg g^{-1} N, 0.44 mg g^{-1} P, 0.21 mg g^{-1} K, 4.62 mg g^{-1} Ca, 2.00 mg g^{-1} Mg, and pH 5.3 (García *et al*. 2009).

Soil sampling

The composite soil sample (LM38-BGBD), was a mixture of 12 subsamples taken with a corer of 5 cm diam × 20 cm height around 2 concentric circles of 3 and 6 m diam, which gave a total sampling area of 114 m^2. The soil of the 12 subsamples was crumbled by hand and mixed. The sample was kept in plastic bags and stored at 4 °C, before spore isolation. The AMF spores present in the sample were morphologically identified according to INVAM keys and the manual of Schenck & Pérez (1990). The same soil samples were also used to inoculate "AMF trap cultures" with *Brachiaria comata* (*Poaceae*). This soil sample (LM28) formed part of the Conservation and Sustainable Management of Below Ground Biodiversity project (CSM-BGBD) of the Los Tuxtlas Biosphere Reserve (http://www3.inecol.edu.mx/csmbgbd/index.php/el-proyecto-bgbd-en-mexico), and is also part of a larger sampling programme of the Lopez-Mateos community at Los Tuxtlas (Varela *et al*. 2009).

AMF pot cultures

The same soil samples were used to inoculate "AMF trap cultures" with *Brachiaria comata* (*Graminae*/*Poaceae*) using the techniques of Sieverding (1991). This plant was chosen due to its wide range of interaction with AMF, as previously reported (de Souza *et al*. 2004). Greenhouse conditions in Xalapa, Veracruz, were set to be similar to those at the sampling site (25–35 °C and 60–80 % relative humidity, with natural daylight and photoperiod). Pots contained autoclaved sterile sand (15 lb, 121 °C, 1 h) as a substrate, and were either irrigated with distilled water every three days, or whenever necessary to preclude drying out. A modified Hoagland's solution (Hoagland & Arnon 1950), containing 20 µM phosphate, was used to fertilise trap cultures once or twice per month, according to the plant developmental stages. Irrigation was stopped after full development of the grass (approx. 3–4 mo), to allow for plant drying and spore production. The *Brachiaria comata* plants were then removed from the pots and a fresh culture was started in a new pot after approx. 3 mo (Burrows & Pfleger 2002). We considered each subculture to be one cycle (Fig. 1). Re-inoculation of pots containing 1 kg of sterile sand was carried out by using ~ 40 g of mixed roots with spores and sand.

Morphological analysis

Morphological identification of AMF from recently sampled field soils was performed in March of 2004 (Varela *et al*. 2009). Trap culture sampling of roots and spores and morphological identification of AMF took place between 2004 and 2012, for a total of nine morphological analyses. Morphological characteristics of sporocarps, spores and root subcellular structures were first observed in water on a glass slide under a dissecting microscope; specimens were later mounted in either polyvinyl alcohol:lactic acid:glycerol (PVLG; 1:1:1; Koske & Tessier 1983), or in a mixture 1:1 of PVLG and Melzer's reagent (lactic acid/water 1:1; Brundett *et al*. 1994). The morphology of the spores was analyzed according to the INVAM (International Culture Collection of Vesicular-Arbuscular Mycorrhizal Fungi, www.invam.caf.wvu.edu) data, and following Schenck & Pérez (1990). Photographs were taken with a digital camera (Motic digital microscope DMB3-223) on a dissecting microscope (Nikon SMZ-2T). Air-dried sporocarps and specimens mounted in either PVLG or a mixture of PVLG and Melzer's reagent were deposited in XAL (Xalapa, Instituto de Ecología A. C., INECOL).

DNA extraction, PCR amplification and sequencing

In order to confirm the final composition of morphotypes (trap culture cycles 13 through 15) we conducted rDNA surveys (~ 20 clones each). AMF colonized roots were cut into 0.5 cm pieces and collected in a 1.5 mL Eppendorf tube. Roots were homogenised in DNAzol® using a pestle. Genomic DNA was extracted in DNAzol® following the manufacturer's instructions. The concentration of genomic DNA was estimated by fluorometry using the Quantit DNA Quantitation kit™ (Cat. No. Q32854, ORE).

Genomic DNA was used for molecular identification of AMF in roots, by a nested PCR protocol. The oligonucleotides used are described by Redecker *et al.* (2000a, b). The mixture for the first PCR reaction contained diluted DNA (1:100 v/v), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer (NS5/ITS4), 0.2 mM of each dNTP, and 1.0 unit of recombinant Taq DNA polymerase (Cat. No. 11615-010, Invitrogen, Mexico City) in 25 µL total volume. The DNA templates were initially denatured at 95 °C for 4 min. The subsequent cycles included a 60 s denaturation step at 94 °C, 60 s of primer annealing at 55 °C, and a 3 min extension step at 72 °C. A final 5 min extension step at 72 °C concluded the programme after 32 cycles. PCR was performed using a MultiGene Thermal Cycler (Model TCP600-G, Labnet International). The first PCR product

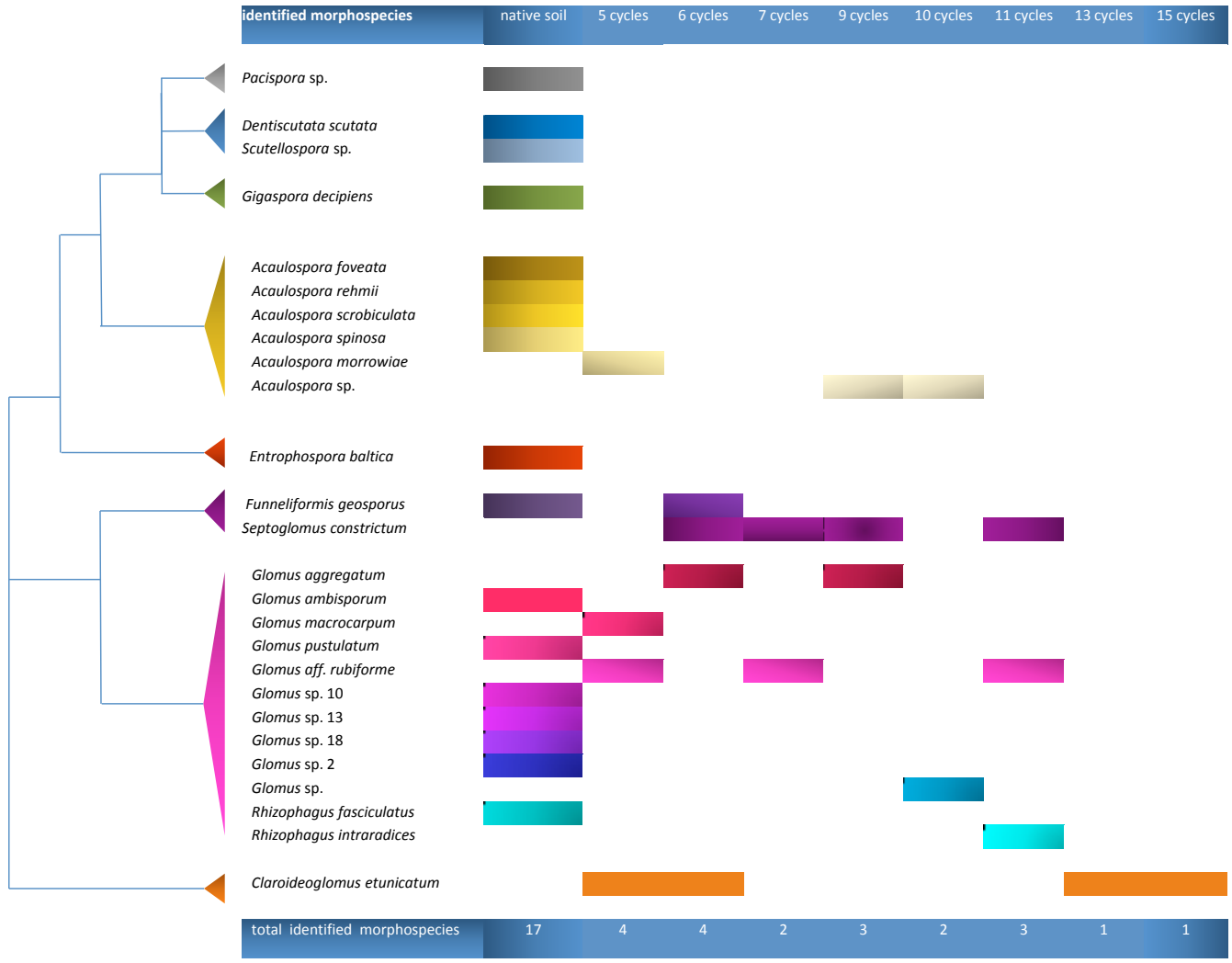

Fig. 1. AMF morphospecies dynamics during trap subculturing of the inoculum. Upper blue bar refers to the identified morphospecies in the native soil (found in the original mixed soil sample), and in the different trap subculturing cycles (indicated by number). Lower blue bar indicates the number of morphospecies found in each subculture cycle. Connecting blue lines and triangles indicates to taxonomic relatedness of the morphospecies found. The presence of a specific morphospecies is indicated by a bar of its designated colour in each cycle column.

was diluted 1:10 v/v with ultrapure distilled water. The dilutions were then used as DNA template for a nested PCR reaction, with primer pairs that amplify different ribosomal DNA regions (LETC1670/ITS4; ITS-1F/GIGA 5.8). The same PCR conditions were used as in the first reaction. Nested PCR bands that were ~ 650 bp and ~ 330 bp were cut out from a 1 % agarose gel, and the DNA extracted with the QIAquick gel extraction kit (Qiagen, Cat. 28704). The purified PCR products were cloned into the pGEM-T Easy Vector II System (Promega, Cat No. A3600, Madison, WI) as described by the manufacturer. Twenty clones were selected to make plasmid minipreps, according to the manufacturer's instructions (QIAprep spin miniprep kit, Qiagen Cat. No. 27106, Mexico City). The amplified and cloned fragments were sequenced using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). PCR products were sequenced in both directions with an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Mexico City). Sequencing was performed using the T7 and SP6 primers. DNA sequences obtained were cleansed of vector sequences, and screened for chimeras, which were excluded from further analysis. The sequences were queried in a BLASTN search in the NCBI database (http://www.ncbi.nlm.nih.gov/; BLASTN v. 2.2.22; Zhang *et al*. 2000). AMF nucleotide sequences derived from cycle 13 were deposited in GenBank (accession numbers: KC841638–KC841656) including one sequence (KC841638) confirming the host plant identity.

RESULTS

Morphological analysis

Morphological analysis of the initial native mixed soil sample (LM 38-BGBD) revealed 17 morphospecies; however, in the López-Mateos area at Los Tuxtlas, 24 morphospecies have been recognized (Varela *et al*. 2009). The AMF morphological analysis in the trap cultures revealed both a decrease in species spore presence and a large variability during subculturing, as compared to the native soil sample. None

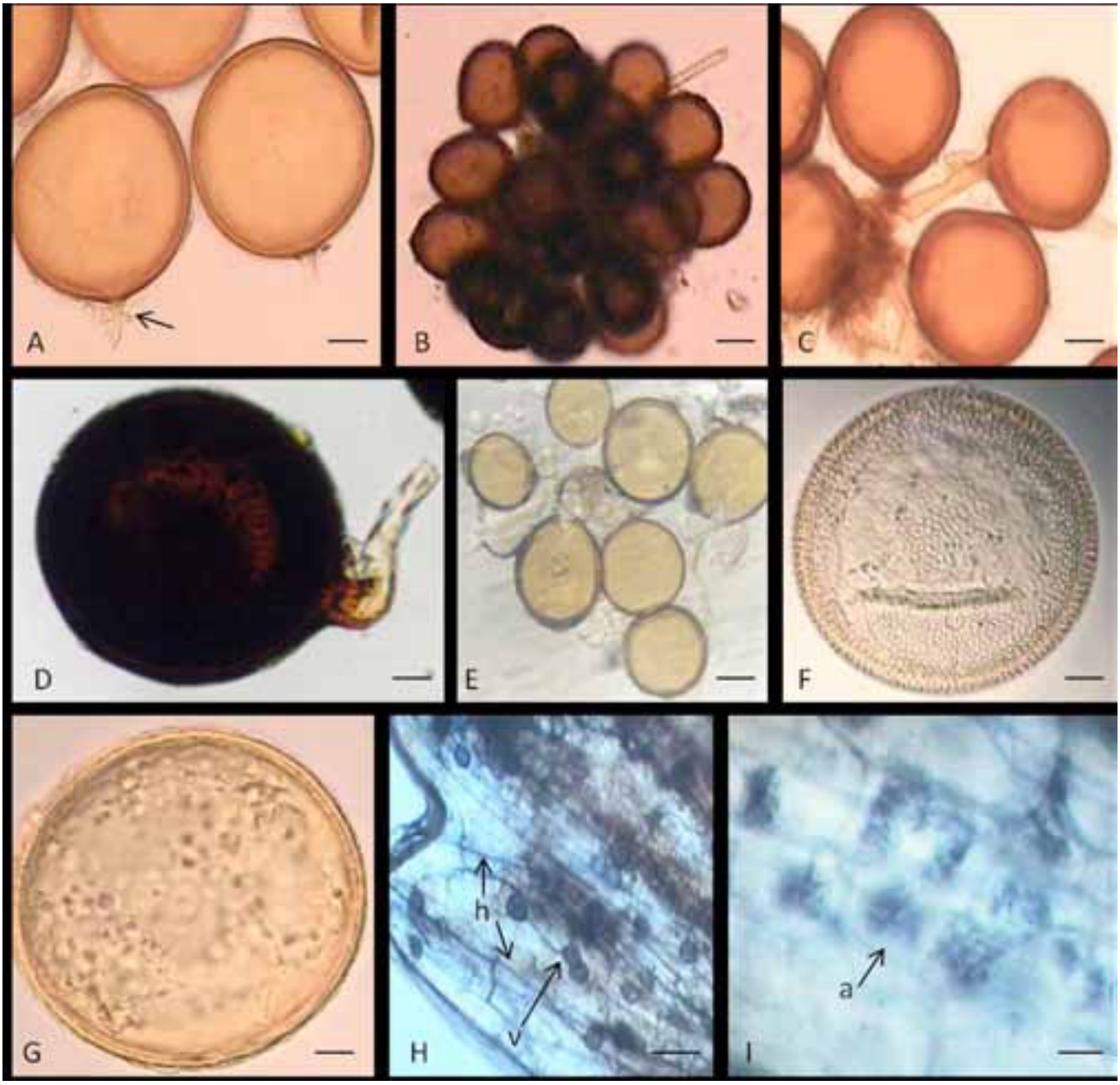

Fig. 2. A sample of the AMF morphotypes observed in *Bracchiaria comata* trap subcultures. **A.** *Claroideoglomus etunicatum*; note the subtending hyphae structure resembling a septum (arrow). **B.** *Glomus* aff. *rubiforme* (syn. *Sclerocystis rubiformis*). **C.** *G. macrocarpum*. **D.** *Septoglomus constrictum*. **E.** *Rhizophagus intraradices* (syn. *Glomus intraradices*). **F.** *Acaulospora scrobiculata*. **G.** *Acaulospora morrowiae.* **H–I.** *Brachiaria comata* AMF-colonized roots showing different internal structures indicated by arrows: hyphae (h), arbuscules (a), and vesicles (v). Bars: A = 23 µm; B = 100 µm; C = 33 µm; D = 100 µm; E = 10 µm; F = 50 µm; G = 5 µm; H = 10 µm; I = 5 µm.

of the field spore species reported in the first morphological soil analysis by Varela *et al.* (2009) were observed at the fifth cycle of successive trap culturing. In fact, four different species were found, including *Claroideoglomus etunicatum*. Morphological screening at the sixth successive trap culture identified *Funneliformis geosporus* (reported at the initial field site soil), along with *C. etunicatum* and two other species not found in the field (Fig. 1).

Species from *Glomerales* (Fig. 2A–E) were consistently found throughout successive cycles of trap culturing. On the other hand, most species of *Diversisporales* that were found in the field were not found in the pot cultures, except for *A. morrowiae* and one *A. scrobiculata* morphotype (Figs. 1, 2F, G). After approximately seven years of maintaining the trap cultures (cycles 13–15), *C. etunicatum* emerged as the dominant species (Fig. 1). *Claroideoglomus etunicatum* spores displayed the characteristic globose to subglobose morphology (Fig. 2A); the spores were mostly orange-brown (0-10-90-5 according to INVAM's colour table), and 80–188 µm diam. Subtending hyphae ranged from cylindrical to slightly flared (19.4–30.2 µm wide). Composite wall thickness (L1, L2 and L3 layers) was < 1 µm, with the innermost layer of the subtending hyphae forming a structure resembling a septum (Fig. 2A). Typical internal structures formed by

C. etunicatum developed in *B. comata* roots during cycle 13 of our subculturing (Fig. 2H, I).

Molecular analysis

All sequences obtained from cycles 13–15 were closely related to *Claroideoglomus etunicatum* and showed high percentages of identity (97–99.8 %) with previously reported sequences from conspecific specimens deposited in the BEG. Sequences obtained from cycle 13 were deposited in GenBank (GenBank Accession nos. KC841639–KC841656).

DISCUSSION

In this study, no *Claroideoglomus etunicatum* spores were found in the native field soil, although they became abundant in the pot cultures. A similar phenomenon has been reported at several sites (INVAM website: http://invam.caf.wvu.edu/), in which AMF are not expressed in trap cultures at the beginning of subculturing, and sometimes require up to two or three subcultures to sporulate. *Claroideoglomus etunicatum* sporulation was inconsistent and the spores present in the soils were not found during several intermediate cycles (e.g. 7–11), although cycles 13–15 were defined by sporulation from this single species. *Claroideoglomus etunicatum* can be considered highly competitive, due to its ability to adapt to extremely different conditions compared to the original sampling sites. The original soil contained 11.51 % organic matter, and by using sterile sand in the trap cultures it changed to an inert substrate having no organic matter and the pH dropped from 5.3 to 4.5. *Claroideoglomus etunicatum* is reported to be tolerant to host plant change, which is an important factor since hosts direct the carbon input to the fungus (Manoharan *et al.* 2008). This species was maintained even after prolonged subculturing in the host plant, preserving its infectivity and sporulation capabilities. These observations are consistent with *C. etunicatum* belonging to a select group of AMF species able to survive in a single host plant (Johnson *et al*. 2005). This species is also reported as one of the most common and ubiquitous AMF species worldwide (Bentivenga & Hetrick 1992).

Our molecular studies confirmed the dramatic drop (by cycle 13) in species richness reported by the morphological observations of the initial soil sample. Others have previously reported that field spores found in their native soil cannot be maintained in trap cultures. For instance, Burrows & Pfleger (2002) demonstrated that after five cycles of trap subculturing, none of the species present in the field soil were found in the trap cultures. A similar phenomenon was reported by Stutz & Morton (1996): in comparison to the first trap subculture, only 75 % of the original species were found after three trap culture cycles. This suggests that a high proportion of AMF present in arid habitats may not sporulate in the field.

Species of *Diversisporales* have been reported to be more susceptible to environmental change than ones of *Glomerales* (Lovera & Cuenca 2007). The latter exhibit shorter sporulation times than *Diversisporales,* considered due to the small size of their spores (Bever *et al.* 1996) which allows them to exhibit enhanced sporulation (Hepper 1984). *Glomerales* species are considered generalists and have been found to be associated with grasslands (Oehl *et al.* 2003). Generally, species belonging to this order are competitive colonisers, which increases their probability of representation in most plant communities (Cordoba *et al.* 2001). Kennedy *et al.* (2002) previously confirmed the dominance of *Glomerales* in trap cultures.

Our study contributes to the growing body of evidence suggesting that the use of a single plant species in trap cultures encourages a decrease in the AMF diversity maintained. This observation may be extrapolated to field conditions where monoculture is practiced for many consecutive years, which could similarly have a negative effect on AMF diversity (Bainard *et al*. 2012, Li *et al*. 2010, An *et al*. 1993, Hijri *et al*. 2006).

Future work will be aimed at testing the efficiency of *C. etunicatum* from Los Tuxtlas on both the development of native plants and in agroecosystem productivity.

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