Incidencia de la degradación del hábitat, la estacionalidad climática y el hábito de crecimiento de la planta hospedadora sobre la diversidad de las comunidades de hongos micorrícicos arbusculares, y su dinámica de sucesión, asociados a plantas características de ambientes mediterráneos

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Memoria presentada para aspirar al grado de Doctor en Biología

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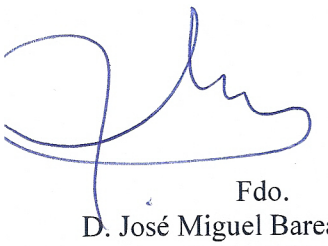

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MARCO CIENTÍFICO-ACADÉMICO INSTITUCIONAL DE LA TESIS

Este trabajo de Tesis Doctoral ha sido realizado en el Departamento de Microbiología del Suelo y Sistemas Simbióticos (Grupo de investigación "Micorrizas") de la Estación Experimental del Zaidín (EEZ-CSIC).

La Tesis forma parte de los proyectos del Plan Nacional de I + D + i CGL-2009-08825 y CGL-2006-02584/BOS desarrollados por el Grupo "Micorrizas", que han sufragado todos los gastos que demanda esta modalidad de investigación.

La Tesis se enmarca en el contexto de los estudios Científico-Académicos que conforman el **Master Universitario en Biología Agraria y Acuicultura** de la Escuela de Posgrado de la Universidad de Granada.

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- A. López-García, J.M. Barea, C. Azcón-Aguilar. Interaction between plant life form and functional traits of arbuscular mycorrhizal fungal community determines the symbiosis assembly. En preparación

Asimismo, parte de los resultados obtenidos durante esta Tesis Doctoral han sido presentados en el III Congreso Nacional Biodiversidad (Granada, 2011) mediante comunicación oral.

El doctorando Álvaro López García y los directores de la tesis D. José Miguel Barea Navarro y Dª Concepción Azcón González de Aguilar, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada a 26 de Junio de 2013

Directores de la Tesis

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Fdo.

Doctorando

Fdo. Álvaro López García

ÍNDICE

I. INTRODUCCIÓN

1. Naturaleza, Interés y Oportunidad de la Investigación

1. Naturaleza, Interés y Oportunidad de la investigación

Las características particulares de los ambientes Mediterráneos, tanto a nivel climático como geográfico, confieren a los ecosistemas desarrollados en tales ambientes una gran originalidad biológica. Debido a ello han sido incluidos entre los "puntos calientes" de biodiversidad a nivel mundial. Sin embargo, esas mismas características también les confieren una baja estabilidad y resiliencia ante las posibles perturbaciones. La marcada estacionalidad, con veranos calurosos y xéricos combinados con una considerable irregularidad y torrencialidad en los eventos pluviosos, limitan en gran medida la capacidad de los ecosistemas semiáridos Mediterráneos para responder ante cambios importantes en el medio. El manejo prolongado por parte del hombre de estos ecosistemas ha desembocado, en muchos casos, en su transformación irreversible hacia ambientes con tendencia a la desertificación, donde la cubierta vegetal y el suelo resultan degradados, con escasas posibilidades de auto-recuperación.

Cuando se producen estas situaciones de deterioro se plantean programas de restauración los cuales, en gran parte, van orientados al restablecimiento o mejora de la cubierta vegetal. Sin embargo, las mismas características climáticas que hacen frágiles estos ambientes, imponen una serie de restricciones fisiológicas para garantizar el éxito de la revegetación. Para paliar este inconveniente se ha recurrido al uso de biotecnologías las cuales promueven el establecimiento y crecimiento vegetal a través del uso de microorganismos que se asocian de forma natural a las plantas.

En efecto, en el estudio de la dinámica de los ecosistemas hay que considerar la existencia, diversidad y actividad de los microorganismos del suelo, así como sus interacciones con otros componentes del ecosistema. Uno de los grupos de microorganismos más extendidos e importantes por su influencia en los procesos que propulsan y rigen la dinámica de los ecosistemas terrestres son los hongos formadores de micorrizas arbusculares (MA). Se trata de organismos que viven en simbiosis con las plantas terrestres. Concretamente, en torno al 80% de éstas se asocian con los hongos MA. La formación de esta simbiosis data de casi 500 millones de años y se reconoce fundamental en el proceso de colonización del medio terrestre por parte de las plantas.

La simbiosis se desarrolla al colonizar los hongos las raíces de las plantas. Una vez establecida la simbiosis, el hongo utiliza su red de micelio extrarradical para facilitar a la planta la captación de nutrientes que difunden lentamente en la solución edáfica, así como la búsqueda de agua. A cambio, la planta cede parte de sus fotosintatos al hongo, ya que éste, al ser heterótrofo, necesita fuentes de C metabolizable. Además, la simbiosis reporta otra serie de beneficios a la planta hospedadora, como son la protección frente a estreses bióticos (patógenos) y abióticos (sequía, salinidad o contaminación por metales pesados o compuestos orgánicos recalcitrantes). Debido a las características de los ambientes mediterráneos, particularmente limitantes del crecimiento de las plantas y de la estabilidad del suelo, estos organismos van a tener un importante papel en la dinámica de las comunidades vegetales y, en consecuencia, pueden influenciar la dinámica global de todo el ecosistema.

En el contexto de la recuperación de los ecosistemas mediterráneos degradados, se ha demostrado la potencialidad del uso de los hongos MA para la restauración de la cubierta vegetal. La utilización de estos microorganismos ha derivado generalmente en la mejora del establecimiento y crecimiento vegetal así como de las propiedades del suelo. Sin embargo, pese a haberse llevado a cabo numerosas investigaciones sobre la prospección y aplicación de los hongos MA, poco se sabe de la dinámica poblacional y estrategias de colonización de estos hongos microscópicos en el ecosistema.

Las características de los ambientes mediterráneos en general y en particular los propios del sureste de la Península Ibérica han condicionado la aparición de diversos tipos de ecosistemas terrestres en los cuales se han originado a lo largo del tiempo una amplia diversidad de formas de vida. Así, la particular climatología, la variada orografía y la historia natural de la región, han derivado en multitud de estrategias adaptativas que facilitan la explotación de los recursos por las especies vegetales. De forma paralela sus compañeros simbióticos, los hongos MA, deben haber desarrollado adaptaciones impuestas por las mismas condiciones ambientales así como por los cambios que éstas producen en sus hospedadores. Aunque hay estudios que evidencian la divergencia en cuanto a estrategias de vida de los hongos MA, poco se ha investigado sobre sus características particulares o como éstas pueden influir en los ecosistemas, siendo la información aun más escasa para los ambientes mediterráneos. Uno de los retos de investigación más importantes en relación con las estrategias de vida de los hongos MA es estudiar el ciclo de vida de los hongos, el tipo de propágulos que producen o como sobreviven entre las

marcadas estaciones climáticas mediterráneas. Así, por ejemplo, uno de los sesgos más importantes que los estudios sobre hongos MA han tenido hasta ahora ha sido la exclusiva utilización de aislados que producen esporas, el único medio de aislamiento certero.

Consecuentemente, el estudio de los ciclos de vida y estrategias de colonización de los hongos MA presentes en ambientes semiáridos mediterráneos podría reportar considerables beneficios por lo que debería ser abordado para diseñar estrategias de restauración de ecosistemas. Los datos obtenidos permitirán conseguir una idea de la diversidad de estrategias de vida de los hongos MA y cómo éstas interaccionan con las características del medio. Ello permitiría la selección de las características que confieran una mayor posibilidad de éxito en las tareas de restauración de estos ambientes, así como asegurar una mayor estabilidad del ecosistema a largo plazo al promover la restauración de sus funciones.

De acuerdo con lo que antecede se propuso la presente Tesis Doctoral, que se enmarca en la línea de investigación del Grupo "Micorrizas" de la EEZ-CSIC sobre uso de hongos MA como herramienta biotecnológica en programas de restauración de la cubierta vegetal en ambientes Mediterráneos.

Para avanzar en esta línea de estudio esta Tesis se diseña con el fin de incrementar los conocimientos sobre el ciclo de vida de los hongos MA, sus estrategias de colonización e interacciones con las plantas hospedadoras. Así mismo, se propone evaluar el efecto de la degradación del hábitat sobre las comunidades de dichos hongos. El impacto de la variabilidad climática estacional, característica de los ecosistemas que modela el "clima Mediterráneo", sobre los hongos MA y sus interacciones con las plantas, será particularmente considerada en este trabajo Doctoral. Los estudios se llevarán a cabo en el Parque Natural "Sierra de Baza", un eco-enclave típicamente Mediterráneo, con un manejo moderado por parte del ser humano. Se asume que los resultados obtenidos pueden ser extrapolables a otras áreas de carácter eco-fisiológico similar.

Concretamente, y para lograr los fines que animan a la presente Tesis Doctoral, se propusieron diversos experimentos, con los siguientes **Objetivos**.

2. Objetivos

2. Objetivos

2.1. Objetivo general

Estudiar el impacto de diversos factores ambientales sobre la diversidad de las comunidades de hongos MA asociados a plantas características de ambientes mediterráneos, y la dinámica sucesional de los mismos.

2.2. Objetivos específicos (operacionales)

(1) Analizar las distintas estrategias que utilizan los hongos MA para la colonización de nuevas plántulas que se incorporan a la comunidad, así como el impacto de la estacionalidad climática en la dinámica sucesional de sus poblaciones.

(2) Estudiar si la relación entre las estrategias vitales de los hongos MA y los caracteres funcionales de sus hospedadores vegetales determinan la comunidad de hongos en las raíces simbióticas, y averiguar en qué medida la estacionalidad climática dirige estas interacciones.

(3) Investigar el impacto de la degradación del ecosistema sobre la diversidad de las poblaciones de hongos MA.

Antes de establecer el **Plan de Trabajo**, para el desarrollo de las investigaciones que permitan alcanzar los anteriores Objetivos, se revisa la literatura científica relacionada para resumir lo que ya se conoce y justificar la necesidad de investigar aspectos que se conocen poco o de forma fragmentaria.

3. Antecedentes biobliográficos

3. Antecedentes bibliográficos

La información que conforma esta Sección, actualizada apropiadamente para esta Tesis Doctoral, y teniendo en cuenta los **Objetivos** de la misma, está basada en la contribución del Doctorando y de los Directores de esta Tesis en el artículo de revisión:

J.M. Barea, J. Palenzuela, P. Cornejo, I. Sánchez-Castro, C. Navarro-Fernández, **A. Lopéz-García,** B. Estrada, R. Azcón, N. Ferrol, **C. Azcón-Aguilar** (2011) Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. Journal of Arid Environments 75:1292-1301

Resumen

Las micorrizas constituyen una asociación simbiótica ampliamente distribuida en los ecosistemas terrestres que se establece entre ciertos hongos del suelo y la mayoría de las plantas superiores. Tienen un papel fundamental en la mejora crecimiento y salud de la planta, y la calidad del suelo, ya que se conoce que la simbiosis micorrícica incrementa la resiliencia de las comunidades de plantas ante estreses ambientales, incluyendo la falta de nutrientes, la sequía o las alteraciones del suelo. Precisamente, son este tipo de estreses los que causan la degradación de los ambientes semiáridos del sureste español. Desde hace años se vienen desarrollando una serie de estudios básicos, estratégicos y aplicados para profundizar en cómo la actividad y diversidad de los hongos micorrícicos afectan a la composición de las comunidades de plantas, su estructura y su dinámica. Estas investigaciones se revisan aquí atendiendo a los siguientes apartados: (i) el análisis de la diversidad de hongos micorrícicos; (ii) el estudio de las interacciones ecológicas y funcionales de las comunidades vegetales y las poblaciones de hongos micorrícicos asociadas; y (iii) el uso de estos hongos como herramienta biotecnológica en la restauración de ambientes semiáridos degradados del sureste español.

En general, las investigaciones realizadas en los ambientes semiáridos del sureste Ibérico evidencian que la degradación de los sistemas suelo-planta disminuye la presencia y diversidad de poblaciones de hongos micorrícicos. Sin embargo, los propágulos micorrícicos no desaparecen completamente, lo que sugiere un cierto grado de adaptación a los estreses lo que permite la utilización de estos ecotipos resistentes para la producción de inoculantes. Se han llevado a cabo numerosos estudios de revegetación en el sureste semiárido de la Península Ibérica, utilizando especies de plantas propias de la sucesión natural de estas áreas, en conjunción con sus comunidades de hongos micorrícicos asociados. Se encontró que este tipo de estrategia ayudó al establecimiento y el desarrollo de las plantas, así como la calidad del suelo, y demostró su potencialidad como herramienta biotecnológica para ayudar a la restauración sostenible de esos ecosistemas. Sin embargo, después de 20 años de estudios, aún no tenemos una comprensión total del potencial de las micorrizas a la hora de mejorar la composición, diversidad, estructura y funcionalidad de las comunidades de plantas de esta región.

Abstract

Mycorrhizas are worldwide symbiotic associations established between certain soil fungi and most vascular plants and are fundamental in optimizing plant fitness and soil quality. Mycorrhizal symbioses improve the resilience of plant communities against environment stresses, including nutrient deficiency, drought and soil disturbance. Since these stresses are paramount in the degradation of semi-arid ecosystems in the SE Spain, a series of basic, strategic and applied studies have been made to ascertain how the activity and diversity of mycorrhizal fungi affect plant community composition, structure and dynamics in this region. These investigations are reviewed here in terms of: (i) analysing the diversity of mycorrhizal fungi; (ii) assessing the ecological and functional interactions among plant communities and their associated mycorrhizal fungal populations; and (iii) using mycorrhizal inoculation technology for the restoration of degraded semi-arid areas in Southeast Spain.

Disturbance of the target semi-arid ecosystems decreases the density and diversity of mycorrhizal fungust populations. Nevertheless, the mycorrhizal propagules do not disappear completely suggesting a certain degree of stress adaptation, and these remaining, resilient ecotypes are being used as plant inoculants. Numerous field experiments, using plant species from the natural succession inoculated with a community of indigenous mycorrhizal fungi, have been carried out in revegetation projects in the semi-arid Iberian Southeast. This management strategy improved both plant development and soil quality, and is a successful biotechnological tool to aid the restoration of self-sustaining ecosystems. However, despite a 20-year history of this work, we lack a comprehensive view of the mycorrhizal potential to improve the composition, diversity, structure and functionality of drought-adapted plant communities in the Region.

3.1. Mycorrhizas: general concepts, types and significance in the soil-plant system

Maintaining the quality and sustainability of soil resources is a key issue, not only for optimizing the stability and productivity of natural ecosystems, but also to prevent erosion and minimize negative environmental stresses (Buscot 2005). Many chemical, physical and biological factors are involved in the framework of interactions involved in ecosystem functioning (Barea 2010). The biological components are based on diverse genetic and functional groups of soil microbial populations (Chaudhary et al. 2009). Soil microbes are responsible for fundamental ecosystem functions such as the biogeochemical cycling of nutrients and organic matter, and the maintenance of plant health and soil quality (Avis et al. 2008). Microbial activities are particularly relevant at the root-soil interface, the rhizosphere, where microorganisms interact with plants and soil constituents (Giri et al. 2005; Lambers et al. 2009; Richardson et al. 2009; Dessaux et al. 2010; Barea et al. 2013a).

Among the most influential members of the soil microbiota are the mycorrhizal fungi, responsible for establishing mycorrhizas with most vascular plant species on Earth (Smith & Read 2008; Brundrett 2009). Mycorrhizas are symbiotic associations established between soil fungi and most vascular plants, where both partners exchange nutrients and energy (Brundrett 2002). It is now universally accepted that mycorrhizal symbioses are fundamental for good plant nutrition and health, and soil quality (Smith & Read 2008; Azcón-Aguilar et al. 2009). The mycorrhizal fungi colonize the root cortex and develop an extraradical mycelium which permeates the soil surrounding the plant roots. This mycelium forms a network specialized for the acquisition of water and mineral nutrients from soil, particularly those whose ionic forms have poor mobility or are present in low concentration in the soil solution, such as phosphate and ammonium (Barea et al. 2005a). Thus, mycorrhiza formation is an adaptive strategy which provides the plant with an increased ability for nutrient capture and cycling in soils with low nutrient availability, particularly in arid and semi-arid ecosystems (Allen 2007). In addition, the mycorrhizal symbiosis improves plant health through increased protection against environmental stresses, either biotic (e.g. pathogen attack) or abiotic (e.g. drought, salinity, heavy metals, organic pollutants), and enhances soil structure through the formation of hydro-stable aggregates necessary for good soil tilth (Rillig & Mummey 2006; Pozo & Azcón-Aguilar 2007; Ruíz-Lozano et al. 2008; 2012; Aroca et al. 2012; Jeffries & Barea 2012; Barea et al. 2013b; Pozo et al. 2013).

The two main types of mycorrhizas are ecto- and endomycorrhizas, which differ considerably in their structure and physiological relationships with symbionts (Barea & Honrubia 2004; Smith & Read 2008; Brundrett 2009). In ectomycorrhizas, the fungus develops a sheath or mantle around the feeder roots. The mycelium penetrates between the cells of the root forming the Hartig net but not forming intracellular penetrations. About 3% of vascular plants, mainly forest trees (Fagaceae, Betulaceae, Pinaceae, Eucalyptus, and some woody legumes) form ectomycorrhizas. In spite of the relatively low number of plant species forming ectomycorrhizas, these mycorrhizal associations and the tree species involved play a key role in forest ecosystems and are widely distributed. The fungi involved are mostly Basidiomycota and Ascomycota. In endomycorrhizas, no sheath is formed and the fungi colonize the root cortex both intercellularly and intracellularly. A few endomycorrhizal types are restricted to species in the Ericaceae ("ericoid" mycorrhiza) or Orchidaceae ("orchid" mycorrhiza), but the arbuscular mycorrhizal (AM) type is the commonest being widely distributed throughout the plant kingdom. This ubiquitous mycorrhizal type is characterized by the tree-like symbiotic structures, the arbuscules, which are formed by the fungus within the root cortical cells. It is here where most of the nutrient exchange between the fungus and the plant is thought to occur (Smith & Read 2008). The AM fungi (AMF) were formerly included in the Zygomycota, order Glomales (see Redecker et al. 2000b), but they now form a new phylum, the Glomeromycota (Schüßler et al. 2001). One further mycorrhizal type, the ectendomycorrhiza, shares characteristics of both groups and is formed by hardy plant species in the Ericales, and in the Monotropaceae and Cistaceae. In ectendomycorrhizas the fungi form both a sheath and intracellular penetrations.

This review will deal exclusively with AM associations because these are the mycorrhizal type formed for the target plants in this Doctoral Thesis

3.2. Phylogeny of AMF, and origin and evolution of AM fungi and symbiosis

The SSU rDNA phylogeny of AMF clearly revealed that they have a monophyletic origin, and were moved to the new phylum Glomeromycota (Schüßler et al. 2001). Analysis of other genes has also corroborated the Glomeromycota as monophyletic, whilst a multi-gene analysis confirmed them as a very old group of terrestrial fungi (James et al. 2006).

A comprehensive view of AM fungi phylogenetic relationships, based on rDNA phylogenies, have recently been established (Schüßler & Walker 2011). They recognized four Orders: Glomerales (with the families Glomeraceae and Claroideoglomeraceae); Diversisporales (with the families Diversisporaceae, Acaulosporaceae, Gigasporaceae and Pacisporaceae); Archeosporales (with the families Geosiphonaceae, Ambisporaceae and Archeosporaceae); and Paraglomerales (with the family Paraglomeraceae) (see Fig. 0.1). In parallel, an rDNA phylogeny developed in combination with morphological characters of AM fungal spores (Oehl et al. 2011), seems to fit properly with previous systematic approaches.

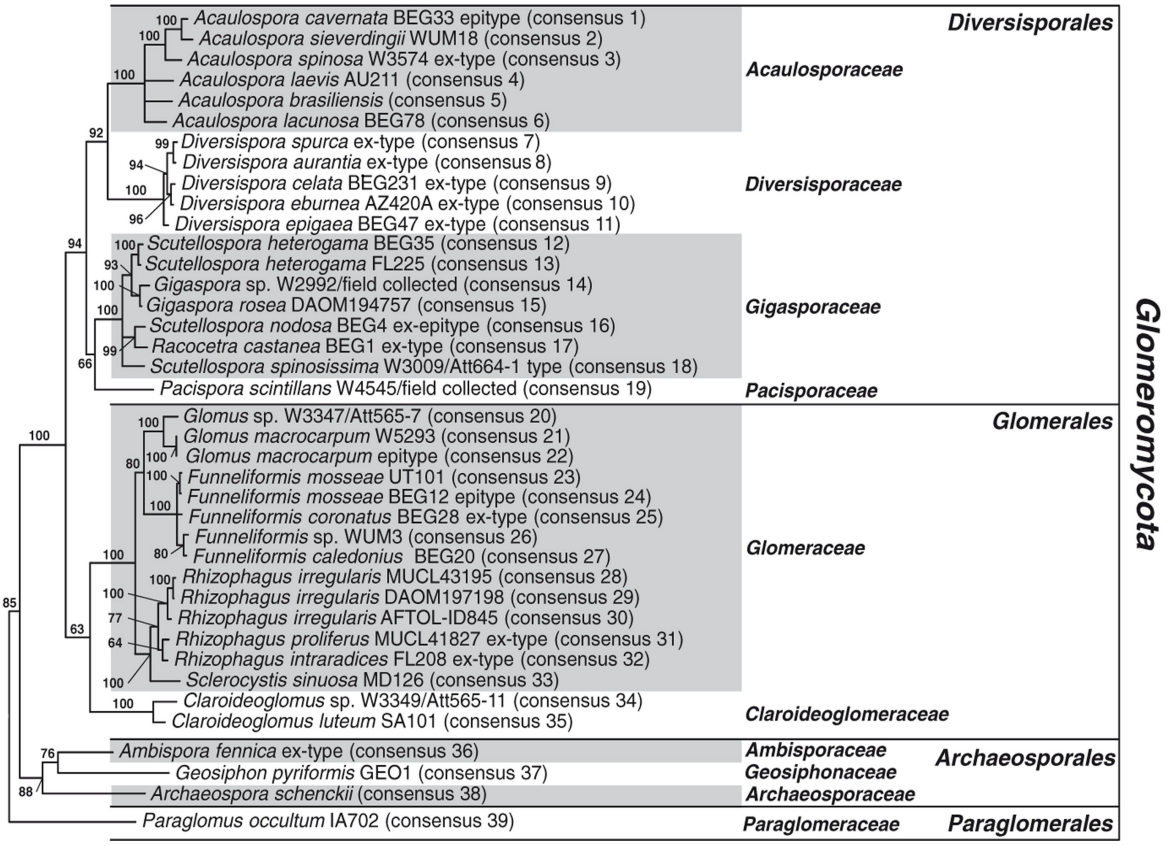

Fig. 0.1 Phylogeny of Phylum Glomeromycota based on small subunit of rDNA. Image source: Krüger et al. 2012.

From a taxonomic point of view and because of their extraordinary genomic features there are difficulties for defining clear species concepts of individuals and also boundaries within populations. However, recent incorporation of molecular approaches into species descriptions, particularly molecular phylogenetic and evolutionary analyses, is incorporating new knowledge on AM fungi

speciation (Rosendahl 2008; Sanders & Croll 2010; Schüßler & Walker 2011). These novel advances refer to topics such as the significance of different polymorphic DNA-sequence variants that are present within a single cell or distributed between genomes or nuclei, the genetic structure of populations, and the definition of an individual, genet or clone (Rosendahl 2008; Sanders & Croll 2010; Schüßler & Walker 2011). The 'molecular species concept' was proposed for AM fungi based on the analysis of their rDNA regions (Schüßler & Walker 2011). As stated by these authors, "…the number of described species in the Glomeromycota is low (~230; see www.amf-phylogeny.com) and only a small proportion of the actual species richness is represented in the molecular database. At the species level, SSU rDNA exceeds its limits of phylogenetic resolution". The use of phylogenetic reference data for systematics and phylotaxonomy of AM fungi has been recently addressed (Krüger et al. 2012).

Land colonization by plants is accepted to have started during the Early Paleozoic with plants at a bryophyte status, apparently having a liverwort grade of organization (Steemans et al. 2009). The fact that AM associations are found in extant species from most groups of bryophytes suggested an early origin of the AM symbiosis with primitive bryophytes (Schüßler & Walker 2011). The discovery of well-preserved fossil plants in the Early Devonian Rhynie Chert, revealed the existence of mycorrhizal associations (Stubblefield et al. 1987; Remy et al. 1994; Kenrick & Crane 1997) in the early evolution of land plants. Fungal structures, such as hyphae and spores, resembling those of extant AMF, were found in fossil records of small 400-million-year-old plants (Kenrick 2003; Honrubia 2009). Furthermore, fungal structures, similar to the arbuscules of extant AM, were found colonizing plant cells of the early vascular land plant *Aglaophyton major* in the Rhynie Chert (Remy et al. 1994; Taylor et al. 2004). The presence of Glomeromycota fungi associated with plants in the Rhynie Chert (Remy et al. 1994) was later confirmed in this ecosystem by the discovery of *Scutellospora*- and *Acaulospora*-like AM spores (Dotzler et al. 2009). Later on, presence of fungal spores clearly resembling those from modern AM fungi was evidenced in a ~460 MY old Ordovician dolomite rock of Wisconsin (Redecker et al. 2000a), pushed back the evolutionary origin of glomeromycotan fungi to a period when land flora was likely to have consisted of plants similar to the modern-day groups of mosses, liverworts and hornworts (Bonfante & Genre 2008).

Based on the analysis from SSU rDNA of several AMF, Simon et al. (1993) reported the first molecular estimates for the age of the Glomeromycota, giving a radiation date of 460 MY ago. They also assumed land plant origin occurred about 420 MY ago. Further studies based on phylogenetic analysis pushed back these numbers for both AM fungi and land plants (Berbee & Taylor 2007). However, a recent molecular clock study (Smith et al. 2010) suggested an origin of land plants around ~477 MY. In summary, evidences based on information from fossil records, paleobotanical studies and phylogenetic analyses of DNA sequences altogether indicate that terrestrial AMF evolved before vascular land plants had emerged. These estimates supports the hypothesis of Pirozynski & Malloch (1975) suggesting that a mycotrophic lifestyle might be fundamental for early land plant to evolve and invade the harsh terrestrial environment. Therefore, it seems acceptable that the AM association could have evolved as a mutualistic symbiosis, facilitating the adaptation of plants to the terrestrial environment (Schüßler 2002). Later on, the primitive roots developed in association with AM fungi and co-evolved with them to build up the mycorrhizal root systems of extant vascular plants (Brundrett 2002). The other types of mycorrhizal associations emerged later in evolution. Published information concerning origin and evolution of Glomeromycota fungi, the AM associations, and land plant co-evolution has been recently reviewed (Barea & Azcón-Aguilar 2013).

3.3. Analysing the diversity of AMF in SE Spain semi-arid ecosystems

Earlier studies of the diversity of AMF communities were largely based on the morphology, wall characters and ontogeny of their large multinucleated spores (Morton 2009; Oehl et al. 2009). However, molecular tools are now available for a challenging dissection of AMF population dynamics (Robinson-Boyer et al. 2009). For molecular identification, the PCR-amplified rDNA fragments of the spores and/or the mycelia from AMF are usually subjected to cloning, fingerprinting and sequencing (Hempel et al. 2007; Öpik et al. 2008; Toljander et al. 2008; Rosendahl et al. 2009; Sonjak et al. 2009; Gamper et al. 2010; Sánchez-Castro et al. 2012a, b; Verbruggen et al. 2012). Alternative molecular tools now exist to quantitatively analyse the effect of environment, management or inoculation of soils on more diverse AMF communities. For example, qPCR can be used for simultaneous specific and quantitative investigations of particular taxa of AMF in roots and soils colonised by several taxa (Gamper et al. 2008; König et al. 2010). In addition, new techniques of high throughput sequencing

(e.g. pyrosequencing) are now being used for AM fungi (Lumini et al. 2010; Lekberg et al. 2012; Lindahl et al. 2013; Salvioli & Bonfante 2013). Despite the advancement in molecular techniques, the identification approaches employed for AMF based on morphological characteristics are still valid and used, being considered complementary to the molecular methods (Morton 2009).

Studies of AMF diversity in SE Spain have been based on the morphological characterization of their large multinucleate spores or on sequence analysis of the small-subunit (18S) ribosomal DNA of the spores and/or the mycelia from these fungi or a combination of both approaches (Barea 2011). Other surveys have used molecular tools to identify the AM fungi actually colonizing plant roots in which single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (TRFLP) as fingerprinting techniques were typically used. Table 2 summarizes the surveys of AM fungi associated with plant communities from semi-arid SE Spain, as a basis for diversity analysis.

As a result of this analysis, a germ-plasm bank of mono-specific cultures of Glomeromycota from SE Spain has been established in the Estación Experimental del Zaidín CSIC, Granada. This comprises almost 200 cultures of ecotypes from diverse morphotypes within eleven genera, with *Glomus* being the most prevalent. Almost all ecotypes have been sequenced and phylogenetically defined as operational taxonomic units (OTUs).

3.4. Ecological and functional interactions between plant communities and their associated AM fungal populations in SE Spain semi-arid ecosystems

The interactions occurring around the plant-mycorrhizal fungus relationship have been the subject of many studies which investigate linking between biodiversity and ecosystem functioning (Bever 1999; van der Heijden & Sanders 2002). Research concerning the impact of mycorrhizal fungi on plant community composition and functioning concludes that the diversity and activity of mycorrhizal fungi is a key mechanism for ecosystem functioning (Read 1998; Hart & Klironomos 2002; Martínez-García et al. 2012). Conversely, diversity and structure of plant cover affects diversity of AM fungal populations (Bever et al. 2002; Read 2002; Wolfe et al. 2005). The nature of the community feedbacks

Table 0.1 Analyzing the diversity of AMF in semi-arid SE Spain ecosystems

Taget AMF propagule	Host plant/region	Methodology	AMF taxa	Reference
Soil-borne spores	Shrub species / Murcia	Spore morphology	*Glomus* sp. *Sclerocystis* sp. *Entrophospora* sp.	Díaz & Honrubia (1993)
	Retama / Granada	Spore morphology	*Glomus* sp.	Herrera et al. (1993)
	Pistacia lentiscus, *Rhamnus lycioides*, *Olea europaea*, *Retama sphaerocarpa* / Murcia	Spore morphology	*Gl. coronatum*, *Gl. constrictum*, *Scutellospora calospora*, *Entrophospora* sp.	Azcón-Aguilar et al. (2003)
	Anthyllis cytisoides / Almería	Spore morphology	Glomus sp., Scutellospora sp., Acaulospora sp.	Requena et al (1996)
	Endangered/endemic flora / Granada	Spore morphology	Genus *Glomus*, *Acaulospora*, *Gigaspora*, *Entrophospora*, *Ambispora*, *Scutellospora*, *Pacispora* and *Diversispora*	Azcón-Aguilar et al. (2010)
	P. lentiscus shrub communiy / Murcia	Spore morphology, molecular characterization (18S rDNA, NS31-AM1 region)	*Gl. mosseae*, *Gl. claroideum*, *Gl. viscosum*, *Gl. constrictum*, *Paraglomus occultum*	Ferrol et al. (2004)
	Shrub communities: dolomitic thyme-, rosemary, broom and sage shrublands	Spore morphology, molecular characterization (18S rDNA, NS31-AM1 region)	26 AMF morphotypes	Palenzuela & Barea (2006, 2009)
	Dolomitic shrubland / Granada	Spore morphology, molecular characterization (18S rDNA, NS31-AM1 region)	*Otospora bareai*, a new species	Palenzuela et al. (2008)
	Endangered/endemic flora / Granada	Spore morphology, molecular characterization (18S rDNA, NS31-AM1 region)	*Entrophospora nevadensis*, a new species	Palenzuela et al. (2010)
Root colonizing AMF (intraradical mycelium)	Shrub species / Granada	PCR-TTGE fingerprinting (18S rDNA, NS31-Glo1)	Managed *Glomus* sp. community	Cornejo et al. (2004)
	Shrub species / Granada	PCR-SSCP fingerprinting (18S rDNA, NS31-AM1 and NS8-ARCH1311)	Eight AMF phylotypes from Glomeraceae and two from Diversisporaceae	Sánchez-Castro et al. (2012a, b)
	Semi-arid shrub and gypsum communities / Murcia	PCR-SSCP (18S rDNA, NS31-AM1, AM2, AM3)	AMF phylotypes from *Glomus* sp. group A and B, *Diversispora* sp. and *Scutellospora* sp.	Alguacil et al. (2009a, b)
	Semi-arid shrub community / Almería	TRFLP (25S rDNA, FLR3-FLR4)	Not identified	Martínez-García et al. (2011)
	Semi-arid shrub and gypsophyte community / Murcia	PCR sequencing (18S rDNA, AML1-AML2)	AMF phylotypes from *Glomus* sp. group A and B, *Paraglomus* sp., *Diversispora* sp., *Archaeospora* sp., *Acaulospora* sp.	Alguacil et al. (2011, 2012)
	Herb community / Murcia	PCR sequencing (18S rDNA, AML1-AML2)	AMF phylotypes from Glomeraceae A and B, Diversisporaceae, Paraglomeraceae, Ambisporaceae	Torrecillas et al. (2012b)
	Semi-arid community / Murcia	PCR sequencing (25S rDNA, LR1-FLR4)	Members of Glomeraceae A and B, Diversisporaceae, Acaulosporaceae	Torrecillas et al. (2012a)

in mycorrhizal associations has been investigated with special emphasis on the several mechanisms/factors responsible for the ecological interactions involved.

These mechanisms/factors include: (i) the functional specificity of the different plant-fungus combinations (van der Heijden et al. 1998; Klironomos 2002; Yang et al. 2012); (ii) the mycorrhizal dependency of the plant species involved (Read 1998; Hart & Klironomos 2002; Ehinger et al. 2009); and (iii) the structure of the individual plant species within the community (O'Connor et al. 2002; van der Heijden 2002; van der Heijden et al. 2006).

Some key concepts can be drawn from the consolidated knowledge on these aspects of mycorrhizal ecology:

(i) Although specificity *sensu strictum* does not exist in mycorrhizal associations (as almost all plants in a community can be colonized simultaneously by several species of mycorrhizal fungi), different mycorrhizal ecotypes are more beneficial to some plant species than others (Sanders 2002). It also seems that not every fungus can colonize every plant in the community (Barea & Azcón-Aguilar 2013).

(ii) In addition to its role in carbon allocation, the establishment of a mycelial web around the roots from the plant community constitutes a diverse inoculum source for the different plant species (Read 1998; van der Heijden 2004).

(iii) The intermingling and extensive extra-radical mycelium allows a more efficient exploitation of soil nutrients and water, thus benefiting the nutrient flow through the soil-fungus-plant system - particularly relevant in arid ecosystems (Allen 2007).

Research investigating these aspects of mycorrhizal ecology and function in the semi-arid SE Spain is summarised subsequently.

3.4.1. Impact of AM symbiosis on the dynamics and functioning of plant communities

Much information on the role of mycorrhizas in SE Spain semiarid ecosystems has been generated during the last two decades (see section 3.6). However, most studies concern the mycorrhizal impact on the establishment and development of individual plant species rather than the functioning of plant communities. However, some experiments can be considered in this context, and are discussed here.

The effect of mycorrhizas at the plant community level was studied by Requena et al. (2001) in a degraded area within the Sierra de los Filabres in Almería. The existing natural vegetation was shrubland, where *Anthyllis cytisoides* L., a drought-tolerant legume able to form symbioses with both rhizobial and mycorrhizal micro-symbionts, was the dominant species. This experiment (further discussed in Subsection 3.6) demonstrated the long term benefits of inoculation not only on plant establishment but also on P acquisition and N_2 fixation by the target legume. The benefits also included increased available P, N and organic matter, and the number of hydro-stable aggregates in the soil supporting the community. Studies using the stable isotope ^{15}N evaluated the amount of N_2 fixed by the shrub legume and showed how this improved N nutrition, via N-transfer to non-N-fixing vegetation grown in association with the inoculated legume. In addition, the mycorrhizal nodulated *Anthyllis* plants behaved as a source of mycorrhizal inoculum for the surrounding area, where new seedlings flourished and accelerated the natural succession. This study showed that the introduction of target indigenous plant species, associated with a managed community of microbial symbionts, could be a successful biotechnological tool to aid the integral recovery of degraded ecosystems.

Navarro-Fernández et al. (2011) studied a dolomitic “thymeshrub” plant community in Sierra de Baza Natural Park, which comprised endemic species dominated by *Thymus granatensis* Boiss. Efficient functioning of the community was dependent on a dolomite-adapted AM fungal community, particularly under the drought stress conditions characteristic of the target area. The presence of AM fungal ecotypes from the high-dolomite environment appeared fundamental for the development of the endemic plant community, as they were involved in the adaptation mechanisms that enable the plant to grow.

Recent studies (Alguacil et al. 2009a, 2009b, 2009c, 2011a, 2011b, 2012; Sánchez-Castro et al. 2012a, 2012b; Torrecillas et al. 2012a, 2012b) have revealed a hidden diversity, with many AMF sequences detected in plant roots that cannot be related to known AMF taxa present in the surrounding soil as spores. These cryptic colonists have also been found for other ecosystems in Europe (Hempel et al. 2007; Öpik et al. 2008). These molecular studies clearly show that populations of spores in soil do not reflect completely the fungi present in roots and vice versa. In fact, the existence of non sporulating fungi has been suggested previously (Denison & Kiers 2011) and their role in well conserved vegetation could be important (Rosendahl & Stukenbrock 2004). The existence of functionally diverse AM fungi has been demonstrated to influence the plant coexistence, improving ecosystem functioning (Klironomos 2000; Maherali & Klironomos 2007). Thus, when a ecosystem restoration programme is planned, it will be necessary to incorporate information about the different life-history strategies of AMF present in the environment and the way in which they interact with plant species. In fact, the use of a wide diversity of AMF life-history strategies would provide a greater variety of services to plant community, which can help at time to improve the ecosystem stability (Klironomos 2000; Maherali & Klironomos 2012).

3.4.2 Influence of the plant species on the structure and composition of their associated mycorrhizal fungal populations

The influence of the plant species on production of mycorrhizal propagules has been investigated in SE Spain. For example, Azcón-Aguilar et al. (2003) studied typical shrubs from semi-arid areas of Murcia and found that *Olea europaea* var. *sylvestris* (Mill.) Lehr and *Retama sphaerocarpa* (L.) Boiss, have a higher capacity to enhance the development of AM propagules in their rhizospheres than *Pistacia lentiscus* L.or *Rhamnus lycioides* L.

Further studies (Sánchez-Castro et al. 2012a) analysed the genetic diversity of the AMF community that colonized the roots in a shrubland species community (*Genista cinerea* (Vill) DC. in Lam. & DC., *Lavandula latifolia* Medicus, *Thymus mastichina* L., *Rosmarinus officialis* L. and *Thymus zygis* L.). The different cooccurring plant species were colonized by AMF communities of different composition. These findings support the earlier contention that there is some level of specificity in mycorrhizal

associations. For methodological approaches and results from this AMF diversity analysis see Table 2. The genetic diversity of AM fungi colonizing the root, the rhizosphere soil and the root-free soil was investigated (Martínez-García et al. 2011). Differences in AMF communities were found in soils under shrubs and in gaps among them, whereas no differences were detected among AM fungal communities colonizing roots. Soil nutrient content drove most of the spatial variations and genetic diversity in the AM fungal community (Fitzsimmons et al. 2008; Johnson 2010). Consequently, it was suggested that different shrub species generate resource islands (Allen 1988), which differ in nutrient content and, therefore, support different AM fungal communities, at least in their associated rhizosphere soil. This increases the AM fungal diversity at the landscape level. Using the same genetic approach Martínez-García & Pugnaire (2011) characterized the AM fungal community colonizing roots of two plant species, *Ballota hirsuta* Bentham and *Lobularia maritima* (L.) Desv., growing under shrubs and in open areas. Differences between AM fungal genetic diversity of the communities associated with the two species were found. In a similar approach in semiarid environments, Alguacil et al. (2011b) analysed the resulting AMF community associated with different plant species used in a restoration project. They found differences relating to AM fungal community composition between plant species and suggested that the use of a higher diversity of plant species in restoration practices could improve the AMF diversity in restored ecosystems.

Otherwise, the nature of this trend was investigated in the subsequent studies of Alguacil et al. (2012) and Torrecillas et al. (2012a). They found interesting differences between the AMF communities associated with annual and perennial plants belonging to the same habitat. This is in agreement with meta-analysis carried out with global data (Hoeksema et al. 2010; Yang et al. 2012), which suggested that the AMF identity responds to the functional characteristics of their hosts.

These data represent new information on the specificity of AMF-plant interactions, mainly in Mediterranean semiarid environments, and suggest a certain relation of AM fungi and plant population and community dynamics in semiarid ecosystems.

3.4.3. Impact of plant cover degradation on AMF diversity

Disturbance of natural plant communities, the first symptom of ecosystem degradation, is often accompanied or preceded by loss of key physical-chemical and biological soil properties such as soil structure, plant nutrient availability, organic matter content and microbial activity (Francis & Thornes 1990). In particular, plant community degradation causes disturbance of mycorrhizal inoculum potential, which is a critical ecological factor to help further plant development in degraded habitats (Requena et al. 2001). The impact of plant cover and/or soil degradation on mycorrhizal fungal diversity has been investigated in SE Spain where drivers of ecosystem degradation include climatic change, mining activities, wild fire, flooding and salinity and land abandonment. For example, in the Sierra de Baza Natural Park, Granada, Palenzuela & Barea (2006, 2009; see Table 2) selected five plant community plots where the vegetation cover was optimal and compared these with plots where diversity and structure of the optimal vegetation cover were degraded. Rhizosphere samples were taken from all the target species and AMF spores isolated and morphologically characterized. The main conclusion was that the degradation of the vegetation cover negatively affected the density (number of spores per 100 g of soil), richness and diversity of AMF.

To assess the impact of drought on AM associations in semi-arid plant communities from SE Spain, Martínez-García (2010) and Martínez-García et al. (2012) measured root length colonization in *Stipa tenacissima* L. and *A. cytisoides* along a natural environmental precipitation gradient, and in *Artemisia barrelieri* Besser growing in several plots subjected to different precipitation regimes. The results showed that the response of the AM associations to precipitation patterns depended on the host species. Natural drought stimulated AM colonization, but artificially-induced drier conditions lowered the AM colonization. It was suggested that arid ecosystems caused by climate change could alter AM interactions in different ways depending both on the host plant and the intensity of the drought, which might then lead to changes in plant communities.

In a study by Díaz & Honrubia (1994), sampling sites with differing degrees of disturbance were established in an area of the SE coast of Murcia that had been severely degraded by mining activities and was covered by waste sediments. The mycorrhizal population level (mycorrhizal root colonization

and the number of spores in the rhizosphere) was adversely affected by soil degradation. However, mycorrhizal propagules did not disappear completely, suggesting a certain degree of adaptation to the soil disturbance suffered by the test area.

The influence of flooding and salinity gradients on the AMF spore counts and root colonization levels of *Inula crithmoides* L. was studied in a transect spanning a gradient from shoreline to interior in La Mata lagoon, Alicante. The plots with high salinity levels and flooding showed decreased spore numbers in soil, a decrease in the percentage of mycorrhization, and a very low number of mycorrhizal fungal propagules as measured by the most probable number test (Roda et al. 2008).

Roldán et al. (1997) studied the AM fungal population in agricultural soils abandoned for different lengths of time (3-45 years) in a semi-arid area of Murcia and found that agricultural use reduced soil fertility and lowered AM fungal populations compared to the soil kept in its natural state. After abandonment, there was a 5-year period when the soils underwent further degradation of their AM potential. After that the AM propagules slowly recovered, reaching values similar to those of the virgin soil after 45 years.

3.5 Prospecting and applying AMF to improve functioning of plant communities in the semi-arid Southeast (SE) Spain

Given their important role in plant evolution, it is now well accepted that mycorrhizas currently continue to help plants to develop in stressed environments (Honrubia 2009; Barea & Azcón-Aguilar 2013) such as those of the Semi-arid Mediterranean ecosystem in SE Spain. In this particular region, mycorrhizal fungi have been shown to help plants to establish and cope with nutrient deficiency, drought, soil disturbance and other environmental stresses characteristically involved in soil degradation (Barea & Honrubia 2004; Barea et al. 2007; Martínez-García & Pugnaire 2009; Palenzuela & Barea 2009; Martínez-García et al. 2012). This ability has promoted investigations of the impact of mycorrhizas in maintaining diversity and functioning of plant communities in arid/semiarid ecosystems under the stress conditions of this region. The related information has been reviewed recently (Barea et al. 2013b).

Applied mycorrhizal research in the SE of Spain began at the end of the 1980s, in the framework of the LUCDEME Project (www.mma.es/portal/secciones/./lucdeme). Many basic, strategic and/or applied studies have been carried out since aimed to use mycorrhizal biotechnology to improve the performance of the regional plant communities.

Loss of AMF following degradation of vegetation cover in Mediterranean ecosystems can inhibit either natural or artificial processes of revegetation. Augmentation of the inoculum potential may be needed (Requena et al. 1996).

Mycorrhizal inoculation in revegetation strategies for degraded areas from SE Spain was first investigated in a semi-arid desertification-threatened environment south of the Sierra Nevada, Granada (Herrera et al. 1993). This assay used woody legumes as plant species symbiotic with both N_2-fixing rhizobial bacteria and AMF, associations which enable the plant to develop in water deficient and low nutrient environments (Azcón & Barea 2010). The target legumes included two native shrubs (*A. cytisoides* and *Spartium junceum* L.), and four non-native tree legumes (*Robinia pseudoacacia* L., *Acacia caven* (Mol.) Mol. and *Prosopis chilensis* (Mol.) Stuntz.and *Medicago arborea* L.). The results of this four-year-old trial showed that: (i) only the native shrub legumes were able to establish and develop under the local environmental conditions; (ii) the biotechnological manipulation of the seedlings, by inoculation with selected rhizobia and mycorrhizal fungi, improved outplanting performance, plant survival, and biomass production.

For revegetation programmes in the water-stressed regions, it is important to evaluate the influence of mycorrhizal inoculation on the water use efficiency (WUE) of the target native plant species. Querejeta et al. (2003) used *O. europaea* var. *sylvestris* and *R. lycioides* to evaluate whether two eco-physiological response variables (foliar carbon isotope ratios ($\delta^{13}C$) and leaf gas exchange) were affected by inoculation with *Glomus intraradices*. They found that the WUE was enhanced under drought conditions by inoculation in *O. europaea* but not in *R. lycioides*. The results suggested that some of the interspecific variability in $\delta^{13}C$ observed for arid land plant communities may be due to different physiological responses to mycorrhization.

Several field experiments have compared the use of autochthonous vs. exotic (and generalist) mycorrhizal fungi for revegetation programmes in the Region. For example, Requena et al. (2001) tested two mycorrhizal inoculation protocols for rhizobium inoculated *A. cytisoides* seedlings that were to be transplanted to a degraded area: (i) an exotic mycorrhizal fungus (*Glomus intraradices*), (ii) a mixture of five taxa of indigenous mycorrhizal fungi representing the natural abundance and diversity in the target site (Sierra de los Filabres, Almería). A long-term improvement of both plant performance and the physicochemical properties in the soil around *A. cytisoides* plants inoculated with indigenous AM taxa was evidenced over a 5-year period, while the exotic fungi were effective only during the first year after transplanting. Similar results were found by Alguacil et al. (2005) and Caravaca (2003, 2005). Thus it appears that native, drought-adapted AMF often improve host-plant performance to a greater extent in the target dry environments than non-native AMF.

3.6. Future trends

Despite the advances in our knowledge of mycorrhizal presence and functioning in SE Spain over the last two decades, further basic, strategic and applied studies are needed to better understand the significance of mycorrhizas in determining biodiversity and function in the semi-arid ecosystems in this region. A key aim would be to use current molecular approaches in the integral analysis of the diversity of all types of mycorrhizal propagules associated with target plant communities. This is particularly critical to detect the “hidden diversity” of AMF populations. In this sense, the dissection of the life-history strategies of AM fungi adapted to Mediterranean environments should be priority at time to exploit all the functions they can offer. A thorough knowledge of this diversity is needed to produce mycorrhizal fungal inoculants representing the integral diversity of the target area. Seedlings with a tailored mycorrhizal status will act as a "resource islands" of inoculum for the surrounding area to benefit plant cover development and for improving soil quality in degraded ecosystems. This would maximize the mycorrhizal benefit in revegetation/conservation programmes facilitating nutrient and water recycling and capture, as well as improving ecosystem services. To carry out field studies to investigate how the diversity, life-history strategies and degradation affect the activity of AMF and in consequence the composition, diversity, structure and functionality of plant communities in semi-arid SE Spain ecosystems will constitute a research priority in the next future.

Una vez definida la problemática ecológica que anima al presente estudio, establecidos sus objetivos y revisados los antecedentes bibliográficos en relación con la temática de investigación propuesta, se planificó el desarrollo de la parte experimental de esta Tesis Doctoral, siguiendo el **Plan de Trabajo** que se detalla a continuación

II. PLAN DE TRABAJO

II. PLAN DE TRABAJO

De acuerdo con lo expuesto anteriormente, para la consecución de los Objetivos Operacionales de esta Tesis Doctoral, se decidió un **Plan de Trabajo**, basado en las siguientes Tareas:

- Investigar las estrategias que utilizan los hongos MA para colonizar nuevas plantas que se incorporan a la comunidad, según las formas de propágulos operativos diponibles, así como la sucesión de los hongos MA en las raíces, afectada por la estacionalidad climática. Se eligió como planta diana el romero, *Rosmarinus officinalis* L., arbusto siempre-verde característico de los ecosistemas mediterráneos, componente importante de una comunidad bien representada en el Parque Natural: el romeral. Los experimentos correspondientes se basarán en un sistema de mesocosmos, en condiciones controladas.

- Averiguar si la interacción entre las características funcionales del biotipo vegetal y de los hongos MA determinan la comunidad simbiótica MA resultante. También se propone investigar el impacto de la variabilidad estacional climática en el desarrollo y sucesión de las comunidades de hongos MA que colonizan las plantas diana. Para ello se elegirán biotipos de plantas, bien representadas en el Parque Natural, de acuerdo con sus características basales: ciclo de vida (anual *vs.* perenne) y hábito de crecimiento (herbácea *vs.* semileñosa). También se seleccionarán dos familias botánicas: Asteraceae y Leguminosae. Concretamente, las especies de plantas seleccionadas fueron: (i) herbáceas anuales, *Trifolium angustifolium* L. (leguminosa) y *Xeranthemum inapertum* (L.) Mill. (asterácea); (ii) herbáceas perennes, *Medicago sativa* L. (leguminosa) e *Inula montana* L. (asterácea); y (iii) semileñosas perennes, *Ononis natrix* L. (leguminosa) y *Santolina canescens* Lag. (asterácea). Los experimentos correspondientes se basarán en un sistema de microcosmos, en condiciones controladas.

- Estudiar la influencia de los procesos de degradación del hábitat en la comunidad de hongos MA asociados a las plantas. Se eligió como planta diana el tejo (*Taxus baccata* L.), una especie vegetal amenazada y en regresión en los ecosistemas mediterráneos del sureste de España. La razones de la elección del tejo no sólo se basan en su carácter de relicto amenazado en la Sierra

de Baza sino, que por su carácter de árbol siempre verde con una prolongada esperanza de vida, podría actuar como refugio para las poblaciones de hongos MA, por lo que resultarían protegidos de la degradación del hábitat. Los experimentos correspondientes se realizarán en condiciones naturales, muestreando raíces de tejos crecidos en el Parque Natural en un rango de niveles de degradación del hábitat.

El material básico de los estudios anteriormente esbozados serán muestras de raíces de las plantas seleccionadas (plantas diana), representativas de la flora del Parque Natural "Sierra de Baza", crecidas en condiciones naturales y/o controladas, de acuerdo con las diferentes Tareas propuestas. En estas muestras se analizarán los filotipos de hongos MA que las colonizan, utilizando "terminal restriction fragment length polymorphism (TRFLP)" como técnica de "fingerprinting" de los genes que codifican para el ARN ribosómico (Dickie & FitzJohn 2007).

III. APORTES EXPERIMENTALES

Chapter 1: Life-history strategies of arbuscular mycorrhizal fungi determine succession into roots of *Rosmarinus officinalis* L., a characteristic woody perennial plant species from Mediterranean ecosystems

Capítulo 1: Las estrategias de vida de los hongos micorrícicos arbusculares como factor determinante de la sucesión de estos hongos en las raíces de *Rosmarinus officinalis* L., planta leñosa perenne característica de los ecosistemas mediterráneos

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Resumen

Objetivos Analizar las estrategias de vida de los hongos micorrícicos arbusculares (HMA) en función de las distintas formas de propágulos que utilizan para colonizar nuevas plántulas de romero (*Rosmarinus officinalis* L.); así como evaluar cómo esas estrategias pueden influenciar la dinámica de sucesión de la comunidad de HMA en las raíces de la planta diana.

Métodos Se diseñó un estudio de mesocosmos donde plántulas de romero no micorrizas (receptoras) fueron expuestas a distintas fuentes de propágulos de HMA: redes de micelio asociadas a plantas de romero vivas y micorrizadas de forma natural en campo o propágulos de resistencia de HMA capaces de sobrevivir en el suelo después de sufrir una alteración severa. Se efectuó un seguimiento trimestral de la comunidad HMA durante dos años, utilizando la técnica de TRFLP para identificar los distintos filotipos de HMA presentes en las raíces de las plantas de romero receptoras.

Resultados Las distintas fuentes de propágulos de HMA determinaron comunidades fúngicas en raíz distintas el primer año. Los HMA que colonizaron preferentemente desde redes de hifas unidas a plantas vivas fueron rápidos colonizadores y estacionales, pero de reducida competitividad. Los hongos procedentes de propágulos de resistencia fueron más frecuentes y mejores competidores una vez dentro de la raíz. La evolución de los HMA en la raíz evidenció distintas estrategias en la sucesión que, además, tenían una base filogenética.

Conclusiones Se describen distintas estrategias de vida en función de la estrategia de colonización y persistencia en el medio para los HMA asociados al romero. Se evidencia una clara sucesión de las comunidades de HMA dentro de la raíz, lo cual tiene importantes implicaciones para el conocimiento y la comprensión de los procesos ecológicos en los ambientes mediterráneos.

Chapter 1: Life-history strategies of arbuscular mycorrhizal fungi determine succession into roots of *Rosmarinus officinalis* L., a characteristic woody perennial plant species from Mediterranean ecosystems

Abstract

Aims To analyze the life-history strategies of arbuscular mycorrhizal fungi (AMF), in terms of whether they spread out from the different propagule types, to colonize new seedlings of rosemary (*Rosmarinus officinalis* L.); and to ascertain how these life-history strategies, putatively contrasting, would influence the successional dynamics of the AMF symbiotic community.

Methods A mesocosm study was carried out. Rosemary seedlings were allowed to be colonized by either the AM hyphal networks coming from either a living rosemary plant, or from AMF resistant propagules resting in soil. The AMF community established in the receiver plants was monitored every three months during two years, using terminal restriction fragment length polymorphism of genes coding for rDNA.

Results Different sources of AMF propagules resulted in a different initial community colonizing rosemary roots. AMF phylotypes spreading out from hyphae seemed to be faster and season-dependent colonizers but have a slight competitive capacity. AMF taxa colonizing from resistance propagules were more frequent and better competitors. The evolution of the AMF community, as symbiotic phylotypes, revealed different strategies in the succession which were also supported by a phylogenetic conservatism.

Conclusions The existence of contrasting life-history strategies in terms of colonization and survival was evidenced for AMF associated with rosemary. The revealed successional dynamics of the AMF symbiotic community has implications in the understanding of ecological processes in Mediterranean environments.

Introduction

One of the most important groups of microorganisms interacting with plants in terrestrial ecosystems is that integrated by arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota). These fungi constitute a widespread group of obligate plant endosymbionts which colonize the roots of approximately two-thirds of terrestrial plant species to form arbuscular mycorrhizas (Barea & Azcón-Aguilar 2013). The association improves the fitness of both partners. AM fungi increase nutrient and water uptake by the plant through the fungal hyphae exploring soil resources, while the host plants supply the fungus carbon compounds from the photosynthesis (Koide & Mosse 2004). Arbuscular mycorrhizas confer other benefits to the plant including protection against biotic and abiotic stresses (Smith et al. 2010; Jung et al. 2012). In addition, and at the ecosystem level, AM symbioses benefit plant diversity, productivity and soil structure (Grime et al. 1987; van der Heijden et al. 1998, 2006; Vogelsang et al. 2006; Bever et al. 2010; Barea et al. 2011). However, the AM fungal autoecology is still poorly understood. Traditionally, they have been considered as generalists, with little or no partner specificity, but recent studies, with more powerful technical resolution, have shown that they exhibit a considerable level of partner selectivity for particular plant species or for ecological plant groups, showing a nonrandom pattern of interactions with plants (Davison et al. 2011; Montesinos-Navarro et al. 2012). Other studies have focused on the influence of environmental factors on the composition of AM fungal communities (Fitzsimmons et al. 2008; Dumbrell et al. 2010; López-García et al. 2013). Although both types of studies have shown interesting insights into the structure and diversity of AM fungal communities in natural conditions, the mechanisms responsible for the detected trends remain unclear.

Studies in recent years have revealed that functional characteristics of the AM fungi (AMF) are important determinants of the formation and functioning of AM symbioses (van der Heijden & Scheublin 2007; Helgason & Fitter 2009). Large contrasts have been shown in terms of colonization strategies (Hart & Reader 2002, 2005), life cycles (Boddington & Dodd 1999) or benefits conferred to the host plants (Munkvold et al. 2004; Avio et al. 2006). Recent research suggests a differentiation in the life-history of AMF in terms of their reproductive strategy (Denison & Kiers 2011). In this context, the existence of AMF spreading out mainly through mycelial networks attached to living plants and

others through resistance propagules was supported by the findings of the presence of glomeromycotan DNA sequences into roots but absent as spore from the same habitat (Hempel et al. 2007; Sánchez-Castro et al. 2012a). In fact, many of the AMF sequences in the databases do not seem to correspond to any known AMF spore (Öpik et al. 2010).

The presence of AMF in plant roots is clearly influenced by their functional characteristics (Hart & Reader 2002). Therefore it is not surprising that, as suggested by Hart et al. (2001), fungi with different life-history strategies appear temporarily distributed within the same root system. Although some studies found no changes over time in the AMF communities into roots (Rosendahl & Stukenbrock 2004; Santos-González et al. 2007), other studies have attributed seasonal changes to host phenology (Merryweather & Fitter 1998) or successionals due to the age of the host plant (Husband et al. 2002). More recently, Dumbrell et al. (2011) proposed temperature as the most influential variable related with the seasonal changes of AMF communities. Thus, it is widely accepted that successional and seasonal changes are probably due to the interaction between abiotic environmental variables and host phenology (Sánchez-Castro et al. 2012a). However, the long period of coevolution of AMF and plants (Redecker et al. 2000a; Schüβler & Walker 2011; Barea & Azcón-Aguilar 2013) have resulted in a wide range of life-history strategies of the fungal partner to exploit all possible niches, both spatial (Hart & Reader 2002) and temporal (Hart et al. 2001). Although some studies have been carried out on the different life-history strategies in terms of spatial colonization (Hart & Reader 2002, 2005; Klironomos & Hart 2002), their influence on the succession of AMF communities has not been addressed experimentally.

The diversity of habitats in Mediterranean environments, together with the climatic regime -including long dry and hot summers and low winter temperatures, with irregular, sometimes heavy, rain events (López-Bermúdez & Albadalejo 1990; Vallejo et al. 1999)- has resulted in a large number of plant strategies (Médail & Quézel 1997). In fact, this great variability has driven the Mediterranean basin to be classified as one of the biodiversity hotspots in the World (Myers et al. 2000). As AMF diversity was shown to promote plant diversity (van der Heijden et al. 1998), it seems feasible that a high plant diversity can facilitate a high taxonomical and functional AMF diversity (Pringle & Bever 2002). Thus, Mediterranean environments appear as good candidates for the dissection of contrasting

life-history strategies in AMF and their implications in the successional dynamics of AMF communities.

To investigate such ecological aspects of AMF in Mediterranean habitats, a representative and well-conserved Mediterranean shrub community, located at the Sierra de Baza (Granada, Southeast Spain), was selected for a series of studies (Sánchez-Castro et al. 2012a, b). For the here reported research, rosemary (*Rosmarinus officinalis* L.), a widespread evergreen sclerophyllus shrub, was chosen as host plant. Rosemary plants form naturally extended vegetation patches, associated with herbaceous, annual, plant species (rosemary groves) (Sánchez-Castro et al. 2012a). They provide an AM inoculum pool responsible of colonizing new plantlets joining the community. We hypothesized that the AMF taxa involved might follow contrasting life-history strategies, with regards to their ability to colonize new plants and to persist in the environment as resistant propagules, which is relevant for understanding the succesional dynamics and functioning of AMF communities.

Accordingly, the objectives of the present study were (i) to test the hypothesis that there are AMF taxa with contrasting life-history strategies, i. e. AMF taxa which preferentially colonize through mycelial networks *vs.* those capable of producing resistance propagules which persist in the soil surviving harsh events; and (ii) to ascertain experimentally how these contrasting life-history strategies influence the successional dynamics of the AMF community. For such purposes a mesocosm trial was designed, trying to mimic the differing situations in the colonization of new roots by AMF. Rosemary rooted cuttings ('receiver plants') were allowed to be colonized by either, or both, types of contrasting AMF propagule types: hyphal networks coming from a living rosemary plant ('donor plant') or resistant propagules from a sieved and dried natural soil. The AMF community established in the receiver plants was monitored every three months during two years, using terminal restriction fragment length polymorphism (TRFLP) of genes coding for ribosomal RNA (Dickie & FitzJohn 2007).

Material and Methods

Experimental design

As indicated before, the questions raised were investigated in a mesocosm study using rosemary (*Rosmarinus officinalis* L.) as host plant. The mesocom units were established in 15 l circular containers with 40 cm diameter and 15 cm depth). Nine rosemary plants were sown in each mesocom unit: one in the center of the container (donor plant) and the other eight (receiver plants) surrounding the central one (at about 12 cm) and equidistant between them. To distinguish between both fungal strategies, i.e. AMF colonizing preferentially through mycelial networks connected to a living plant vs. those colonizing from resistant propagules, two different sources of mycorrhizal inoculum were tested: natural soil from the target area, having only resistant AMF propagules (either spores or mycorrhizal root fragments) and a field-grown rosemary plant, the mycorrhizal donor plant, colonized by the AMF taxa naturally associated with rosemary. In the first case, we expected to select fungi with the highest survival capacity. In the other case, the field rosemary plant would contain its natural AMF assemblage, including fungi with different life-history strategies. Thereby, depending on the source of mycorrhizal propagules, three different treatments were established: 1) soil containing resistant mycorrhizal propagules with a non-mycorrhizal plant as donor plant ('soil propagules'); 2) sterilized soil (without mycorrhizal propagules) with a field rosemary plant as mycorrhizal donor plant, containing the natural community of AMF colonizing the plant ('plant propagules') and 3) the combination of the previous treatments, i. e. soil containing resistant mycorrhizal propagules and the mycorrhizal donor rosemary plant ('soil+plant propagules').

Mycorrhizal propagule source

Both the soil and donor plants used as source of AMF propagules in the experiment were obtained from the Sierra de Baza Natural Park, where rosemary plants grow naturally. For this purpose, an area of about 100 m^2 comprising a representative, well-conserved, Mediterranean shrub community at Sierra de Baza was selected. The vegetation is dominated by patchily distributed shrubs of the Labiatae family: *Rosmarinus officinalis*, *Lavandula latifolia*, *Thymus zigys* and *T. mastichina* associated with

herbaceous, annual, plant species. The soil is a calcaric cambisol. On March 2008, six random points were selected to collect 300 l of soil. The soil was collected at a depth of ca. 5-30 cm and sieved through a 5 mm sieve.

Before setting up the experiment, the soil was dried to eliminate the AMF propagules more susceptible to soil degradation and consequently to select fungi with the highest survival capacity. For the desiccation process, the soil was extended in a 3 cm layer inside a greenhouse without temperature control, but isolated from the external environment. During eight months (March to November) the soil was weekly flipped to get a uniform desiccation. The mycorrhizal colonization potential of the soil was measured weekly by quantifying the number of AMF entry points in a trap plant, *Sorghum vulgare*, following the protocol by Franson & Bethlenfalvay (1989). Prior to drying, one-third of the collected soil was steam sterilized (1 h for 3 consecutive days).

Two to three-years-old rosemary plants were randomly chosen from the target area in Sierra de Baza on November 2008. Twelve of the plants were used as mycorrhizal donor plants and another six to analyze the natural AMF assemblages colonizing their roots. The plants were extracted from the soil trying to get the maximum of their root system. The root system of the field plants was minutely washed with tap water to remove extraradical hyphae and spores before sowing.

Set up of the experiment

The experiment was set up on November 2008. A field-grown rosemary plant was established in the center of each mesocosm unit as mycorrhizal donor plant. In the treatment in which the mycorrhizal propagules should come only from the soil, the donor plant was replaced by a non-mycorrhizal plant, obtained from sterile rooted rosemary cuttings. All mesocosm units containing steam-sterilized soil were supplied with a filtrate of the non-steamed soil to restore natural microbial communities, but free from AMF propagules.

One month later (December 2008) eight non-mycorrhizal rooted rosemary cuttings (receiver plants) were established in each mesocosm unit surrounding the donor plants. These individuals were the

subject of the different measurements carried out during the experiment. Six replicate mesocosm units were established per treatment. The mesocosm units were maintained for two years in a greenhouse without light supplement. Day/night temperatures were adjusted to 25/18ºC only in summer using a cooler system. One receiver plant was harvested every three months, beginning in March 2009, to determine the mycorrhizal colonization level and to characterize the associated AMF community.

Mycorrhizal colonization

Rosemary roots were cut in 1 cm pieces and thoroughly mixed. Two g of roots were stained with tripan blue (Phillips & Hayman 1970) and were examined under a compound light microscope to determine the percentage of mycorrhizal root colonization and the frequency of intraradical fungal structures, arbuscules and vesicles (McGonigle et al. 1990). The remaining roots, in aliquots of 200 mg, were immediately liquid nitrogen-frozen and stored at -80ºC until use for the molecular analysis to determine AMF diversity.

AMF gene library

To apply the TRFLP database approach (see Dickie & Fitzjohn 2007), a previous gene library containing a major part of the sequences of glomeromycotan fungi included in the whole study was constructed. For that purpose, DNA from 200 mg of roots per sample was extracted using a DNeasy plant mini-kit (Qiagen Inc., Mississauga, ON, Canada) and eluted in 75 µl ddH_2O. Partial ribosomal SSU DNA fragments were amplified using the primer set AML1-AML2 (Lee et al. 2008), a specific pair of primers for AMF DNA amplification. The Polimerase Chain Reactions (PCR) were carried out using the illustra Pure-Taq Ready-To-Go PCR beads (GE Healthcare UK Limited, Buckinghamshire, UK) and 5 µM of each primer. PCR conditions were: one minute at 94ºC, 30 cycles at 94ºC for 1 min, 62ºC for 1 min, and 72ºC for 1 min, followed by a final extension period at 72ºC for 5 min. As a template, 1 µl of extracted DNA previously diluted 1/10 was used in all reactions.

An equimolecular mix of all PCR products was cleaned up (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare Life Sciences, Freiburg, Germany), cloned into the pCR®2.1 vector

following the protocol recommended by the manufacturer of the TA Cloning® Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into One Shot® TOP10F' Chemically Competent *Escherichia coli* cells (Invitrogen Life Technologies). Each clone was reamplified using the primer pair NS31 (Simon et al. 1992), a universal eukaryotic primer, and Glo1 (Cornejo et al. 2004), a primer designed for a nested amplification of glomeromycotan fungi. The resulting PCR product of around 230 bp has an ideal size to be discriminated by Single Strand Conformational Polymorphism (SSCP, see Kjoller & Rosendahl 2000). In addition, this internal region of AML1-AML2 is a highly variable area allowing the discrimination of a majority of sequences of AMF (Cornejo et al. 2004). Clones for SSCP were prepared by mixing 4 µl of the PCR product with 4 µl of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF and 20 mM EDTA). Samples were then denatured for 5 min at 95ºC and immerse on ice before loading. The Dual Adjustable Mega-Gel unit (C.B.S. Scientific company, Del Mar, CA) was used to carry out the electrophoresis. Samples were separated in discontinuous 50 cm x 22 cm x 0.7 mm gels with 25% (w:v) MDE acrylamide, 5% glycerol, 0.05% ammonium persulfate (APS), 0.0125% N-Tetramethylethylenediamine (TEMED) and 0.5 x TBE buffer (separation gel) and 75% (w:v) formamide, 6% acrylamide (19:1), 0.15% APS, 0.1% TEMED and 0.2 x TBE buffer (stacking gel), using 0.5 x TBE as running buffer. Gels were run for 3 h at 20ºC (gel temperature). Bands were visualized by silver staining (Bio-Rad Silver Stain, California, USA). To ensure a good coverage of the AMF diversity in the rosemary plants more than one clone of the AML1-AML2 fragment was sequenced per SSCP profile. The sequencing was done by the sequencing services of the Estación Experimental del Zaidín (Granada, Spain) using the set of vector binding primers M13F-M13R. The abundance of DNA sequences indicated by their profiles in the SSCP fingerprinting was used to carry out the rarefaction analysis.

Phylogenetic analysis

The phylogenetic analysis was carried out using the sequences obtained in the study and a representative group of sequences of all major glomeromycotan groups from GenBank (references KC665640-KC665707). Sequences were aligned with MAFFT version 6 and similarities excluding the primer sequence were determined using BioEdit software. The outgroup used was *Mortierella polycephala* X89436. The phylogenetic analysis was computed in MEGA4 (Tamura et al. 2007) using

the neighbor-joining algorithm with Kimura-2 parameters as the model of substitution and 1,000 replications to obtain bootstrap values. Phylotypes were determined using a minimum of 97% sequence similarity and a high bootstrap value as indicators (Öpik et al. 2010). A blast search in the Maarj*AM* database (Öpik et al. 2010) was used to name the sequences with the number code of the closest virtual taxon. The prefix of the phylotype name used below corresponds with the glomeromycotan families (Krüger et al. 2012): Cla-Claroideoglomeraceae, Div-Diversisporaceae, Glo-Glomeraceae, Pac-Pacisporaceae, Par-Paraglomeraceae and Sac-Sacculosporaceae (Oehl et al. 2011).

TRFLP analyses

REPK online software (Collins & Rocap 2007) was used to select a combination of four enzymes able to discriminate between the found phylotypes in the sequence library. These enzymes were: HinfI, MboI, AvaII and BstXI (New England Biolabs). In order to carry out the TRFLP analysis, PCR reactions were redone as described above. Two reactions were performed per each sample, the first one with the fluorescent label 6-FAM and the second one with HEX, both attached to the forward primer AML1. Forty ng of each PCR product were digested with the corresponding restriction enzyme for 2 hours at 37ºC. HEX PCR products were digested with HinfI and AvaII and 6-FAM PCR products with MboI and BstXI. Every digestion was cleaned (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare Life Sciences, Freiburg, Germany) before sending it to the fragment length determination analysis. Since the analysis was carried out in multiplex, the digestions were mix as follows: HEX PCR products digested with HinfI in combination with 6-FAM PCR products digested with MboI on one side; and, on the other side, the combination of the other two digestions (BstXI and AvaII). Samples were analyzed in the Unidad de Genómica y Síntesis de DNA, Instituto de Biomedicina y Parasitología López Neyra (Granada, Spain). Data were processed using GeneMapper software version 3.7 (Applied Biosystems, 2004). In order to get the empirical TRF profiles of the detected sequences in the database, the clones selected for the gene library construction were re-amplified, digested and analyzed in the same way as explained for the samples. The R (R Development Core Team, 2010) package, TRAMPR (FitzJohn & Dickie 2007), was used for matching TRFLP profiles of the samples with the sequence database profiles, which allowed us to allocate the phylotypes present in the roots of each plant.

Statistical analyses

Repeated measure ANOVA with Greenhouse-Geisser adjusted probability was used to analyze the mycorrhizal colonization data in order to test propagule source and time effects. A split-plot design with time as the within-subject factor and mycorrhizal propagule source as the between-subject factor was used; mesocosm units were considered as subjects. Due to the lack of normality of phylotype richness data, non parametric Kruskal-Wallis tests were carried out to check for effects of time and propagule source.

The diversity of sequences resulting from the cloning was analyzed by rarefaction analysis using the Analytic Rarefaction freeware program (Holland 2008). Based on the assumption that rarefaction curves generally show an exponential rise to an asymptote, the results were fitted to the formula $y = y0+ax/(b + x)$.

A Non-metric Multidimensional Scaling (NMDS) ordination, using Jaccard distance as community dissimilarity measurement, was carried out to reduce AMF community variation to two axes. Function *envfit* (vegan package in R statistics, Oksanen et al. 2011) was used to fit the experimental variables as vectors (plant and soil propagules and time) or centroids (seasons) onto the NMDS ordination. Permutational analyses were carried out for testing the significance of the effect of the experimental variables. These analyses were stratified according to the date of sampling (time). Consequently the effect of time was extracted from the effect of the rest of variables. The goodness of fit was provided by the squared correlation coefficient (r^2).

Since the data obtained from the TRFLP database approach consisted of a presence/absence matrix, only richness (S) as diversity index was calculated. In order to assess the differences in phylotype composition found in the rosemary roots colonized from different propagule sources during the experiment, the frequency of detection was calculated as the percentage of replicates containing each phylotype.

Dufrêne-Legendre indicator species analysis (Dufrêne & Legendre 1997) was carried out using function *indval* from labdsv package in R statistics (Roberts 2010) to identify AMF tied to specific levels of tested variables that could serve as indicator species. This index varies from 0 to 1 and is maximal if all individuals of a particular phylotype are present only among every sample of a particular treatment. As a reference value 0.20 was used as the threshold to consider a species indicator of a certain treatment. The different levels of each variable were tested separately. In the case of the time variable, different periods were tested.

Results

As a preliminary step it was necessary to apply a drastic treatment to the soil to ensure that only the AMF propagules resistant to disturbances would remain viable. This drastic treatment consisted in the initial sieving of the soil, followed by a drying process during eight months. Surprisingly, the mycorrhizal inoculum potential of the soil did not significantly vary during the desiccation period, keeping an average value of 0.68 (± 0.02 SE) colonization units per cm of root during the eight months of drying (Fig. 1.1). The soil moisture at the beginning of the desiccation period was 3.75 % (± 0.12). However from the second measure (second week) moisture was maintained around 1.73 % (± 0.02) until the end of the process (Fig. 1.1). The elimination of the most sensitive mycorrhizal propagules to soil alteration occurred probably during the soil sieving and its subsequent transfer to the laboratory to proceed to the desiccation period.

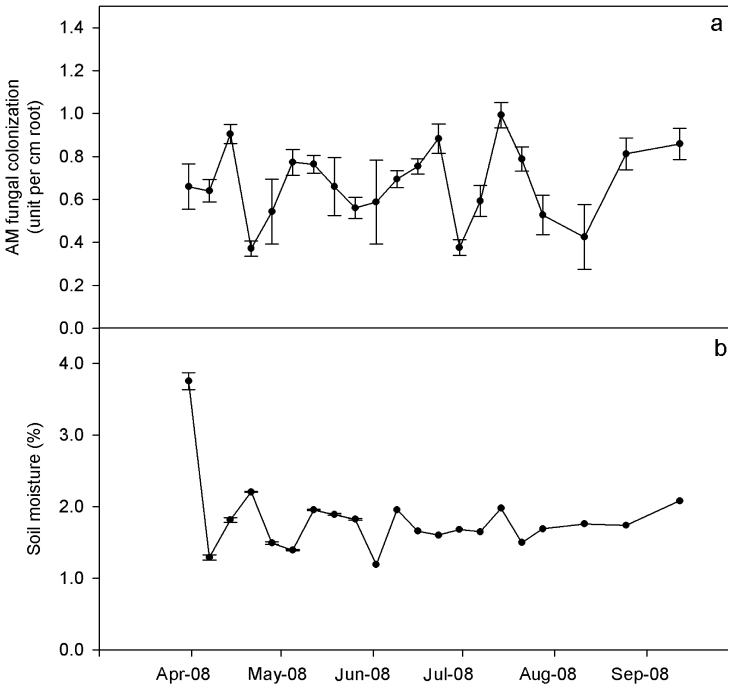

Fig. 1.1 Evolution of a) the mycorrhizal potential of the soil treated to contain only resistant AMF propagules and b) the moisture level of the soils used for the mesocosm units

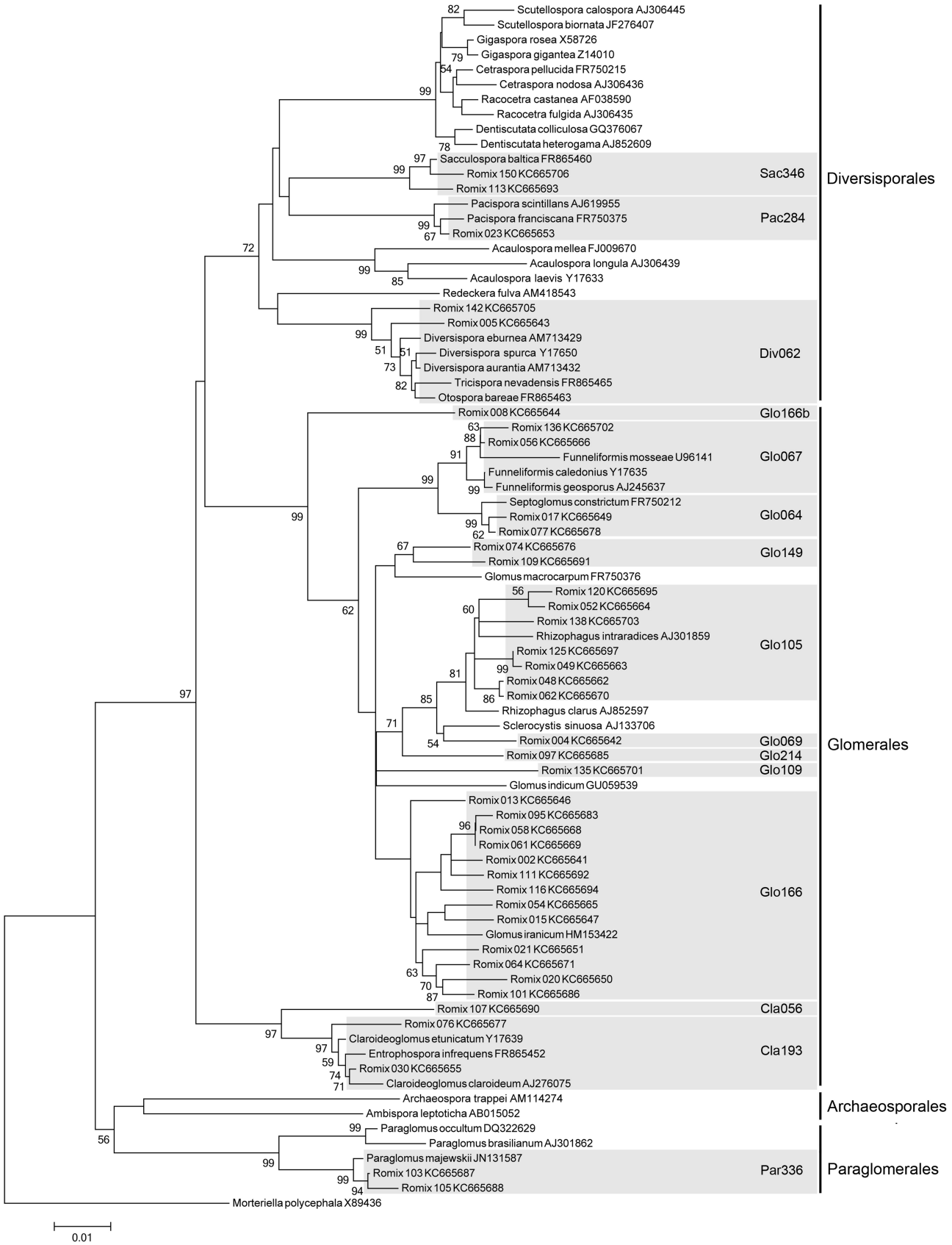
Scutellospora calospora AJ306445
Scutellospora biornata JF276407
Gigaspora rosea X58726
Gigaspora gigantea Z14010
Cetraspora pellucida FR750215
Cetraspora nodosa AJ306436
Racocetra castanea AF038590
Racocetra fulgida AJ306435
Dentiscutata colliculosa GQ376067
Dentiscutata heterogama AJ852609
Sacculospora baltica FR865460
Romix 150 KC665706
Romix 113 KC665693
Sac346
Pacispora scintillans AJ619955
Pacispora franciscana FR750375
Romix 023 KC665653
Pac284
Acaulospora mellea FJ009670
Acaulospora longula AJ306439
Acaulospora laevis Y17633
Redeckera fulva AM418543
Romix 142 KC665705
Romix 005 KC665643
Diversispora eburnea AM713429
Diversispora spurca Y17650
Diversispora aurantia AM713432
Tricispora nevadensis FR865465
Otospora bareae FR865463
Div062
Diversisporales
Romix 008 KC665644
Glo166b
Romix 136 KC665702
Romix 056 KC665666
Funneliformis mosseae U96141
Funneliformis caledonius Y17635
Funneliformis geosporus AJ245637
Glo067
Septoglomus constrictum FR750212
Romix 017 KC665649
Romix 077 KC665678
Glo064
Romix 074 KC665676
Romix 109 KC665691
Glo149
Glomus macrocarpum FR750376
Romix 120 KC665695
Romix 052 KC665664
Romix 138 KC665703
Rhizophagus intraradices AJ301859
Romix 125 KC665697
Romix 049 KC665663
Romix 048 KC665662
Romix 062 KC665670
Glo105
Rhizophagus clarus AJ852597
Sclerocystis sinuosa AJ133706
Romix 004 KC665642
Glo069
Romix 097 KC665685
Glo214
Romix 135 KC665701
Glo109
Glomus indicum GU059539
Romix 013 KC665646
Romix 095 KC665683
Romix 058 KC665668
Romix 061 KC665669
Romix 002 KC665641
Romix 111 KC665692
Romix 116 KC665694
Romix 054 KC665665
Romix 015 KC665647
Glomus iranicum HM153422
Romix 021 KC665651
Romix 064 KC665671
Romix 020 KC665650
Romix 101 KC665686
Glo166
Romix 107 KC665690
Cla056
Romix 076 KC665677
Claroideoglomus etunicatum Y17639
Entrophospora infrequens FR865452
Romix 030 KC665655
Claroideoglomus claroideum AJ276075
Cla193
Glomerales
Archaeospora trappei AM114274
Ambispora leptoticha AB015052
Archaeosporales
Paraglomus occultum DQ322629
Paraglomus brasilianum AJ301862
Paraglomus majewskii JN131587
Romix 103 KC665687
Romix 105 KC665688
Par336
Paraglomerales
Morteriella polycephala X89436
0.01

Fig. 1.2 Neighbor-joining phylogenetic tree based on the AML1-AML2 fragment of the SSU rDNA gene of AMF. Reference sequences from GeneBank are showed together with sequences obtained from rosemary root samples. Numbers near branches indicate the bootstrap values. Only topologies with values ≥ 50% are shown (1000 replicates). Sequences are labelled according to the clone identity number. Sequences having a pairwise similarity higher than 97% were clustered as phylotypes (delimited by grey boxes). Phylotypes are named following the closest virtual taxa code of Maarj*AM* database (Öpik et al. 2010). The prefix corresponds to the glomeromycotan family (following Krüger et al. 2012): Div-Diversisporaceae, Cla-Claroideoglomeraceae, Glo-Glomeraceae, Par-Paraglomeraceae, Pac-Pacisporaceae and Sac-Sacculosporaceae (Oehl et al. 2011). *Mortierella polycephala* was used as out-group

The cloning and sequencing of glomeromycotan DNA sequences to create a database for the whole experiment revealed a high diversity of phylotypes. The resulting phylogenetic tree is shown in Fig. 1.2. Members of six glomeromycotan families were detected (Glomeraceae -9-, Pacisporaceae -1-,Claroideoglomeraceae -2-, Diversisporaceae -1-, Sacculosporaceae -1- and Paraglomeraceae -1-) reaching a total of 15 phylotypes. The rarefaction analysis, based on the SSCP profile of the different clones, showed a maximum of 17 phylotypes (Fig. 1.3) in the case that 1,300 clones were analyzed (R^2=0.996). The most abundant glomeromycotan DNA sequences in this study corresponded to members of the Glomeraceae family (particularly to Glo166 -35.3 %-, Glo105 -17.4 %- and Glo067 -10.4 %-). Div062, Par336 and Glo064 were detected in approximately 7 % of the clones; Cla193 and Pac284 in around 3 % and the remaining phylotypes were detected in less than 2 % of the clones. A 2.7 % of non specific amplifications were detected, which included rosemary sequences.

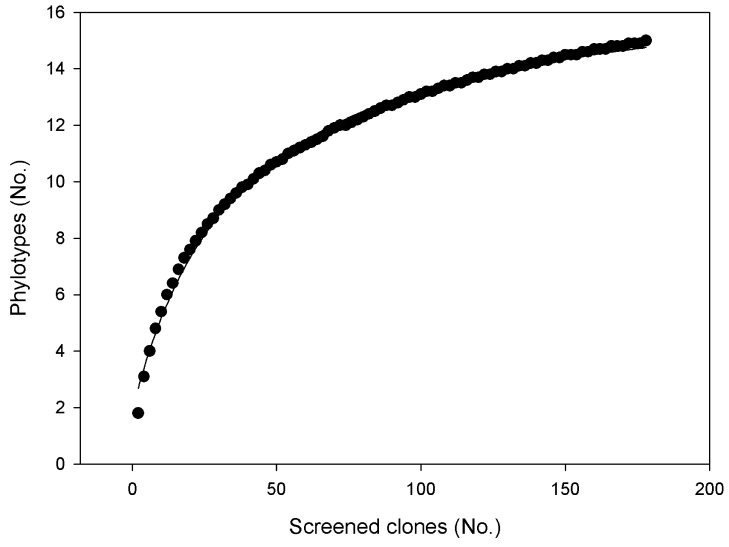

Fig. 1.3 Rarefaction analysis performed on the 18S sequence data of AMF obtained from the clone library of rosemary roots. A 97% sequence similarity threshold value was used to define phylotypes

Mycorrhizal propagule source significantly affected the AMF community composition in the rosemary roots as showed by the fitting of environmental variables onto the NMDS ordination (k=2, Stress = 0.121; R^2 = 0.105 and P = 0.001 for

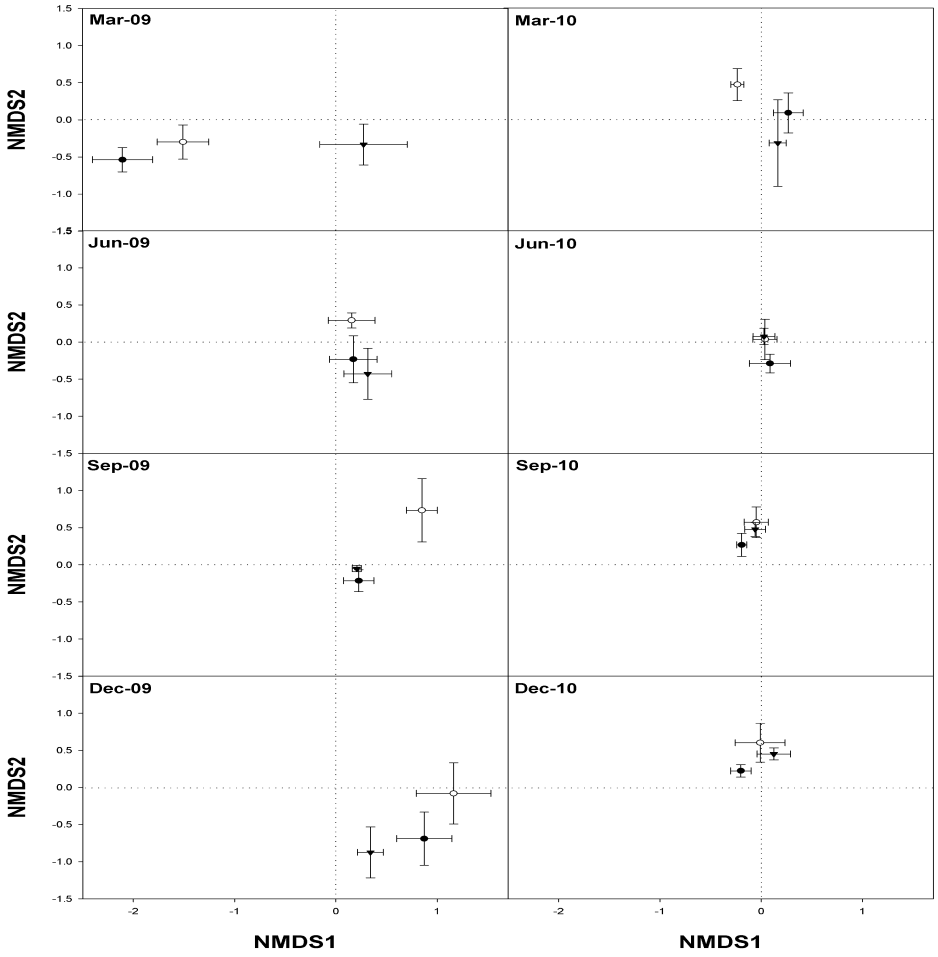

Fig. 1.4 Time-course evolution of the AM fungal community composition. NMDS ordination (k=2, stress=0.121) was represented by date of harvesting. Standard error bars are represented for each treatment

the vector representing resistant propagules from the soil and $R^2 = 0.081$, $P < 0.01$ for the vector representing propagules from the plant). In addition, the effect of time revealed a successional pattern ($R^2 = 0.164$, $P = 0.001$) influenced by seasonality ($R^2 = 0.147$, $P = 0.001$).

The evolution of the AMF community composition, as NMDS ordination, is shown in Fig. 1.4. From the second sampling time, the treatment including mycorrhizal propagules coming from both the plant and the soil showed a tendency to be close, and in consequence similar, to the treatment including only 'soil propagules'. In the three last sampling times the AMF community appeared very similar in all treatments. AMF phylotype richness was influenced by propagule source ($\chi^2 = 9.2556$, $P<0.01$) as well as time ($\chi^2 = 19.3504$, $P<0.01$). In general, plants colonized by 'soil propagules' had a higher richness than those mycorrhizal from 'plant propagules' all along the experiment (Fig. 1.5). Plants colonized by propagules from both plant and soil presented values more similar to those of plants colonized only from 'soil propagules' during the first year of the study; however, at the end of the experiment they became more similar to those colonized from 'plant propagules'.

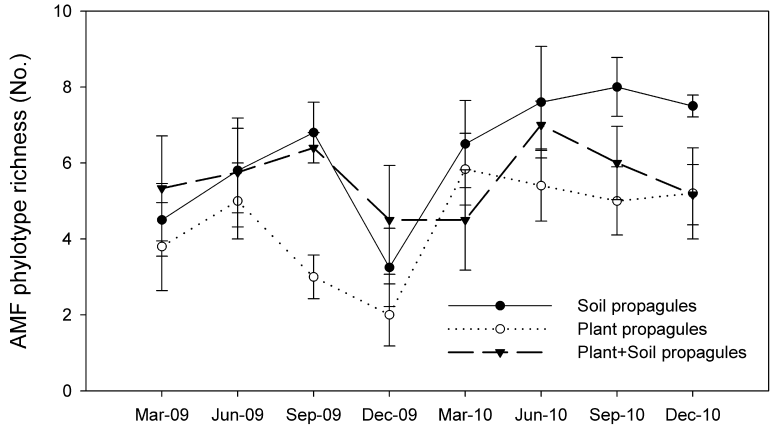

Fig. 1.5 Time-course evolution of AMF phylotype richness

The fit of experimental variables (propagule source -soil or plant-, time and seasonality) onto NMDS ordination is represented in Fig. 1.6. In this graph the effects of the experimental variables onthe presence of the different AMF phylotypes is revealed by the location of each phylotype.

The clearest pattern is shown by members of Claroideoglomeraceae, which appear to be associated to the variable representing 'soil propagules'. In a lesser extent, members of the Glomeraceae and this

Table 1.1 Indicator Value calculated for phylotypes related to each level of the different experimental variables. Indicator values higher than 0.20 were considered as indicative of the presence of the phyotype (Dufrêne & Legendre 1997). Signif. codes: *p<0.05; **p<0.01;***p<0.001

	Soil		Plant		Time period (months)				Season			
	+	-	+	-	0-6	7-12	13-18	19-24	Mar	Jun	Sep	Dec
Par336				0.26**								
Div062		0.21*			0.31***				0.29***			
Sac346					0.24***				0.23***			
Pac284												
Glo166b							0.26**	0.33**				
Glo166							0.21**	0.28**				
Glo149							0.27**	0.31**		0.23**	0.31**	
Glo067								0.36***				
Glo064	0.35***			0.40***				0.22**				
Glo105								0.25***				
Glo069												
Glo214												
Glo109												
Cla193				0.32*								
Cla056	0.50***			0.34*	0.22*							

glomeromycotan order (fam. Paraglomeraceae families seem more influenced by time or 'plant propagules' variables. It is noteworthy that the Diversisporales phylotypes detected in this study are negatively related to time. In addition, the location of the season factor corresponding to March was the nearest to the members ofDiversisporaceae, Sacculosporaceae and Pacisporaceae). The proximity between members of the same glomeromycotan order or family supports that functional traits of AMF have a taxonomical basis.

To corroborate the patterns revealed for the different AMF phylotypes, data obtained were submitted to a Dufrêne-Legendre indicator analysis (Dufrêne & Legendre 1997) (Table 1.1). The Table is organized by experimental variables. Two phylotypes were indicators of the presence of 'soil propagules' and the absence of 'plant propagules' (Cla056 and Glo064, Table 1.1). In fact, both AMF phylotypes were very frequent in plants colonized from 'soil propagules', and almost absent when the source of mycorrhizal propagules was the donor plant. Other two phylotypes correlated better with the absence of a certain type of propagules than to the presence of their own source of propagules. This was the case of Div062 and Par336. These AMF phylotypes colonized preferentially from the 'plant propagules' (Div062) or almost exclusively from the 'soil propagules' (Par336); but they were indicators of the absence of propagules coming from the soil or the plant, respectively (Table 1.1).

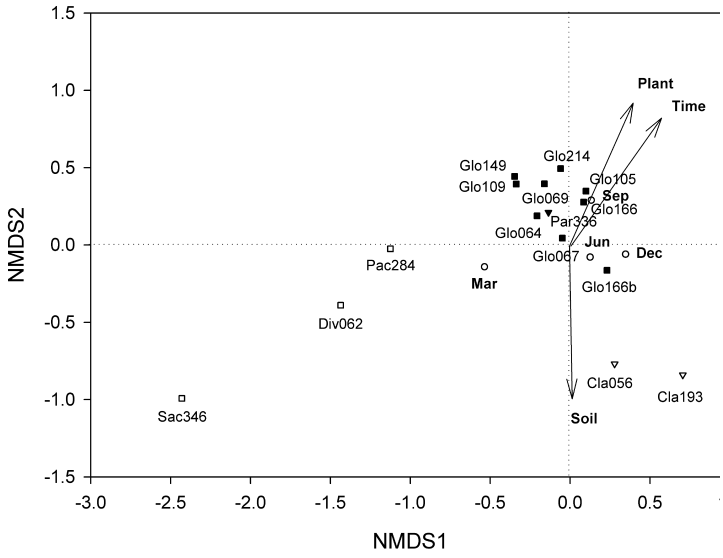

Fig. 1.6 Fitting of experimental variables onto the NMDS ordination (k=2, stress=0.121). Vectors show the direction of the influence of the represented variables (propagules of plant –Plant- or soil –Soil- origin and time). The theoretical location of seasons in the ordination gives an idea of their influences (white circles). White squares represent AMF phylotypes belonging to the Diversisporales order, black squares to the family Glomeraceae (Glomerales), white triangles to the Claroideoglomeraceae (Glomerales) and the black triangle corresponds to the Paraglomerales member. The number indicates the code of the closest virtual taxa (Öpik et al. 2010, see also Fig. 1.2)

Different time periods were analyzed to study the temporal succession of AMF and the one showing the clearest pattern was six month periods. In overall, Diversisporales members (comprising both the *Diversispora* and *Sacculospora* phylotypes) appear as early colonizers with a high frequency of detection during the first sampling times (Fig. 1.7, Table 1.1).

Although Pac284 did not show a statistically significant trend, probably due to its low level of detection, it showed a similar behavior to the other Diversisporales phylotypes. Claroideoglomeraceae appeared best represented in the central time periods, while during the second half of the study, the dominating phylotypes were members of Glomeraceae. Concerning seasonality, Diversisporales appear as indicators of March (end of winter time), while Glo166, the most abundant phylotype in this study, resulted indicator of June and September (spring and summer harvestings). The different patterns of temporal dynamics followed by the different group of AMF phylotypes are recorded in Fig. 1.7.

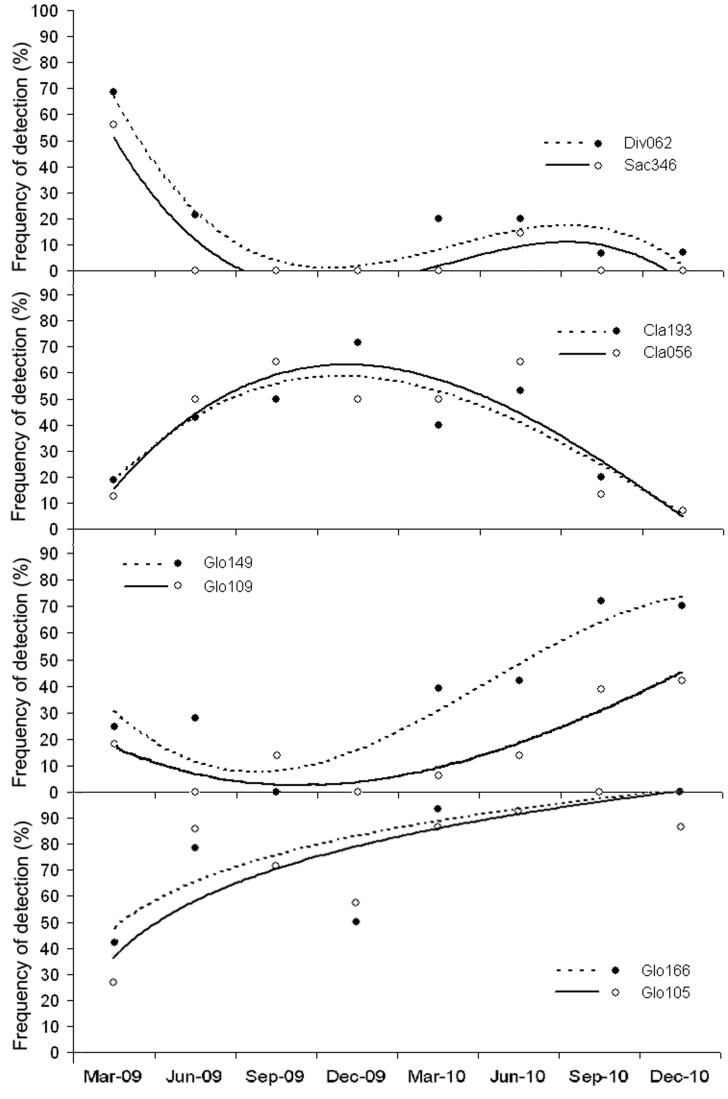

Fig. 1.7 Temporal dynamics of colonization followed by different AMF phylotypes, grouped according to their colonization trend

Apparently there was no correspondence among the AMF phylotypes colonizing the plants growing in natural conditions ('donor plants') and those colonizing preferentially from plant propagules. In fact, Div062, the AMF phylotype colonizing preferably from the plant was not detected in any of the analyzed donor plants, while Par336 and Glo064, which colonize almost exclusively from the soil,

were detected in 50 % of the plants growing in field conditions (data not shown). A higher level of concordance was found for other phylotypes, since Cla056, colonizing almost exclusively from the soil was not detected in the donor plants, and Glo166 and Glo105, the most abundant phylotypes in the 'receiver plants' were detected in 50 and 100 % of the donor plants, respectively.

Propagule source and time also influenced significantly mycorrhizal colonization. The effect of the propagule source on total mycorrhizal colonization ($F=8.51$, $P<0.01$, $\varepsilon=0.45$) was mainly due to differences in the first sampling time since plant colonized only from 'plant propagules' had a quite low rate of mycorrhizal colonization (6.40%, Fig. 1.8). From the second sampling time mycorrhizal colonization levels were similar among treatments and no significant differences could be detected. The extent of arbuscular colonization in the roots was also influenced by time ($F=38.48$, $P<0.001$, $\varepsilon=0.51$) while the source of propagules had no statistically significant effects. The frequency of arbuscule detection in the root system over time showed a cyclic pattern which can be easily attributed to seasonality (Fig. 1.8). Finally, the frequency of vesicle detection was clearly influenced by both, time ($F=11.73$, $P<0.001$, $\varepsilon=0.36$) and propagule source ($F=5.10$, $P<0.05$,

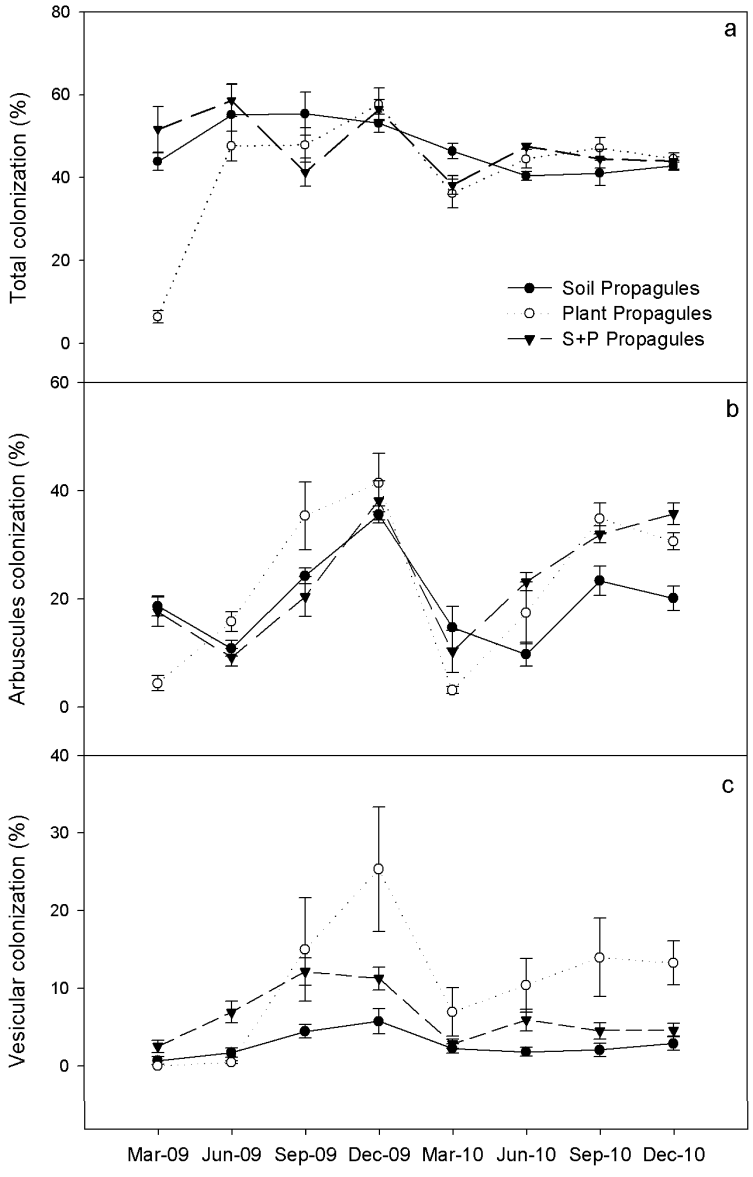

Fig. 1.8 Mycorrhizal colonization dynamics over time. Total (a), arbuscular (b) and vesicular (c) colonization were calculated as the percentage of the root system showing hyphae, arbuscules or vesicles, respectively

ε=0.36). Plant colonized only from 'plant propagules' presented higher levels of vesicle formation than those colonized only from 'soil propagules' (Fig. 1.8). Plants colonized by both types of propagules showed intermediate values.

Discussion

This study has set up an experimental approach that would allow us to reveal different life-history strategies of AMF in relation to their colonization strategies, the way in which they persist between growing seasons under unfavourable conditions (as soil propagules or attached to living roots) and, in consequence, the successional dynamics of their communities. Basically, AMF could follow two contrasting strategies: (i) AMF taxa which spread out mainly through the production of resistant propagules able to survive disturbances, i. e. spores or fragments of colonized roots; and (ii) AMF taxa which remain in the roots of living plants during unfavourable seasons and that use hyphae, rather than spores, as the main strategy for root colonization. The strategy based on the production of resistant AMF propagules can be very important where climate has great variations between seasons, as it is the case of the Mediterranean environments, thereby ensuring the survival of AMF populations until next favourable season (Tommerup & Abbott 1981). The alternative strategy would be best suited to environments with less climatic variation (Rosendahl & Stukenbrock 2004). In fact, for ectomycorrhizal fungi a high rate of hyphal death during the dry season in Mediterranean environments has been shown (Allen & Kitajima 2013). The treatment applied to the soil to eliminate the mycelium connected to living roots (sieving and subsequent drying for eight months) was strong enough to ensure that only spores and some small fragments of infected roots could persist. There are evidences that mycelial networks cannot maintain their infective potential after a similar process of soil disturbance (Jasper et al. 1989).

The colonization of new plants from the different types of propagules clearly influenced the resulting AMF community during the first year of the study. Only one phylotype (Div062) was preferentially detected when the mycorrhizal plant was the only source of propagules. This suggests that this phylotype disperses through mycelium rather than spores and also that it is a poor competitor with other phylotypes colonizing mainly from resistant propagules in the soil. As it has already been

described for ectomycorrhizal fungi (Peay et al. 2011), the formation and maintenance of hyphal networks by AMF is a way of dispersion which would not depend on spore formation (Denison & Kiers 2011). Mycelial growth could be favoured by the permanent growth of the roots of perennials (Rosendahl & Stukenbrock 2004) as rosemary is. It has been suggested that mycelium connected to living roots is generally more efficient than spores in colonizing new roots because it has a much larger carbon budget than a spore, and can use it to effectively explore soil and encounter uncolonized roots (Peay et al. 2011). In fact, Div062 was the fastest colonizer in this study, and was detected in almost all plants at the first sampling time. It is noteworthy that plants colonized from 'plant propagules' showed a significant increase in the abundance of AMF vesicles in the roots. This increase was especially prominent in December, at the beginning of winter time, what can be an indication of the resource hoarding needed by this and other AMF, mainly those relying on mycelial-based dispersal, for the upturn in growth experienced in the following spring.

Few studies have been carried out on the colonization strategies of AMF. Most of these studies were based on fungi belonging to the Glomerales and Diversisporales, particularly to the Glomeraceae, Acaulosporaceae and Gigasporaceae families (Klironomos & Hart 2002; Hart & Reader 2002, 2005; Powell et al. 2009). As far as we know, no information is available on other Diversisporales. It is interesting to highlight that the three phylotypes belonging to this order detected in the present study (Div062, Sac346 and Pac284) shared some specific functional traits: they were effective colonizers from plant propagules, minority in the root system (especially Pac284) and showed a seasonal behaviour.

The rest of AMF phylotypes associated to the rosemary rhizosphere in field-grown plants seemed to colonize the new roots more effectively from the soil propagules. This could be explained by the dominance in these environments of sporulating fungi that preferentially colonize through spore germination ('spore colonizers') as it has been suggested for some degraded habitats (Jansa et al. 2003; Oehl et al. 2011). In general terms, the ecology of the rosemary shrublands supports the here found patterns. Although rosemary is a perennial woody species, the ecological characteristics of its communities could be unfavourable to the development of AMF spreading by their mycelial network. This vegetation community is not the climactic one, being an earlier step in the succession of the

Mediterranean forest (Valle 2003). Its scarce plant cover and poor soil development can promote sporulating AMF (Rosendahl & Stukenbrock 2004; Schnoor et al. 2011). In any case, for certain AMF, dead AM roots can also act as mycorrhizal propagules (Tommerup & Abbot 1981); however their infective potential after a long period of soil drying remain to be elucidated.

Par336, Glo064 and Cla056 were mainly detected in the receiver plants when they grew in the soil containing only resistant propagules. This suggests that these AMF phylotypes have difficulties to colonize new roots from hyphae attached to living roots. This could be due to a poor development of the external mycelium, as suggested for some AMF particularly in the Glomeraceae family (Hart & Reader 2005), or with a limited exploration ability to colonize new roots, as it is has been shown for nutrient uptake (Smith et al. 2000). We cannot discard that the mycorrhizal donor plant were not colonized with these fungal phylotypes; however, this possibility does not seem very likely, at least for Par336 and Glo064, since they were detected in 50 % of the plants naturally growing in the field. The detection level of these phylotypes was even reduced in the presence of the mycorrhizal donor plant, what suggests competitive interactions with some of the AMF colonizing from the plant-attached mycelia. Competitive interactions have been shown among certain AMF (Pearson et al. 1993) and they play a role in shaping the community structure of AM and ectomycorrhizal fungi (Koide et al. 2005; Maherali & Klironomos 2012; Pickles et al. 2012).

On the other hand, a clear pattern of AMF succession was found. Three different colonization strategies, explained by differences in dispersal ability (of both spores and mycelium) and competition, appear to determine the succession of the AMF community. The revealed trends have a taxonomical basis, in agreement with previous findings by Hart & Reader (2002) and Powell et al. (2009):

• Diversisporales members seem to be fast colonizers. They experience a seasonal dynamics, appearing mainly in March. It can be hypothesized that their strategy consists in an earlier colonization before best competitors can displace them. These phylotypes are subsequently replaced by members of the Glomerales. This seasonal trend was described for other *Diversispora* sp. in a previous study in Sierra de Baza Natural Park (Sánchez-Castro et al. 2012b).

• Members of the Claroideoglomeraceae, within the Glomerales, appear somewhat later than Diversisporales in the succession. Their dynamics showed a plateau being present in the central periods of the study. Finally, they were displaced by members of the Glomeraceae family.

• The phylotypes with higher persistence ability in this study were members of the Glomeraceae. In spite that they are frequently considered r-strategists (IJdo et al. 2010), they were not the first colonizers in this study. However, once they had colonized, were those who best persisted and, as a result, the best competitors once inside the root. This can be explained by two concomitant facts. Firstly, Glomeraceae fungi have been repeatedly described as extensive root colonizers (Hart & Reader 2002), what to some extent may have introduced a bias and hindered the detection of the more minority fungi. Secondly, it is possible that the experimental conditions during this study have restricted root growth, especially during the second year. It is well accepted that a more constrained niche space can exclude species with poor rates of root colonization (van der Heijden & Scheublin 2007; Maherali & Klironomos 2012), as it can be the case of the Diversisporales members detected in this study.

In general, it seems clear that the initial AMF community was replaced by competitively stronger AMF species, leading to community convergence in all treatments. This structuring of AMF assemblages could respond to two mechanisms: either selection by host plant of the most compatible/beneficial AMF by the preferential allocation of resources to preferred fungal partners (Bever et al. 2009; Grman 2012), or the natural succession of AMF communities based in their life-history strategies (Hart et al. 2001). However, we cannot exclude that the convergence of the AMF community shown in the present study is partially due to the restriction imposed on the rosemary growth by the mesocosm units.

In summary, we can state that differences in the formation and presence of different type of AMF propagules have implications in the successional colonization pattern in roots. The inverse relationship between colonization ability and persistence reveals a trade-off that helps the understanding of the mechanisms governing AMF succession in natural communities and the structuring of AMF assemblages. This succession in plant colonization is particularly important to consider for planning, setting up and inoculating experiments using multispecies AMF consortia.

Capítulo basado en:

A. López-García, J. Palenzuela, J.M. Barea, C. Azcón-Aguilar. Life-history strategies of arbuscular mycorrhizal fungi determine succession into roots of Rosmarinus officinalis L., a characteristic woody perennial plant species from Mediterranean ecosystems. Artículo en revisión

Chapter 2: Interaction between plant life form and functional traits of arbuscular mycorrhizal fungal community as determinant of the resultant symbiotic assembly, as affected by the Mediterranean climate seasonal variation

Capítulo 2: Interacción entre el biotipo vegetal y las características funcionales de los hongos micorrícicos arbusculares como factor determinante de la comunidad simbiótica resultante, bajo la influencia de las variaciones estacionales del clima Mediterráneo

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Resumen

Objetivos Analizar la evolución, a lo largo del tiempo, de las características funcionales de hongos micorrícicos arbusculares (HMA) presentes en el suelo, de acuerdo con su capacidad de supervivencia, modulada por las variaciones climáticas estacionales del clima Mediterráneo, y evaluar si tales características funcionales contrastantes determinan la asociación simbiótica resultante de su interacción con distintos biotipos vegetales.

Métodos Como medida de la evolución estacional de las comunidades de HMA se utilizó su capacidad de supervivencia. Dicha medida se cuantificó a través de la determinación del potencial de colonización del suelo natural y tras tamizarlo. A través de un sistema de planta trampa (*Sorghum vulgare*), se compararon ambas comunidades de HMA, utilizando TRFLP (ADN ribosomal). Se aplicó la misma técnica en un segundo experimento para comparar la composición de las comunidades HMA en el suelo fruto de la interacción entre biotipos vegetales y la estacionalidad climática.

Resultados Se observó la alternancia de una comunidad de HMA con una capacidad de supervivencia alta con otra más sensible a la alteración, lo cual parece deberse a la estacionalidad del clima. Además, distintos biotipos vegetales dieron lugar a comunidades de HMA diferentes entre ellos, aunque sólo ocurrió cuando la estacionalidad climática no produjo un sesgo de las características funcionales de la comunidad.

Conclusiones La estacionalidad climática Mediterránea determina las características funcionales dominantes de la comunidad de HMA. Además, las características funcionales de hongos y plantas interaccionan para determinar la simbiosis resultante, hecho que tiene importantes consecuencias en la determinación de las funciones ecosistémicas derivadas de la presencia de distintos biotipos vegetales.

Chapter 2: Interaction between plant life form and functional traits of arbuscular mycorrhizal fungal community as determinant of the resultant symbiotic assembly, as affected by the Mediterranean climate seasonal variation

Abstract

Aims To ascertain the temporal evolution of the functional characteristics of arbuscular mycorrhizal fungal (AMF) present in the soil -their survival capacity- throughout the Mediterranean climatic seasonal variation. To assess how different plant life forms (PLF) can interact with AMF communities with different functional characteristics to determine the symbiotic assembly.

Methods The seasonal evolution of AMF communities in a natural area was assessed by measuring their survival capacity in the soil. Measure of natural colonization potential and that after sieved-disturbed soil was used to evaluate this variable. A trap plant system (*Sorghum vulgare*) was used to compared, via TRFLP of AMF rDNA, the AMF community composition. A second experiment compared the AMF communities in the soil as promoted by different PLFs using AMF inoculum of different seasons. The same trap plant system was chosen to compare of AMF community composition.

Results The alternation of AMF communities with higher survival capacity with those more sensible was dependent on Mediterranean climatic seasonality. Besides, different PLFs promoted a distinct AMF communities composition only when they were inoculated with an AMF community whose functional characteristics were not biased by climatic seasons.

Conclusions The Mediterranean climatic seasonality determined the functional characteristics of the dominant AMF community, finding two extreme situations: end of the summer, dominated by more resistant AMF community, and end of spring, dominated by most sensible AMF. The functional characteristics of AMF and that of PLFs influence the symbiosis assembly. This fact can have important implications on the ecosystem functions altered by changes in the dominant PLF.

Introduction

Arbuscular mycorrhiza is probably the most important and widespread symbiotic association in nature (Brachmann & Parniske 2006). Actually, about 80% of land plants are able to establish this mutualistic association with the so-called arbuscular mycorrhizal (AM) fungi (Brundrett 2009), being the AM symbiosis fully recognized as a biological entity of major concern for terrestrial ecosystems functioning (Smith & Read 2008). The ancient AM fungi (phylum Glomeromycota), which date back more than 500 million years, have coevolved with plants as a symbiotic assembly highly specialized to facilitate plant adaptation to the primitive harsh terrestrial environment, being commonly accepted that AM fungi and symbiosis played a crucial role in dry land colonisation by plants (Schüßler & Walker 2011), an ability which continue being operative in modern terrestrial ecosystems where the AM symbiosis exerts crucial ecological impacts (Barea & Azcón-Aguilar 2013).

In the AM symbiosis, the microscopic fungus colonizes plant roots and develops an extraradical mycelium expanding into the soil microhabitats surrounding the roots. This mycelial network contributes to increase water and nutrient availability to plant, which, in turn, derives a part of their carbon compounds from photosynthesis to the fungus (Koide & Mosse 2004). The AM symbiosis produces other benefits to plants such as protection against biotic and abiotic stresses, thereby helping plants not only to survive but also to be productive under adversity (Barea et al. 2013b). Additionally, the presence of AM fungi (AMF) influences some ecosystem properties: promoting plant diversity, increasing productivity and improving soil structure (Grime et al. 1987; van der Heijden et al. 1998, 2006; Vogelsang et al. 2006; Rillig & Mummey 2006; Bever et al. 2010; Barea & Azcón-Aguilar 2013). Conversely, some studies have demonstrated how the environment can shift the distribution, identity and activity of AMF symbionts, thus, soil characteristics can influence the diversity and functions of the AMF community (Fitzsimons et al. 2008; Oehl et al. 2010) either as a consequence of degradation processes (López-García et al. 2013) or due to the existence of nutrient gradients (Egerton-Warburton & Allen 2000; Johnson et al. 2003).

Traditionally the symbiosis has been considered to be generalist however recent studies are revealing another trends and suggest a certain level of specificity in the relation between AMF and their

plant hosts. This fact has been attributed to the identity of a given plant species (Vandenkoornhuyse et al. 2003; Martínez-García & Pugnaire 2011; Sánchez-Castro et al. 2012), and/or to the functional or ecological groups to which the plant belongs (Scheublin et al. 2004; Öpik et al. 2009). Nevertheless, only few studies have been focused on the influence of plant life form (PLF) on AMF community composition. PLF is the most common and simplest method for classifying plants. Essentially, it is based on morphological features easy to determine (Schulze 1982) which can separate plants into trees, shrubs or forbs including different life cycles (Raunkiaer 1934). Furthermore, while these characters are insensitive to environmental changes, PLFs can be useful for functional grouping because they imply estimates of physiological variables (Brooks et al. 1997). In this sense, Cakan & Karatas (2006) established a certain relationship between the presence of different PLF in dune succession and the extent of AMF colonization in each plant community. More recently, in a global meta-analysis, Yang et al. (2012) found a high level of host specificity for its associated AMF at different scales, including plant functional type, selected on the basis of their PLF. However Urcelay et al. (2009) did not find differences between AMF spore communities when they carried out a removal experiment of similar plant groups.

Studies concerning AM fungal diversity in Mediterranean regions, specifically in southeastern Spain, have been focused mainly on perennial woody plants (Caravaca et al. 2005; Sánchez-Castro et al. 2012a, b). However, other studies include host plants with different life cycles (annual versus perennial), which constitute different PLFs, and have found differences in community composition of their associated AMF (Alguacil et al., 2012; Torrecillas et al., 2012a, b). These findings clearly drive to the hypothesis that AMF community can be affected by the life cycle of the host plant. There is also evidence supporting a seasonal behavior of AMF communities (Merryweather & Fitter 1998; Oehl et al. 2009; Dumbrell et al. 2011; Sánchez-Castro et al. 2012b) as well as a differentiation in terms of reproductive strategies of AMF suggesting the existence of sporulating and non-sporulating taxa (Hempel et al. 2007; Schnoor et al. 2011; Sánchez-Castro et al. 2012a). Although an adaptation between plant and fungus life cycles could be feasible due to the long time of coevolution of AM symbiosis, there are no studies focused on the relationships among the involved traits.

Despite of the influence of seasonality, there could be other multiple plant functional traits that can interact with fungal life-history strategies to bias the AMF community. For example, a longer plant life span could benefit the presence of more persistent AMF (López-García et al. in preparation). On other hand, as proposed by Torrecillas et al. (2012a), fungi approaching the roots more rapidly have a higher chance to colonize earlier active roots which belong to the herbaceous annuals (Hooper & Vitousek, 1998). In other words, the identity of the partners in the AM symbiosis can be leaded by the interaction between plant and fungal functional traits.

Taking into account these considerations, the aim of this study was to assess whether the feedback between the functional traits of plants and AMF influences the preferences in the AM symbiosis assemblages. As an initial hypothesis we propose that: (a) the Mediterranean climate seasonal variation will induce changes in the functional characteristics of the AMF community present in the soil throughout the year. In this sense, the variation of survival capacity of AMF can be indicative of differences in the fungal life cycles and consequently affects the presence of AMF in the soil throughout the year. The production of spores, as resistance propagules, can increase their survival capacity and extend their temporal presence in the soil in comparison with fungi with less sporulating capacity (Rosendahl & Stukenbrock 2004). As a consequence, it is also hypothesized that (b) the association of different PLFs with AMF with contrasting life cycles will show certain preferences since their functional characteristics, mainly in terms of life cycle, were related. Thus, the inoculation with AMF belonging to different seasons -late summer (September), late autumn (December), late winter (March) and late spring (June)-, and, at the same time, with different functional characteristics, can address the selection of the PLFs.

For this purpose a seasonal characterization of the AMF community, aimed to assess the impact of the Mediterranean climate seasonal variation on fungal populations, was first approached. Afterwards, a feedback experiment using different PLFs was designed to assess the influence of plant type over AMF soil community in term of establishing a given AM symbiotic assemblage. A trap plant system, combined with applying the TRFLP fingerprinting technique (Dickie & FitzJohn 2007), were used to compare the AMF communities of the target root samples. The results will provide an interesting contribution to the understanding of the AM symbiosis, both in highlighting the processes that cause

seasonal dynamics of AMF populations and the factors that determine the guest-host identity.

Material and Methods

Study site and sampling

The study site is located in Sierra de Baza Natural Park, at 1,500 m.a.s.l. An area of 15,000 m^2 was selected using ArcGIS 9.3 (ESRI, Redlands, USA) keeping a constant slope (15-20%) and orientation (east), being the soil a calcaric cambisol. The vegetation is an open autochthonous Mediterranean forest combined with naturalized pines coming from restoration plans. Components of both supramediterranean and mesomediterranean levels are well represented including *Quercus ilex*, *Pinus* sp., *Juniperus oxycedrus*, *Rosmarinus officinalis*, *Berberis hispanica* or *Rosa* sp. associated with herbaceous, annual, plant species. The climatic conditions are characterized by a scarcely and irregular precipitation with an annual mean of 385 mm, the temperature has a mean of 25ºC in summer and 6ºC in winter.

In order to get a representative AMF soil community, 20 random points were selected in the target area. After removing the first soil layer, 7 l aprox of soil, from a layer situated in between 2-20 cm, were collected. The soil was kept for 24 h in bags of 30 l capacity. All the soil samples were pooled and mixed in a concrete mixer. The sampling was repeated at the end of each climatic season, i.e. 18th December 2009, 19th March 2010, 18th June 2010 and 20th September 2010.

Experimental designs

Impact of climate seasonal variation on AMF communities

The experimental design was oriented to get information on two parameters: the AMF community composition and its survival/resistance capacity against disturbance throughout the year (see Fig. 2.1).

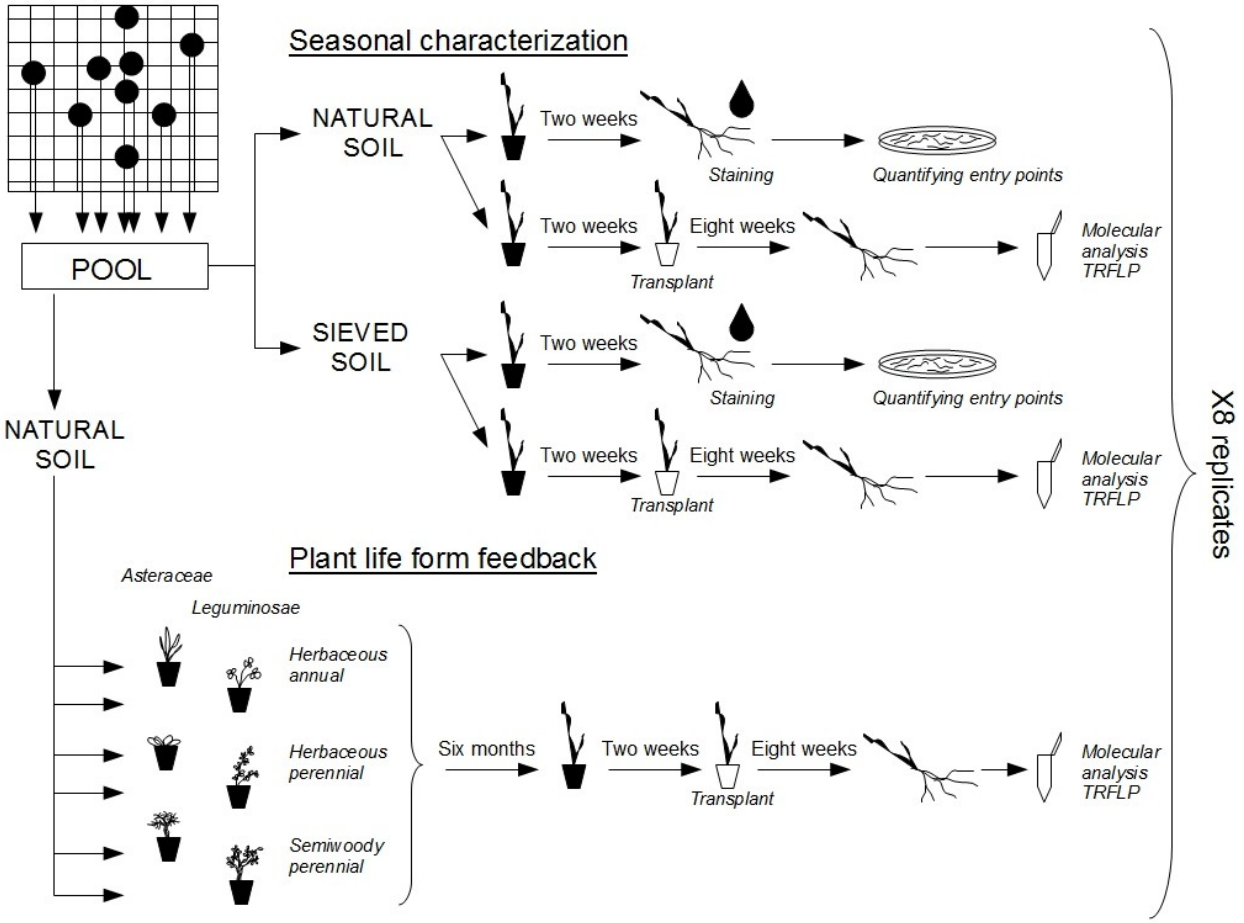

Fig. 2.1 Schematic diagram of theexperimental design

To assess the survival capacity of the AMF community, as affected by season, the colonization potential of the target soil, either natural or sieved (through a 5 mm sieve), was measured following the protocol of the "infection unit method" described by Franson & Bethlenfalvay (1989). This technique is based on the quantification of AMF entry points established on the roots of a model trap plant (*Sorghum vulgare*), during two weeks of growth.

Similarly, a comparison between the AMF community composition of natural and sieved soil was carried out. For that purpose, a bioassay based on a trap plant system, modified from that by Johnson et al. (2004), was used to "capture" most of the AMF taxa. To ensure that data of community composition

were fully related to those concerning inoculum potential assessment, the same plant species and timing (*Sorghum vulgare* and two weeks) as in the "infection unit method" (Franson & Bethlenfalvay 1989) were used. Although two weeks is time enough for sorghum plant to be colonized by a large number of AMF propagules, it may be a short time for the fungi to be subsequently detected in the molecular analysis. Thus, 80 ml pots were filled with natural or sieved soil, from samples taken at each season. One sorghum plant per pot was grown under controlled greenhouse conditions (16/8 h day-night light, 24-18 ºC). Once sorghum plants were kept in contact with the soil containing AMF propagules for two weeks, they were harvested, their root thoroughly cleaned and the plants transferred to a new 80 ml pot filled with steamed soil to let AMF spread out inside roots. This will avoid the entrance of other AMF taxa which would not be represented by the colonization potential data. After a further eight-week-period, plants were harvested and their roots cuts in one cm pieces mixed and kept in 200 mg aliquots at -80ºC for the molecular characterization of their AMF symbiotic assemblages.

Plant life form and AMF community feedback experiment

The same soil used for studying the impact of climate seasonal variation on AMF populations was used to set up this experiment (Fig. 2.1). This study was carried out using 1 l pots filled with the natural soil. A pre-germinated seedling belonging to each one of the selected plant species was sown per pot. The chosen plant species were present at, or close to, the study area. Thus they share habitat with the AMF community naturally living in the target area so that, being both partners ecosystem-adapted, the target plant species can be naturally colonized by these AMF. Two families and three life forms were selected with the aim of having into account taxonomic diversity, legumes versus other families (Scheublin et al. 2004), and two PLF characteristics: growth form and life cycle (Table 2.1). Eight replicates per target plant species were established. Seeds were provided by Real Jardín Botánico (Madrid, CSIC) and Banco de Germoplasma Vegetal Andaluz (Córdoba). The seedlings were pre-germinated one week before each seasonal setting up of the experiment in sterilized wet filter paper in growth chamber following protocols described by Royal Botanic Gardens of Kew (www.kew.org). After a growth period of six months (controlled conditions 16/8 h day-night light, 24-18 ºC, watered three times per week until field capacity) the pots were harvested. The entire plants were removed and

Table 2.1 Selection of plant life form in terms of growth form, life cycle and plant family

Plant Life Form	Family	
	Leguminosae	Asteraceae
Herbaceous annual	*Trifolium angustifolium* L.	*Xeranthemum inapertum* (L.) Mill.
Herbaceous perennial	*Medicago sativa* L.	*Inula montana* L.
Semiwoody perennial	*Ononis natrix* L.	*Santolina canescens* Lag.

the soil was minutely mixed and further prepared to be analyzed as described in the bioassay test to assess the *impact of climate seasonal variation on AMF communities*.

Molecular analysis

T-RFLP approach was used to fingerprint AMF communites by analysing gene polymorphism in a 800 bp approximately section of small subunit (SSU) rDNA. For that purpose, DNA from 200 mg of roots per sample was extracted using a DNeasy plant mini-kit (Qiagen Inc., Mississauga, ON, Canada) and eluted in 75 µl ddH_2O. Partial ribosomal SSU DNA fragment was amplified using a nested PCR. First, an initial reaction with the universal eukaryotic primer NS1 and NS4 amplified a 1,000 bp aprox region. PCR were carried out using the illustra Pure-Taq Ready-To-Go PCR beads (GE Healthcare UK Limited, Buckinghamshire, UK) and 5 µM of each primer. As a template, 1 µl of extracted DNA previously was used in all reactions in a final volume of 25 ml. The program was: an initial denaturation at 94 ºC for 3min, followed by 30 cycles at 94 ºC for 30 s, 40 ºC for 1min, 72 ºC for 1min, followed by a final extension period at 72 ºC for 10 min.

The resulting amplicons were used to carry out a nested PCR. The primer set used was AML1-AML2 (Lee et al. 2008), a specific pair of primers for AMF DNA amplification. This primer set have been found to be very specific in previous studies of the research group (López-García et al. in preparation; Varela-Cervero et al. in preparataion) using the following conditions: one minute at 94ºC, 30 cycles at 94ºC for 1 min, 62ºC for 1 min, and 72ºC for 1 min, followed by a final extension period at 72ºC for 5 min. Since TRFLP profiles were analyzed in multiplex, two independent and analogous reactions were done for each sample, one with 6-FAM labelled AML1 primer and other with HEX labelled AML1 primer. One µl of the product of the universal PCR previously diluted 1/10 was used as

template. The PCR was carried out using the illustra Pure-Taq Ready-To-Go PCR beads (GE Healthcare UK Limited, Buckinghamshire, UK) and 5 μM of primer in the 6-FAM reactions and 20 μM of primer in the HEX reactions. This difference in the protocol was based in a differential amplification capacity of both reactions, using that conditions both obtained a similar final concentration of DNA. Afterwards, they were cleaned using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Freiburg, Germany).

The digestion of the PCR products were carried out using 1.5 units of MboI (for 6-FAM labelled products) and HinfI (for HEX) restriction enzymes. Both have been widely used in TRFLP analysis of AMF (Dickie & FitzJohn, 2007). The combination of digestion with MboI and HinfI were found to have a similar sizes variability in the 800 bp rDNA fragment (AML1-AML2) as found for typically used 550 bp fragment (NS31-AM1) (data not shown). Both digestion used 40 ng of PCR product and were mixed and cleaned together (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare Life Sciences, Freiburg, Germany). TRF size determination was carried out in the Unidad de Genómica y Síntesis de DNA, Instituto de Biomedicina y Parasitología López Neyra (Granada, Spain). Profiles were processed using GeneMapper software version 3.7 (Applied Biosystems).

Peaks were filtered by setting the minimal cut-off height under 100 fluorescence units. TRFs smaller than 50 base pairs (bp) and bigger than 800 bp were not considered. TRFs occurring in only one sample were considered artifacts of the T-RFLP procedure and were also excluded. Although a similar fluorescence level was found in each sample, peak areas were normalized as the percentage of the total fluorescence in each sample and those which contribute less than 1% to the total peak area were excluded. The diversity and composition of AMF community of each sample was estimated from the TRF abundance.

The use of peak area as a measure of relative abundance is a controversial issue, due to the bias caused by the PCR (Dickie & FitzJohn 2007). However, this measurement is widely accepted (e.g. Mummey et al. 2005; Mummey & Rillig 2008; Martínez-García & Pugnaire 2011) and empirical data suggest that peak heights are correlated with abundance of mycorrhizal fungi (Burke et al. 2006).

Data analysis

As a measure of AMF diversity, richness was calculated by counting the number of OTUs per sample. Simpson dominance index was calculated in the form of 1-D, this implies that a major index value means a major diversity. Both indices were calculated by combining peak areas detected in the TRFs determination of both enzymes.

Differences in colonization potential of the AMF in soil and calculated diversity indices for each treatment were tested by two-way ANOVA. In the case of lack of normality in the variables, arcsin transformation was applied to the selected variable. Tukey´s post-hoc test was used to analyze found differences.

Effects of the experimental variables on the AMF community composition were investigated by permutational multivariate analysis of variance (PERMANOVA, McArdle & Anderson 2001) using Bray-Curtis distance as a measure of community dissimilarity. To ensure that found effects were not a consequence of a different dispersion of the AMF communities between samples of each treatment, differences in beta dispersion were evaluated as a complement. The Bray-Curtis similarity matrix was used to perform a Non-metric Multidimensional Scaling ordination (NMDS) to graphically visualize community composition patterns. The same matrix was used to perform two-way analysis of similarity (ANOSIM) pairwise comparison to test for significant differences in AMF community composition associated with the different treatments (Clarke 1993). P values were subsequently ajusted applying Bonferroni correction. All functions used to develop these analyses belong to vegan package (R project, Oksanen et al. 2011). Data concerning each experiment: *Impact of climate seasonal variation on AMF communities and Plant life form and AMF communities feedback experiment*, were analyzed separately.

Results

Impact of climate seasonal variation on AMF communities

As expected for an area with Mediterranean climate, the contrasting conditions of different seasons caused significant differences in term of the colonization potential of AMF present in the soil ($F=7.391$, $P<0.001$) (Fig. 2.2). Sieving had also an effect on the colonization potential ($F=22.862$, $P<0.001$), as well as the interaction between sieving treatment and season ($F=5.041$, $P<0.01$) (Fig. 2.2). It seems that summer conditions, with drought and high temperatures, were responsible for the major reduction in the natural AMF colonization potential in the soil at the sampling time of September. In addition, the sieving treatment did not change significantly the AMF colonization potential at that date (Fig 2.2). From September onward, natural colonization potential increased to get a maximum in June (Fig 2.2), which is the most favorable season in Mediterranean climate, i.e. moderate temperatures and presence of spring rainfall (López-Bermúdez & Albadalejo 1990).

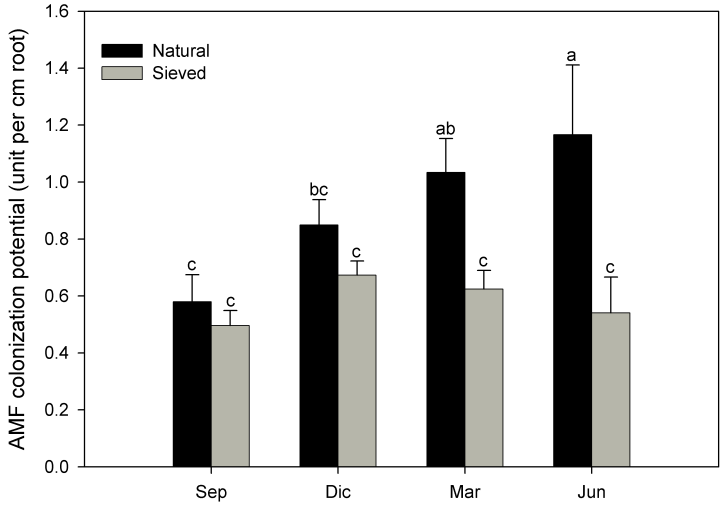

Fig. 2.2 Evolution of AMF colonization potential of natural and sieved soil through Mediterranean climatic seasons

Colonization potential after sieving kept constant throughout the year (Fig. 2.2). However its relation with the natural colonization potential implies that the major part of AMF propagules present in the soil in September survived after sieving and, from December onward, the natural colonization potential was progressively more affected by sieving. Thus, as a continuum, it seemed that a most resistant AMF community dominated the end of Mediterranean summer (September) in contrast to a much more sensible AMF community at the end of spring (June), with intermediate situations between both extremes (December and March).

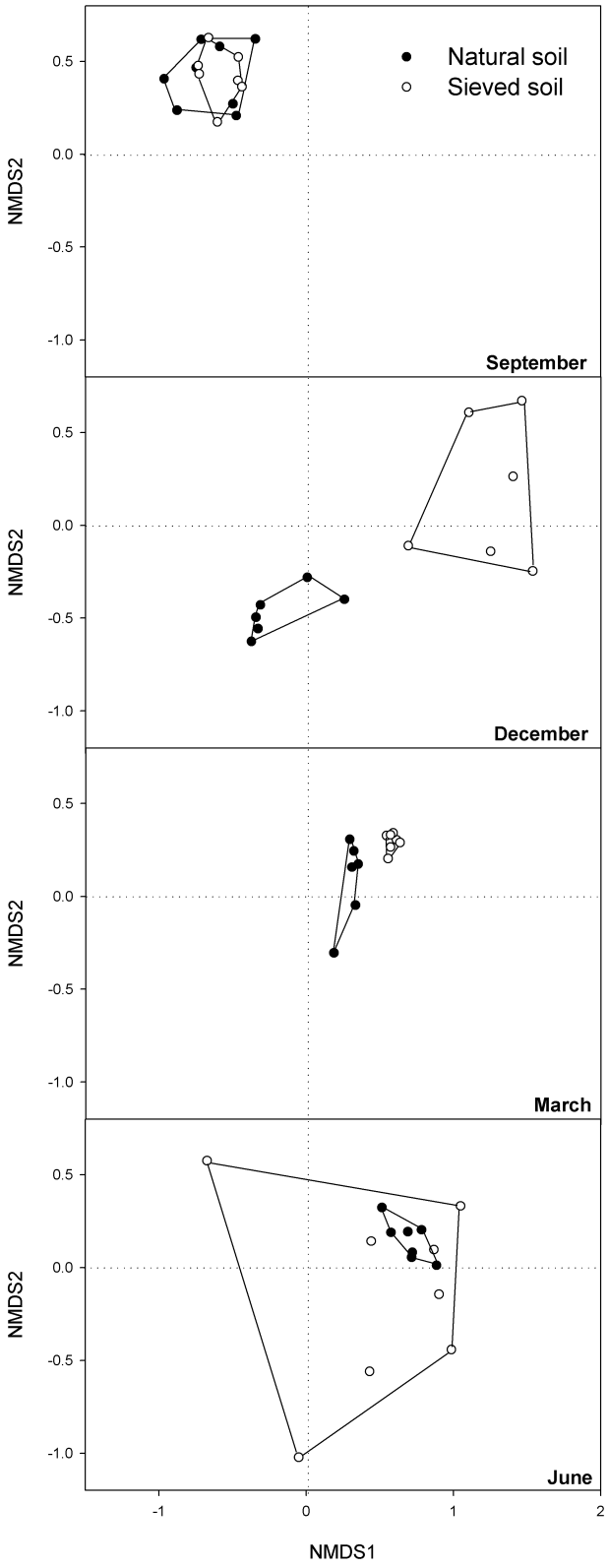

Fig. 2.3 Seasonal evolution of AMF community composition for natural and sieved soil, represented as Non-metric Multidimensional Scaling ordination (k=2, stress=0.196)

PERMANOVA showed a significant influence of season and the interaction season:sieving on AMF community composition (R^2=0.454, P<0.001 and R^2=0.125, P<0.001 respectively). ANOSIM pairwise comparison revealed differences in the AMF community composition between sieved and natural soil in December (R=0.5884, P<0.05) and March (R=0.5884, P<0.05), as appreciated in the NMDS representation (Fig 2.3). Since beta diversity analysis showed significant differences along treatments (F=8.1271, P<0.001), could bias the result of PERMANOVA. However, the post-hoc comparison revealed differences in beta diversity inside the same season exclusively in June sampling date (P<0.001).

Diversity indices, richness and Simpson dominance (1-D) in natural soil, tend to decrease from September to June (Fig 2.4) (Richness: F=0.096, P<0.001; Dominance: F=11.558, P<0.001). Sieving treatment also shown differences in their AMF diversity associated between seasons (Richness: F=18.692, P<0.001; Dominance: F=7.858, P<0.01), as well as the interaction season:sieving (Richness: F=7.148, P<0.001;

Dominance: F=7.124, P<0.001). As shown in Fig 2.4, the effect of sieving was patent in December when the diversity decreased after the treatment. Nonetheless, the evolution of the sieved soil AMF diversity showed an increasing pattern from December to get a maximum in September.

Plant life form and AMF community feedback experiment

PERMANOVA evidenced a clear influence of season on the AMF community composition (Table 2.2). Part of this variation was explained by the differences in the beta dispersion (F=8.8096, P<0.001) that from September showed asignificantly less dispersion than the rest of the sampling times (data not shown). None of the variables related to host characteristics (neither taxonomical family nor plant life-form) turned out to be determinant for the AMF community in general (Table 2.2). However, a significant interaction was found between season and PLF (Table 2.2). No differences in beta dispersion were patent throughout life-form treatments. ANOSIM pairwise comparisons showed no significant differences between AMF communities associated to PLFs except in March (R=0.2558, P<0.001). At this time, the resulting AMF community was different in perennial semi-woody plants and the two herbaceous (P<0.01 for the two comparisons). ANOSIM also showed that AMF communities linked to each PLF had different trends throughout the year. Thus, as shown in Fig. 2.5, semi-woody plants

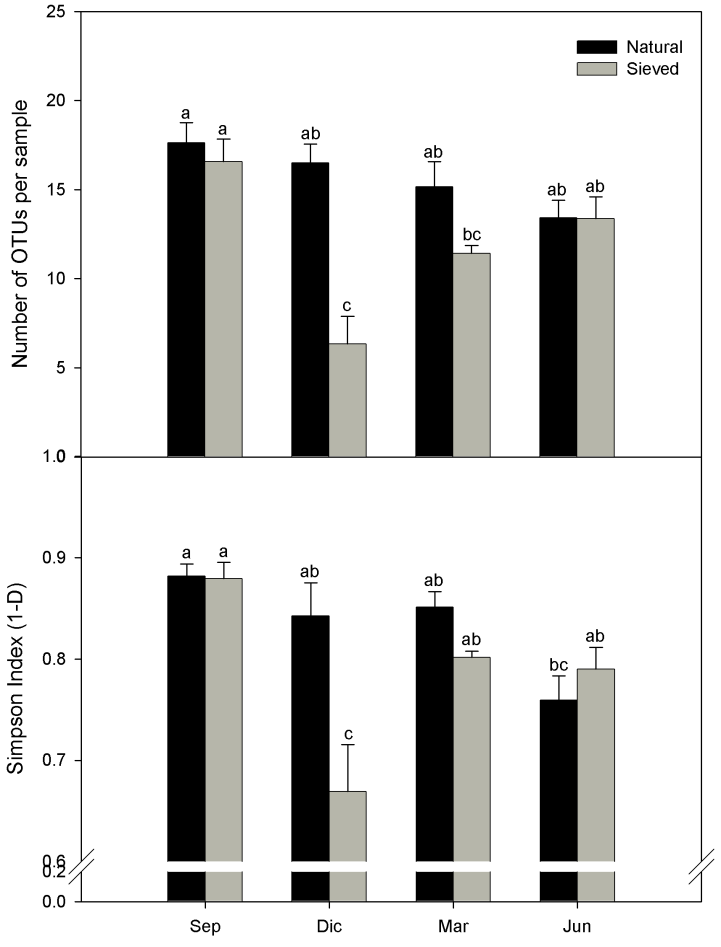

Fig. 2.4 Seasonal variation of diversity indices: richness of TRFs and Simpson dominance index (1-D) of AMF communities present in natural and sieved soil through seasons. Different letters indicates significant differences in the mean as found by ANOVA. Bars denote means ± S.E.

showed wider differences between seasons, having a particular AMF community composition at each time. Herbaceous annual and perennial had a less variable behavior, they had only two different communities (Fig. 2.5) being September markedly different from the other seasons.

The analysis of diversity indices did not show significant differences between PLF treatments as well as when compared with the *climate seasonal variation on AMF communities* data.

Table 2.2 Summary of results of permutational multivariate analysis of variance (PERMANOVA)

Source of variation	Df	MeanSq	F	P
Season	3	2.156	10.512	0.001***
Family	1	0.411	2.004	0.068
Family:season	3	0.288	1.404	0.135
PLF	2	0.330	1.607	0.073
PLF:season	6	0.325	1.586	0.021*

Discussion

Our results show that in a Mediterranean environment there was an annual evolution of AMF community in terms of both, species composition and functional characteristics. Our experimental approach demonstrated that different PLFs have the capacity to promote a specific AMF community on the basis of their functional characteristics but this capacity is biased by the climate seasonality since it has been demonstrated to drive the AMF functional characteristics. Thus, we provided evidence supporting that AM symbiosis assembly is dependent on both plant and fungal functional traits.

It was already shown that seasonality can alter soil microbial communities in semiarid climates (Cregger et al. 2012). A seasonal variation on AMF community composition have already been found (Oehl et al. 2009; Dumbrell et al. 2011; Sánchez-Castro et al. 2012b), and the effect of soil disturbance on AMF communities has been also reported, in terms of species richness, community composition or AMF phenology (Jansa et al. 2003; Violi et al. 2008; Oehl et al. 2010). However, the assumption that an alternation between favorable and more exigent climate seasons could cause similar effects over AMF in soil than a disturbance event has not been found previously. In the current study, mechanical

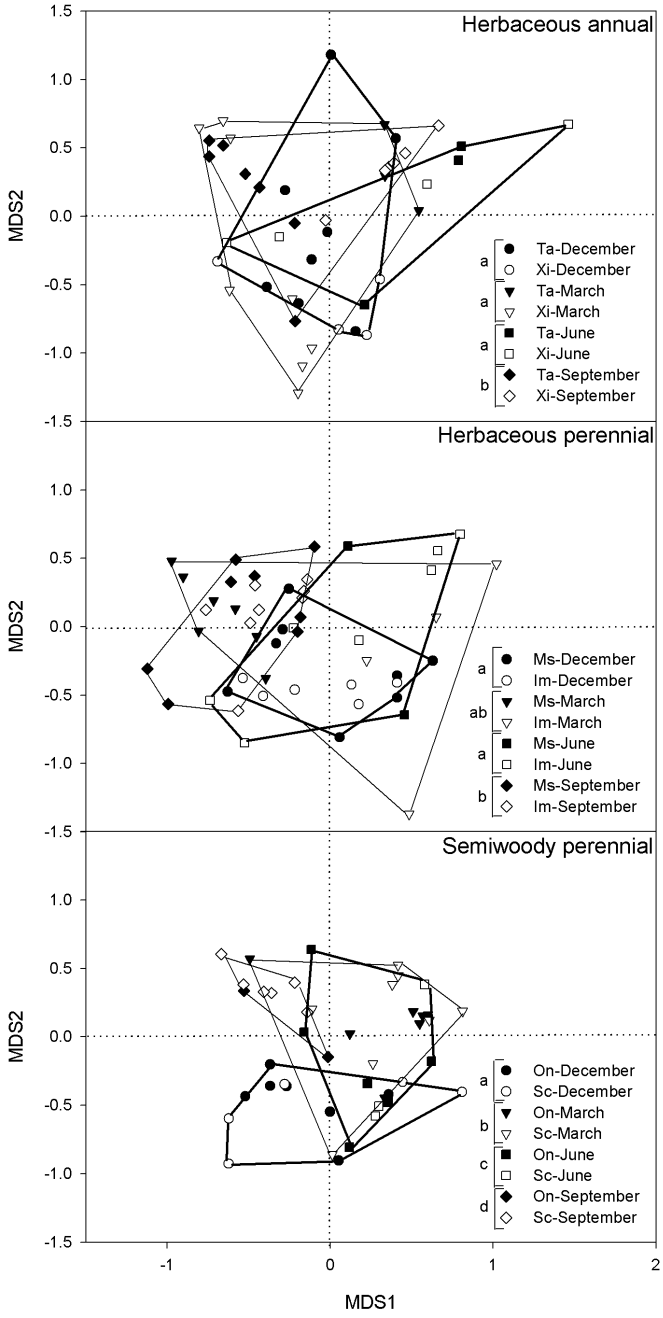

Fig. 2.5 Non-metric multidimensional scaling ordination (k=2, stress=0.196) of AMF communities promoted in different seasons by different plant species, grouped according to plant life form. Different letters indicate the existence of significant differences in the community composition as found by ANOSIM pairwise comparison. Ta, *Trifolium angustifolium*; Xi, *Xeranthemum inapertum*; Ms, *Medicago sativa*; Im, *Inula montana*; On, *Ononis natrix*; Sc, *Santolina canescens.*

disturbance of soil (sieving) demonstrated to reduce AMF colonization potential in a similar extent to dry seasons of Mediterranean climate does and, consequently, to be a valid tool to assess the AMF survival capacity. Thus, we found that summer biased the AMF community favoring the persistence in the soil of AMF with higher resistance ability. Meanwhile, most sensible AMF taxa were favored by the season combining moderate temperature with higher water availability -corresponding to June- (López-Bermúdez & Albadalejo 1990). Differences in terms of fungal characteristics were also supported by the here found differences in the species composition of AMF community in more favorable seasons as caused by sieving. The natural and sieved communities were clearly different on December and March, but in June the change consisted in a increasing of the variation of OTUs (β-diversity) in the sieving treatment. This trend was similar to that described by Schnoor et al. (2011) in

other scenarios. They found that, the variability of the AMF community composition in disturbed plots was higher than that found in natural soils. Thus, they proposed that this was caused by more spurious associations between AMF taxa and individual plants established after the disturbance event, which results in a higher influence of stochastic processes during early successional stages (Peet & Christensen 1980; Christensen & Peet 1984).

The annual evolution of the diversity of AMF was in accordance with the results of Hawkes et al. (2011), in their study of rainfall manipulation. They found more diverse communities under drought conditions and attributed this to a moderate competition due to the stress level. In our study, the highest richness level was located after the summer season, being progressively reduced towards spring. In addition, Hawkes et al. (2011) suggested that AMF hyphae in the soil could stop growing or contract in low soil water conditions. Similarly, the monitoring study of Allen & Kitajima (2013) for ectomycorrhizal fungi corroborated these facts in real-time conditions. Therefore both studies supported the diminished AMF colonization potential after summer drought. In addition, the increasing in the dominance in the structure of the AMF community on greater resource availability, was expected for the growing season (June), as found by Dumbrell et al. (2011) for another scenario. However, they attribute differences in AMF community composition to temperatures. Our results indicate that, in Mediterranean climate, the increase of summer drought was the most limiting factor influencing the AMF community, as happen with plant communities (Prentice et al. 1992; Miranda et al. 2011).

In summary, climate regime shifts the AMF community functional characteristics, which was, in turn, conditioned by a differential AMF community composition. Although we only supported information on survival capacity, as a functional trait, it has been demonstrated that AMF taxa differ considerably in other fungal characteristics as the colonization strategies (Hart & Reader 2002, 2005) or the functions that they provide to the host plants (López-Ráez et al. 2010; Sikes et al. 2010). Whether or not PLFs are related to AMF communities with different functional characteristics could be only tested using as inoculum an AMF community with a wide range of strategies. No consistent results were found when PLFs were inoculated with homogeneous AMF communities, i.e. dominated by resistant AMF in September or by sensible taxa in June, due to plants have no opportunities to choose more beneficial or adapted AMF partners. In contrast, the AMF community promoted by

different PLFs, at the March sampling time, were notably distinct in their OTUs composition.

The use of a high mycotrophic trap plant (*Sorghum vulgare*) responds to two different reasons. Firstly, using the same biological host has the advantage to homogenize the AMF community to make them comparable (Johnson et al. 2004). Secondly, the AMF community colonizing sorghum plants will reflect the fungal community present in the soil which has been promoted by the PLFs, in other words, those which has the potential to colonize other plants and affect stronger the ecosystem dynamics.

Some studies have found a differential AMF community for legumes with respect to other plant species (Scheublin et al. 2004; König et al. 2010). However, we only found changes in AMF communities that can be attributed to seasonality and PLF. This is an interesting and novelty result, if plant life form can have a higher influence in the AMF community determination than legumes versus non-legumes, future experiments on legumes should incorporate PLF variables to search for stronger and consistent ecological patterns.

Plant functional grouping has been demonstrated to determine ecosystem properties (McLaren & Turkington 2010). Its influence in the soil dynamics is clear, e.g. controlling the litter decomposition (Cornwell et al. 2008) or directly changing the soil carbon content (Gill & Burke 1999). Although they have been found to drive microbial communities in general (de Vries et al. 2012), its influence on AMF could have not been revealed in previous studies (Urcelay et al. 2009). In contrast, our results strongly support that PLFs have an important impact on the AMF community composition associated and, even more important, that their and their symbionts functional characteristics interact to determine the AMF symbiosis assembly. Once recognized that AMF are key ecosystem components and have a great role in the ecosystem properties determination (van der Heijden et al. 1998; Vogelsang et al. 2006), this finding adds new insights in the understanding on the consequence of changes observed in plant species diversity on AMF diversity and functioning (Bever et al. 2010).

From an eco-systemic point of view, the natural promotion of different AMF communities by PLFs on March can have important ecological consequences. This is the starting of growing season in Mediterranean climates (López-Bermúdez & Albadalejo, 1990), when the new seedlings of a major

part of plant species germinate. This means that AMF best adapted to each PLFs are present and plant species have the chance to be colonized by their preferred fungal symbionts from their initials. Thereby the adapted AMF taxa can benefit naturally both the stability and the coexistence of plant communities in natural conditions (Hart et al. 2003). Our results are in accordance with the findings of Schnoor et al. (2011) that *disturbance appeared to be a stronger structuring force than host preference* on AMF assemblages. Consequently, if a strong enough disturbance event occurs, AMF communities could be irreversible functionally biased and consequently have different effect on the PLFs associated with them. In this sense, we found that annual herbaceous plants promoted an AMF community more constant between seasons than semi-woody, suggesting the capacity of their preferred associated to persist in the soil throughout the year. This higher persistence ability was suggested for sporulating AMF (Schnoor et al. 2011). In contrast, Sikes et al. (2012) and Oehl et al. (2011), found a dominance of AMF developing a more extended hyphal network in the soil in advanced successional stages. This trait has been considered to be more sensitive to alteration and related with perennial plant species (Rosendahl & Stukenbrock 2004) as we have corroborated in the present study.

Interaction between plant life form and functional traits of arbuscular mycorrhizal fungal community as determinant of the resultant symbiotic assembly, as affected by the Mediterranean climate seasonal variation

Capítulo basado en:

A. López-García, J.M. Barea, C. Azcón-Aguilar. Interaction between plant life form and functional traits of arbuscular mycorrhizal fungal community as determinant of the resultant symbiotic assembly, as affected by the Mediterranean climate seasonal variation. Artículo en preparación

Chapter 3: The influence of environmental degradation processes on the arbuscular mycorrhizal fungal community associated with yew (*Taxus baccata* L.), an endangered tree species from Mediterranean ecosystems of Southeast Spain

Capítulo 3: Influencia de los procesos de degradación del hábitat sobre la comunidad de hongos micorrícicos arbusculares asociados al tejo (*Taxus baccata* L.), una especie vegetal amenazada en los ecosistemas Mediterráneos del sureste de España

Capítulo 3: Influencia de los procesos de degradación sobre la comunidad de hongos micorrícicos arbusculares asociados al tejo (*Taxus baccata* L.), una especie vegetal amenazada en los ecosistemas Mediterráneos del sureste de España

Resumen

Objetivos Investigar si las raíces de tejo, las cuales ofrecen un ambiente estable poco variable debido a su longevidad, pueden mantener la comunidad de hongos micorrícicos arbusculares (HMA) originalmente asociada a esta planta durante un evidente proceso de degradación ambiental en la zona de estudio.

Métodos La diversidad HMA que colonizan las raíces de tejo se analizó utilizando la secuencia de ADN de la subunidad pequeña del ARN ribosómico para construir una base de datos que representara la comunidad en general de HMA en el área de estudio. La técnica de TRFLP (terminal restriction fragment length polymorphism) se utilizó para identificar las comunidades de HMA en las raíces de tejo. Se cuantificaron variables fisiológicas y ambientales relacionadas con la topología y las características del suelo y la planta como marcadores del nivel de degradación.

Resultados Las comunidades de HMA en las raíces de tejo fueron dependientes de las variables relativas al suelo, la planta y la topología que indicaban cambios en el estado de degradación del hábitat de cada tejo. Se evidenció que la diversidad filogenética de los HMA asociados al tejo se reducía en los habitats más expuestos a la degradación, con respecto a los mejor conservados.

Conclusiones Los tejos en estudio se pudieron agrupar en dos niveles de degradación, lo cual fue base para el investigar el impacto de la degradación sobre la diversidad de los HMN. En efecto, la comunidad HMA se afectó negativamente por el proceso de degradación que sufrían sus hospedadores. Este resultado desecha el posible papel de estos árboles como refugio para sus poblaciones originales de HMA, lo que debe ser tenido en cuenta al diseñar biofertilizantes a base de HMA para los programas de reintroducción de especies amenazadas.

Chapter 3: The influence of environmental degradation processes on the arbuscular mycorrhizal fungal community associated with yew (*Taxus baccata* L.), an endangered tree species from Mediterranean ecosystems of Southeast Spain

Abstract

Aims To assess whether the yew roots, which are able to provide a very constant environment due to their long life-span, can maintain the original arbuscular mycorrhizal (AM) fungal community during yew population decline.

Methods The diversity of AM fungi (AMF) colonizing the roots of yew was analyzed by selecting the small subunit ribosomal RNA genes to construct a database of the overall community of AMF in the experimental area. A terminal restriction fragment length polymorphism (TRFLP) approach was used to identify the AMF communities present in yew roots. Physiological and environmental variables related to topology and soil and plant characteristics were determined as markers of habitat degradation.

Results The AMF communities within yew roots were found to be dependent on soil, plant and topological variables indicative of habitat degradation surrounding the yew. The phylogenetic diversity of AMF associated to the yews was lower in habitats more exposed to degradation than in those better conserved.

Conclusions The target yews can be grouped into two degradation levels. AMF communities were also affected by the degradation processes affecting their hosts. This finding rules out the role of these trees as refugia for their original AMF community, a fact that should be considered in plant reintroduction programs using AMF as bioenhancers.

Introduction

Mediterranean ecosystems are characterized by particular climate conditions which include a dry and hot summer and irregular, sometimes heavy, rain events concentrated in spring and autumn. In the Southeast of the Iberian Peninsula these conditions result in a semi-arid climate, causing ecophysiological constraints for a wide range of plant species. Such stress situations are exacerbated by the negative impact of anthropogenic activities which produce disturbances that fragment and decrease plant populations (Kéfi et al. 2007). The arbuscular mycorrhizal (AM) symbiosis is known to have an ecological and functional impact on plant performance in water-deficient and degraded habitats (Allen 2007), particularly in semi-arid ecosystems (Barea et al. 2011), and this impact must be considered in the restoration of these biomes.

AM fungi (AMF) associate with most (between 70-80%) vascular plants (Smith & Read 2008; Brundrett 2009), improve their growth and nutrition (Barea et al. 2005b) and promote ecosystem parameters such as plant diversity, productivity and soil aggregation (Grime et al. 1987; van der Heijden et al. 1998, 2006; Rillig & Mummey 2006; Barea et al. 2011). AMF can colonize individual roots as assemblages consisting of several AMF species and some host preference has been shown (Öpik et al. 2009). Since AMF can increase the success of establishment and survival of seedlings in the field and improve soil quality (Requena et al. 2001), they have been proposed as bio-enhancers in the restoration of populations of endangered plants (Barea et al. 2011). The reliance of vegetation stability with stability of AMF has been highlighted by several studies (Jeffries & Barea 2012) revealing the need to restore the original AMF populations as well as plant cover in order to regenerate degraded habitats.

Yew (*Taxus baccata* L.) is a plant species which typically experiences the stress situations characteristic of Mediterranean ecosystems in South-East Spain. It is widely distributed in northern and central European countries, but where the Mediterranean climate represents its southern limit, such as in Southern Iberia, it has been relegated to certain mountain areas (Thomas & Polwart 2003). This is due to the pressure of human practices (deforestation, overgrazing, increases in wildfire events, etc.) which usually have a higher impact on plants near to the boundaries of their distribution area (Pons

1981). These practices have caused yew populations to be reduced and fragmented (García et al. 2000). Since the natural regeneration of yews is limited (García et al. 2000), pro-active restoration of populations of yew is necessary to ensure their viability in Southern Spain. As this plant species has been demonstrated to form an AM symbiosis (Harley and Harley 1987), the impact of AMF on the restoration of yew populations needs consideration (Barea et al. 2011). However, information on the diversity of root-colonizing AMF associated with yew, and on potential changes in this community under anthropogenically-altered situations, is poorly understood.

Interest in the structure of AMF communities has increased recently and molecular tools are now available for a challenging dissection of AMF population dynamics (Robinson-Boyer et al. 2009) or for analyzing the distribution of AMF in ecosystems (Öpik et al. 2009). Since this group of organisms is cryptic, i.e. do not form taxonomically informative structures in roots, molecular methodologies are needed to study their presence inside plant roots. Terminal restriction fragment length polymorphism (TRFLP) of genes coding for ribosomal RNA has been widely used to assess AMF diversity (Dickie & FitzJohn 2007; Mummey & Rillig 2008; Antunes et al. 2009). This method was applied to identify the AMF community associated with individual yew trees since it provides a high throughput and reproducible profiling of samples containing complex microbial communities.

The aim of this study was to test whether or not the AMF community associated with yew trees was conserved on isolated individuals during yew population decline. Accordingly, two alternative hypotheses can be formulated: (a) the diversity of AMF is maintained in yew roots growing in degraded habitats as in those growing in better preserved conditions. In that case, it could be expected that these trees would act as refugia for a continuous and homogeneous assemblage of AMF even during their population decline. (b) Factors which drove yew populations to decline may have also altered AMF community composition, as shown by differences in the AMF communities between plants growing under different degraded conditions. In ascertaining which of these alternative hypotheses is true, different issues need to be tested: i) the existence of differences in the degradation levels of the habitats of each individual yew, as expressed by changes in soil properties, the physiological status of each tree and the composition of the surrounding vegetation and ii) the variation in the AMF community according to the conservation status of the habitat in which each individual plant lives.

The results of this study will help to the appropriate management of AMF in the restoration programs of yew in an area of particular ecological interest, the Sierra de Baza Natural Park (Granada province, Spain).

Material and methods

Study site and sampling procedure

The Sierra de Baza Natural Park has been historically managed by humans. Its use to feed cattle and the deforestation for the local use of wood were the main sources of degradation during last centuries. Since 1950, these mountains have been reforested for timber production. However the conformation of too dense areas of pines did not allow a good production of wood and their exploitation was abandoned. These wide mono-specific woodlands of pines replaced indigenous vegetation and increased the fire risk causing some fire events in recent years.

Under these circumstances, the population of *Taxus baccata* L. has been affected similarly as the rest of the vegetation of Sierra de Baza. However, the slow regeneration rate of yew trees makes them more vulnerable to the degradation of their natural habitats. Nowadays only more or less isolated trees persist in the highest area of the mountains in contrast with a more wide and homogeneous distribution in the past.

The studied population is located at altitudes between 1800-2000 m a.s.l., where the mean precipitation is about 600 mm per annum (Blanca & Morales 1991). The bedrock is calcareous forming litosols, regosols and cambisols. The population is situated within an area of 4 km^2 (Fig. 3.1) with 23 trees, 13 of which were sampled. On July 2010, two subsamples of 5 g fine roots, at the north and south side of each tree, carefully tracing roots from the stem, were collected and immediately put on ice.

Two subsamples of soil (500 g) and plant material (branches with leafs) were also collected for soil nutrient determination and Specific Leaf Area (SLA) measurements.

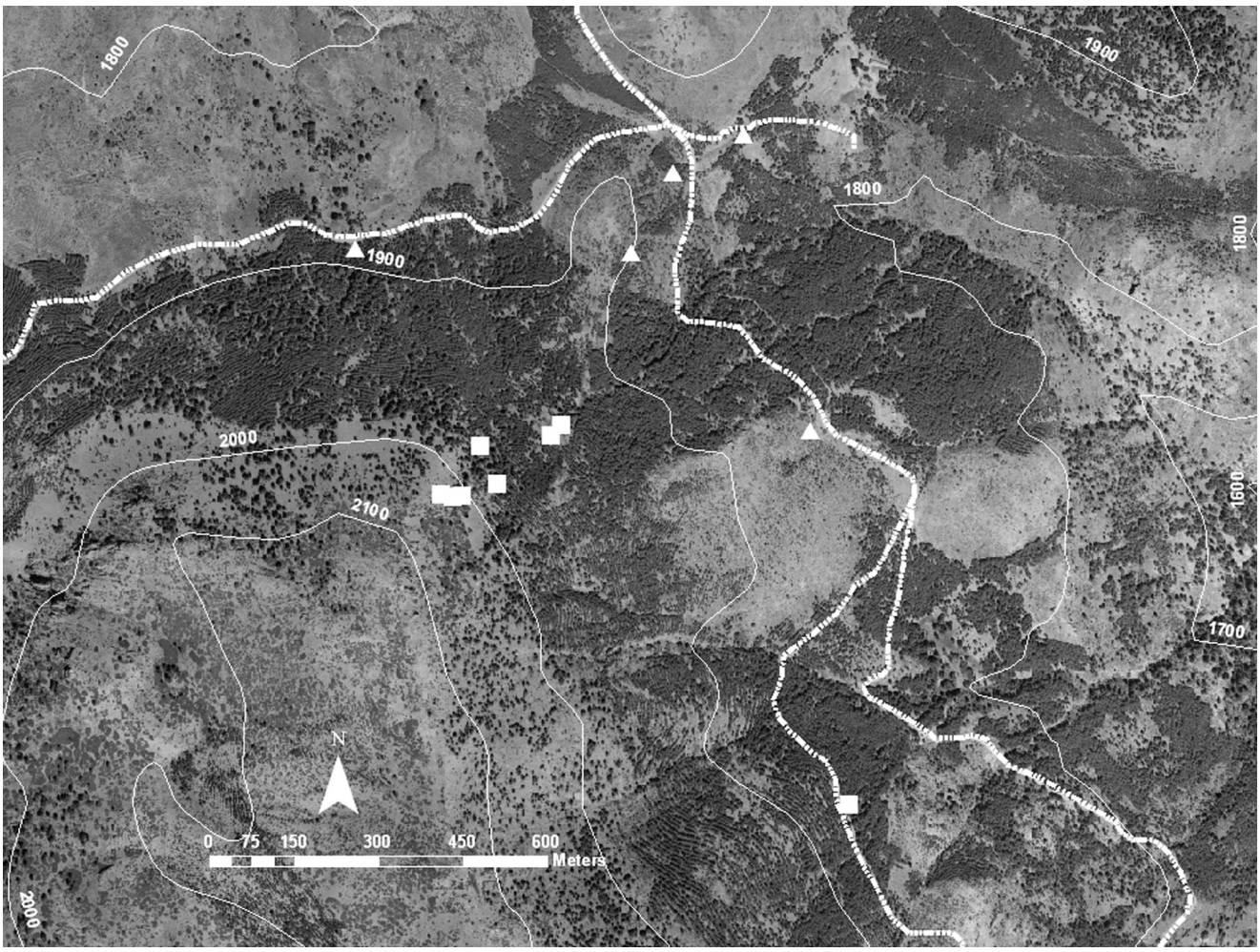

Fig. 3.1 Spatial distribution of the sampled yews in the Sierra de Baza Natural Park. Triangles represent yews belonging to

Vegetation analysis, plant physiological status and mycorrhizal colonization

The presence and cover of plant species in a 5 m radius surrounding the trees were determined. To assess the level of ecosystem degradation, the coverage of taxa indicating an alteration of the natural habitat of yew as well as taxa belonging to a well-conserved plant community growing with yew were determined following the description of habitat requirements by Tutin et al. (1980) and the description of the natural vegetation community of yew in García et al. (2000) (Table 3.1). Shannon index (H') and richness (S) were calculated to be used in subsequent analyses.

Table 3.1 Plant species indicating altered and well-conserved habitats. Habitat indicates the typical one in which every plant species appears

Plant species of Altered Habitats	Habitat
Artemisia campestris L. subsp. *glutinosa* (DC.) Batt.	Nitrophilous
Bromus tectorum L.	Ruderal
Carduncellus monspelliensium All.	Nitrophilous
Carduus platypus Lange subsp. *granatensis* (Willk.) Nyman	Ruderal
Carlina hispanica Lam.	Nitrophilous
Cirsium sp.	Ruderal
Galium verum L. subsp. *verum*	Ruderal
Mantisalca salmantica (L.) Briq. & Cavill.	Ruderal
Marrubium supinum L.	Ruderal
Medicago sativa L.	Ruderal
Mentha longifolia (L.) Huds.	Nitrophilous
Ononis pusilla L.	Ruderal
Plantago lanceolata L.	Ruderal
Sanguisorba minor Scop.	Pasture
Santolina rosmarinifolia L.	Nitrophilous
Torilis arvensis (Huds.) Link	Ruderal
Trachynia distachya (L.) Link	Pasture
Plant species of Well Conserved Habitats for yews	
Acer opalus Mill. subsp. *granatense* (Boiss.) Font Quer & Rothm.	
Juniperus communis L.	
Juniperus sabina L.	
Ononis aragonensis Asso	
Pinus sylvestris L.	
Pinus nigra Arnold subsp. *salzmannii* (Dunal) Franco	

Specific leaf area (SLA) is a good indicator of potential relative growth rate, as individuals growing in resource-rich environments tend to have larger SLA than those in environments with limited resources (Cornelissen et al. 2003). Two distal branches per individual from the same aspect of each yew plant were collected and kept in a cool box. At the laboratory, ten leaves were removed, scanned with a portable scanner (Canoscan N656U, Canon) at 300 dpi, and the projected area measured (Midebmp v.4.2, Ordiales-Plaza 2000) to calculate leaf area. Due to their small size, leaves of each plant were measured together, first scanned and then weighed after drying at 72ºC for 48 h. Specific leaf area (SLA, $m^2\ kg^{-1}$) was computed as the ratio between leaf area and mass. Average SLA was calculated for each yew.

Roots (2 g) from each subsample were cut in 1cm pieces, mixed and stained (Phillips & Hayman 1970). The percentage of mycorrhizal colonization was determined by using the magnified intersection method (McGonigle et al. 1990) under a compound light microscope.

Soil and topological data

A subsample of 10 g of the collected soil was sieved to 2 mm and milled. Total soil nutrients (Table 3.2) were determined by the Servicio de Ionómica of Centro de Edafología y Biología Aplicada del Segura (CSIC) by ICP-OES (ICAP 6500 Duo/Iris Entrepid II XDL). An elemental analyzer C/N (Flash EA 1112 Series-Leco Truspec) was used to measure total and organic C and total N. Available P was also measured (Olsen et al. 1954). Soil pH was determined in a 1:2.5 (w/v) suspension of sieved soil in dH_2O.

A Digital Elevation Model was provided by the Centro Nacional de Información Geográfica (2004) and processed using ArcGIS 9.2 (ESRI, 2006) to obtain topological data: latitude, longitude, altitude, slope, orientation and distances to the nearest yew and to a 3.5 m wide sandy forest road through the natural park.

Molecular methods

The remaining roots were frozen (-80ºC) in aliquots of 200 mg. DNA from 200 mg of roots per subsample was extracted using a DNeasy plant mini-kit (Qiagen Inc., Mississauga, ON, Canada) and eluted in 50 µl ddH_2O.

The database TRFLP approach (see Dickie & FitzJohn 2007) was used to characterize the AMF community of yew. To construct a TRFLP database, sequences derived from AMF colonizing yew roots were produced. Partial ribosomal SSU DNA fragments were amplified using the primer set NS31 (Simon et al. 1992), a universal eukaryotic primer, and AML2 (Lee et al. 2008), a specific primer for AMF DNA amplification. Polymerase Chain Reactions (PCR) were carried out in a final volume of 25 µl using the Illustra Pure-Taq Ready-To-Go PCR beads (GE Healthcare UK Limited, Buckinghamshire,

Table 3.2 List of variables included in the three independent Principal Components Analyses

Plant data	Soil data	Topological data
Coverage (%) of every plant taxa 5m surrounding yews	Nutrient content: Ca, Mag, Zn, Pb, S, B, P, Na, Fe, K, Cu, Ni, Al, Cr, Mn, Cd	Distance to nearest yew
Total coverage (%) of plant taxa indicating altered habitats	Total C (%), organic C (%), total N (%), C/N	Distance to trackway
Total coverage (%) of plant taxa indicatind conserved yew´s habitat	Available P	Altitude
Shannon diversity index (H')	pH	Orientation
Richness (S)		Slope
Specific Leaf Area (SLA)		UTM West
		UTM North

UK) and 20 µM of each primer. PCR conditions were: an initial minute at 94ºC, 30 cycles at 94ºC for 30s, 52ºC for 30s, and 72ºC for 40s, followed by a final extension period at 72ºC for 5 min. As a template, 1 µl of extracted DNA was used in all reactions.

A equimolecular mix of all PCR products was purified using QIAquick Gel Extraction Kit (QIAGEN Iberia, Madrid, Spain) and cloned into the pCR®2.1 vector following the protocol recommended by the manufacturer of the TA Cloning® Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into One Shot® TOP10F' Chemically Competent *Escherichia coli* cells (Invitrogen Life Technologies). Ninety six clones were sequenced. The sequencing was done by the sequencing service of the Estación Experimental del Zaidín (Granada, Spain) using the set of vector binding primers M13F-M13R. Rarefaction analysis of the sequences was done using the Analytic Rarefaction 1.3 software (Holland 2008).

To obtain a good coverage of the AMF diversity present in the Sierra de Baza Natural Park and to complete the sequence database produced by cloning AMF sequences from the yew roots, additional sequences of AMF derived from roots, soil and spore samples from the Sierra de Baza Natural Park were added to the database (Sánchez-Castro et al. 2012a, b; Palenzuela et al. unpublished).

Sequences were aligned with MAFFT version 6 and similarities excluding the primer sequence were determined using BioEdit software. Phylogenetic analysis was carried out using the sequences obtained in the study and in the previous database of the Sierra de Baza Natural Park as well as a representative group of sequences of all major Glomeromycota groups from GenBank. The outgroup used was *Morteriella polycephala* X89436. The phylogenetic analysis was computed in MEGA4 (Tamura et al. 2007) using the neighbour-joining algorithm with Kimura-2 parameters as the model of substitution and 1000 replications to obtain bootstrap values.

Phylotypes were determined using a minimum of 97% sequence similarity and a high bootstrap value as indicators (Öpik et al. 2010). A blast search in the Maarj*AM* (Öpik et al. 2010) database was used to name the sequences with the number code of the closest virtual taxon when showing similarities higher than 97%.

REPK online software (Collins & Rocap 2007) was used to select a set of four enzymes which allowed discrimination between the phylotypes found in the database. These enzymes were: MboI, VspI, TaqI and Bme1390I (New England Biolabs, Ipswich, USA). Sequences were subjected to in-silico T-RFLP analysis using TRiFLe (Junier et al. 2008).

PCR reactions were redone as described above with the exception of a fluorescent label (6-FAM) attached to the forward primer NS31. After purification of the PCR products using NucleoSpin Extract II (Macherey-Nagel GmbH, Düren, Germany), 80 ng of each amplified product were individually digested for 2 hours at 37ºC with 1.5, 1.5, 3 and 6 units of MboI, Bme1390I, VspI and TaqI, respectively. Fragment lengths were determined at the SMB Service GmbH (Berlin, Germany) using a customized ROX size standard as marker size on a ABI 3100 capillary sequencer (Applied Biosystems Carlsbad, CA, USA). Data were processed using GeneMapper software version 3.7 (Applied Biosystems 2004) excluding fragments shorter than 50 bp and longer than 600 bp. The amplitude threshold was 20 RFU and the calling method “Local Southern Method”. The R (R Development Core Team, 2010) package, TRAMPR (FitzJohn & Dickie 2007), was used with three nucleotides mismatch as threshold level for matching TRFLP profiles with the T-RFLP fragment sizes obtained during the in-silico analysis of the sequences of the constructed database.

Since the data obtained from the TRFLP database approach consisted of a presence/absence matrix, only richness (S) was calculated. In order to assess the differences in phylotype composition in the two groups of yews identified in the study, the frequency of detection was calculated as the percentage of trees containing certain phylotypes. Species accumulation curves were also calculated by group to ensure that a similar coverage was obtained in the TRFLP approach.

Statistical analyses

In order to reduce the dimensionality of predictor variables, one PCA (Principal Component Analysis) per group of variables (plant, soil and topological related variables, see Table 3.2) was carried out. Since they represent the major part of variation, the first two axes of each PCA were selected to be used in the subsequent analyses. To ensure that collinearity between PCA axes of each variable set did not confuse the results, Pearson correlations were used to evaluate this possibility.

A cluster based on Euclidean distance was used to set up classes in terms of environmental conditions affecting the yews. Since the separation by this method was not totally clear, k-means was used to check the possibility of having two or three groups and to define them. Both clustering processes included the six PCA axes and were carried out using PAST 2.08 (Hammer et al. 2001). Mean values of the most relevant variables were also calculated to characterize the groups of yews found in the clustering and a t-test was carried out to check for significant differences between groups.

Effects of the six environmental axes on the AMF community were investigated by permutational multivariate analysis of variance (permanova, McArdle & Anderson 2001) embedded in the function *adonis* from vegan package in R (Oksanen et al. 2011), using Jaccard distance as a measure of community dissimilarity. This methodology was also applied to study differences in AMF community between both groups of yews.

In order to analyze the partial influence of each type of variable on the AMF community, a variance partitioning analysis (*varpart* function from vegan package) followed by a redundancy analysis (RDA) test for significance was used. Again, we only applied the first two axes of each explanatory PCA.

Results

Conservation status of the yew environments

Two groups of yews were defined by euclidean and k-means clustering (Fig. 3.1). They showed different levels of habitat degradation based on vegetation, soil characteristics and topological data (Table 3.3). Group 1 (six individuals) showed a greater degree of alteration with respect to group 2 (seven individuals), according to the accompanying vegetation (Table 3.3). Although no differences were found in the total coverage of well-conserved habitat indicators, the presence of *Ononis aragonensis*, a well-conserved habitat indicator, is significantly higher in the group of yews living in better conserved habitat. On the other side, *Ononis pusilla* and *Galium verum*, indicators of altered conditions, were more frequent in the group of yews with altered habitat. While no significant differences were found in the rest of indicator plant species, *Santolina rosmarinifolia* and *Plantago lanceolata*, altered conditions indicators, were absent surrounding yew plants growing in better conserved habitats.

Specific Leaf Area was found to be significantly higher in the group of yews from the better conserved habitat. Among soil data, pH and available P were found to be very similar for both groups but Ca, Mg, total N and organic C contents were higher in the soils corresponding to the better conserved habitats, however only significant differences were found for Ca (Table 3.3).

The distance between yews was almost ten times higher in the group developing in altered habitats (group 1). In contrast, the distance to the road track and the altitude were higher in the group of yews growing in better conserved habitats (group 2). Longitude had a similar pattern revealing the distribution of yews along a slope (Table 3.3).

Characterization of AMF community composition and influential environmental variables

A total of 96 sequenced clones obtained from the pooled PCR products were analyzed and eight different phylotypes were found. Of these 96, 33 corresponded to non-specific amplifications of plant

Table 3.3 Mean values for measured variables of altered (Group 1) and well-conserved habitat (Group 2) groups of yews

	Variable	Group of yew		*t*	*P*
		Altered (Group 1)	Conserved (Group 2)		
PCA Plant related variables	Coverage plants altered (%)	6.9 (2.5)	0.9 (0.5)	2.531	0.028*
	Coverage plants conserved (%)	22.9 (8.7)	47.7 (9.5)	−1.902	0.084
	SLA (m^2kg^{-1})	5.2 (0.3)	7.0 (0.5)	−3.271	0.007**
	Ononis aragonensis (%)	–	1.5 (0.5)	−3.154	0.020*
	Pimpinella tragium (%)	–	0.2 (0.1)	−2.121	0.078
	Galium verum (%)	0.3 (0.1)	–	2.434	0.033*
	Santolina rosmarinifolia (%)	0.5 (0.4)	–	1.225	0.275
	Lonicera splendida (%)	0.2 (0.1)	–	1.581	0.175
	Plantago lanceolata (%)	0.2 (0.1)	–	1.581	0.175
	Rhamnus saxatile (%)	5.0 (3.2)	–	1.581	0.175
	Koeleria vallesiana (%)	1.3 (0.5)	0.1 (0.1)	2.373	0.062
	Ononis pusilla (%)	0.3 (0.1)	–	2.434	0.033*
PCA Soil components	pH	7.4 (0.1)	7.4 (0.1)	−0.287	0.779
	Ca (%)	2.7 (0.8)	7.9 (2.0)	−2.200	0.050*
	Mg (%)	1.7 (0.6)	3.3 (0.8)	−1.572	0.144
	Nt (%)	0.6 (0.1)	0.9 (0.1)	−2.135	0.064
	C organic (%)	9.2 (1.4)	14.8 (2.3)	−2.083	0.065
	Available P (ppm)	6.1 (0.7)	5.4 (0.3)	1.103	0.333
PCA Topological variables	Dist. nearest yew (m)	324.5 (89.7)	35.7 (8.8)	3.205	0.023*
	Dist. road track (m)	25.6 (10.3)	407.3 (30.5)	−11.868	0.000***
	Altitude (m)	1858.0 (12.7)	1926.0 (7.3)	−4.814	0.001***
	Longitude (UTM)	2833476.7 (1393.7)	6892.9 (330.4)	−2.569	0.026*
Mycorrhizal variables	Extent of colonization (%)	20.2 (3.9)	19.5 (2.9)	0.155	0.880
	Richness of phylotypes (S)		6.6 (0.9)	−0.279	0.785
	No. glomeromycotan families		4.4 (0.4)	−2.459	0.032*

Mycorrhizal parameters and only highly correlated variables (correlation value ≥0.5) belonging to PCA axes with significant effects on AM fungal community are considered. Standard errors are shown in brackets. Student'st-test has been used for mean comparisons. Signif. codes: '*' p<0.05; '**' p<0.01; '***' p<0.001

and saprotrophic fungi, and 63 belonged to glomeromycotan fungi. Through a rarefaction analysis, 12 phylotypes were predicted as the total in the mix of all PCR products, with 8 phylotypes representing 65.6% (Fig. 3.2). The analysis also implied that it would be necessary to sequence 100 additional clones to increase the number of phylotypes by one and an overall of ~1500 clones to cover the

expected diversity.

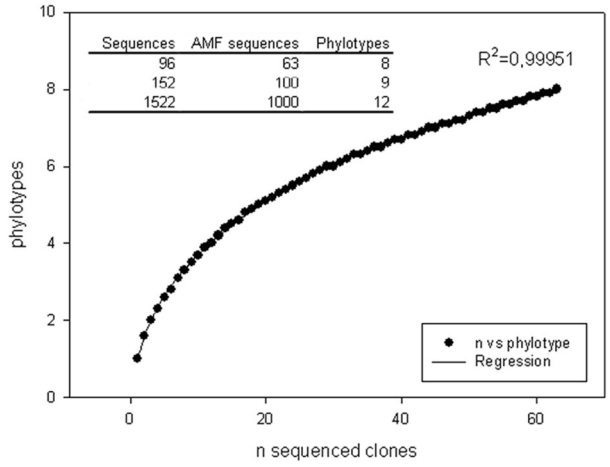

Fig. 3.2 Rarefaction analysis performed on the 18S sequence data of AMF obtained from the clone library of yew roots. A 97 % sequence similarity threshold value was used to define phylotypes

The eight phylotypes found in the clone library were added to another 16 phylotypes from previous studies of Sierra de Baza Natural Park (Sánchez-Castro et al. 2012a, b; Palenzuela et al. unpublished). Thus, a total of 24 phylotypes composed the final TRFLP database. The TRFLP analysis found 17 of these phylotypes in the studied samples, belonging to seven glomeromycotan families: Acaulosporaceae (2 phylotypes), Claroideoglomeraceae (1), Diversisporaceae (1), Gigasporaceae (1), Glomeraceae (9), Pacisporaceae (1) and Paraglomeraceae (2) (Fig. 3.3).

Species accumulation curves based on the phylotypes presented by both groups of yews showed a similar coverage of the total predicted diversity (74.4% for group 1 and 76.9% for group 2) (Fig. 3.4).

The AMF community associated with yew in Sierra de Baza was significantly influenced by plant-related variables (axis 1, permanova: F=0.23, p=0.0149), topological variables (axis 1, F=0.12, p=0.0499) and soil characteristics (axis 2, F=0.17, p=0.033). Pearson correlations between the first two PCA axes of each variable set and the rest of PCA axes were not important (less than 0.6 or higher correlation with a PCA axis with a low percentage -less than 5%- of explained variance). This avoids the possibility of misinterpreting the results due to the influence of PCA axes not included in the analysis.

Analysis of variance partition revealed a higher influence of plant related variables (R^2=0.34, F=3.08, p=0.005), followed by topological ones (R^2=0.16, F=1.96, p=0.025) and soil characteristics (R^2=0.14, F=1.85, p=0.05), with a total of 64 % explained variance in the whole analysis. Mean values

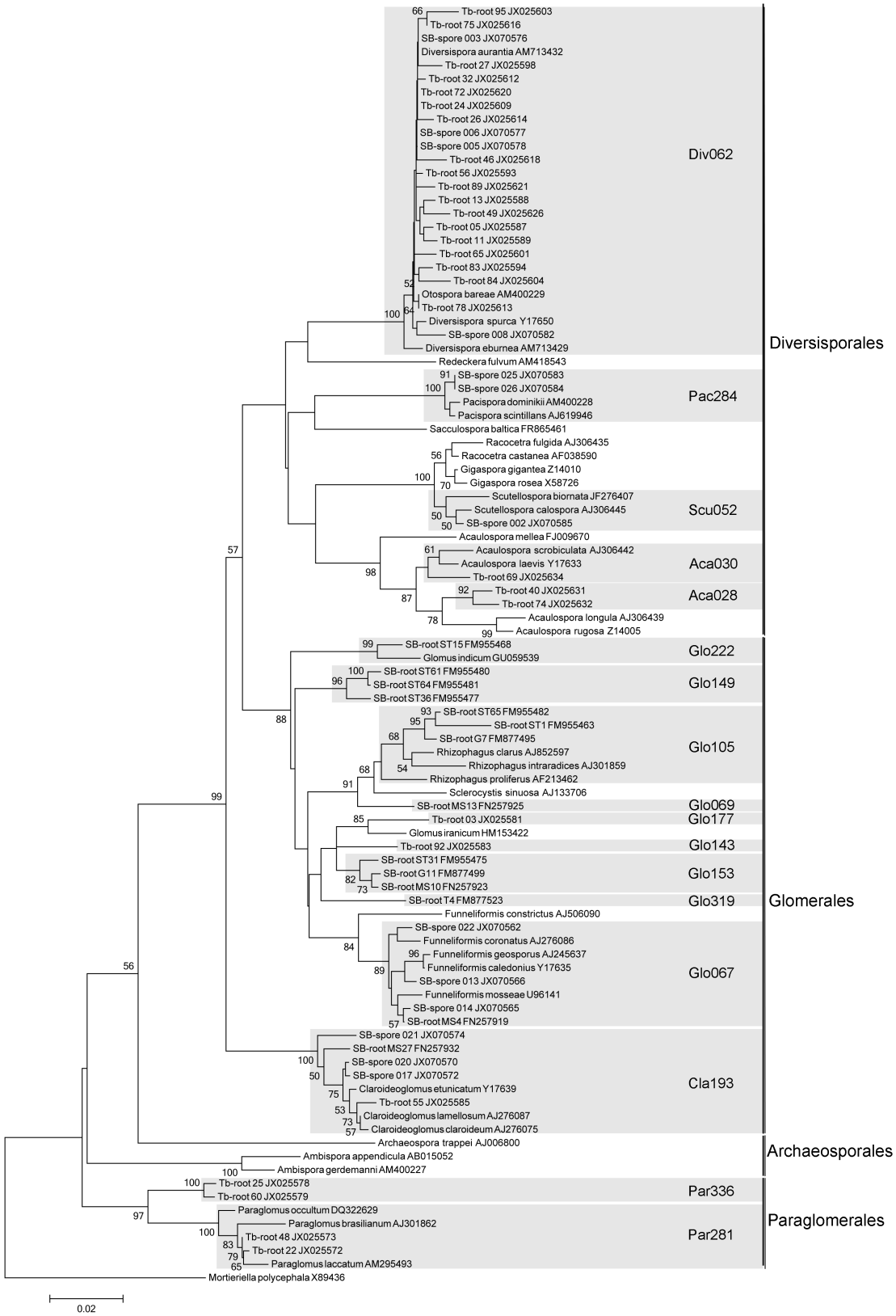
Tb-root 95 JX025603
Tb-root 75 JX025616
SB-spore 003 JX070576
Diversispora aurantia AM713432
Tb-root 27 JX025598
Tb-root 32 JX025612
Tb-root 72 JX025620
Tb-root 24 JX025609
Tb-root 26 JX025614
SB-spore 006 JX070577
SB-spore 005 JX070578
Tb-root 46 JX025618
Tb-root 56 JX025593
Tb-root 89 JX025621
Tb-root 13 JX025588
Tb-root 49 JX025626
Tb-root 05 JX025587
Tb-root 11 JX025589
Tb-root 65 JX025601
Tb-root 83 JX025594
Tb-root 84 JX025604
Otospora bareae AM400229
Tb-root 78 JX025613
Diversispora spurca Y17650
SB-spore 008 JX070582
Diversispora eburnea AM713429
Div062
Redeckera fulvum AM418543
SB-spore 025 JX070583
SB-spore 026 JX070584
Pacispora dominikii AM400228
Pacispora scintillans AJ619946
Pac284
Sacculospora baltica FR865461
Racocetra fulgida AJ306435
Racocetra castanea AF038590
Gigaspora gigantea Z14010
Gigaspora rosea X58726
Scutellospora biornata JF276407
Scutellospora calospora AJ306445
SB-spore 002 JX070585
Scu052
Acaulospora mellea FJ009670
Acaulospora scrobiculata AJ306442
Acaulospora laevis Y17633
Tb-root 69 JX025634
Aca030
Tb-root 40 JX025631
Tb-root 74 JX025632
Aca028
Acaulospora longula AJ306439
Acaulospora rugosa Z14005
Diversisporales
SB-root ST15 FM955468
Glomus indicum GU059539
Glo222
SB-root ST61 FM955480
SB-root ST64 FM955481
SB-root ST36 FM955477
Glo149
SB-root ST65 FM955482
SB-root ST1 FM955463
SB-root G7 FM877495
Rhizophagus clarus AJ852597
Rhizophagus intraradices AJ301859
Rhizophagus proliferus AF213462
Glo105
Sclerocystis sinuosa AJ133706
SB-root MS13 FN257925
Glo069
Tb-root 03 JX025581
Glo177
Glomus iranicum HM153422
Tb-root 92 JX025583
Glo143
SB-root ST31 FM955475
SB-root G11 FM877499
SB-root MS10 FN257923
Glo153
SB-root T4 FM877523
Glo319
Funneliformis constrictus AJ506090
SB-spore 022 JX070562
Funneliformis coronatus AJ276086
Funneliformis geosporus AJ245637
Funneliformis caledonius Y17635
SB-spore 013 JX070566
Funneliformis mosseae U96141
SB-spore 014 JX070565
SB-root MS4 FN257919
Glo067
SB-spore 021 JX070574
SB-root MS27 FN257932
SB-spore 020 JX070570
SB-spore 017 JX070572
Claroideoglomus etunicatum Y17639
Tb-root 55 JX025585
Claroideoglomus lamellosum AJ276087
Claroideoglomus claroideum AJ276075
Cla193
Glomerales
Archaeospora trappei AJ006800
Ambispora appendicula AB015052
Ambispora gerdemanni AM400227
Archaeosporales
Tb-root 25 JX025578
Tb-root 60 JX025579
Par336
Paraglomus occultum DQ322629
Paraglomus brasilianum AJ301862
Tb-root 48 JX025573
Tb-root 22 JX025572
Paraglomus laccatum AM295493
Par281
Paraglomerales
Mortierella polycephala X89436
0.02

Fig. 3.3 Neighbor-joining phylogenetic tree based on the NS31-AML2 fragment of the SSU rDNA gene. Sequences from single AMF spores and root samples from the Sierra de Baza Natural Park are showed together with reference sequences from GeneBank. Numbers above branches indicate the bootstrap values. Only topologies with values ≥50 % are shown (1,000 replicates). Sequences are labelled according to the data set from which it originated (Tb-root = obtained from T. baccata roots; SB-root = from roots of other plants characteristic of the site; SB-spore = from spores isolated from the Sierra de Baza Natural Park), followed by the clone identity number. Sequences having a pairwise similarity higher than 97 % were clustered as phylotypes (delimited by vertical lines). Phylotypes are named following the closest virtual taxa code of MaarjAM database (Öpik et al. 2010). The prefix corresponds to the glomeromycotan family (following Krüger et al. 2012): Aca-Acaulosporaceae, Glo-Glomeraceae, Cla-Claroideoglomeraceae, Par-Paraglomeraceae, Pac-Pacisporaceae, Div-Diversisporaceae and Scu-Scutellosporaceae. *Mortierella polycephala* was used as out-group. To reduce the size of the tree, half of the sequences were removed

for the most influential variables on the AMF communities, which correspond to variables related tosignificant axes of each PCA with a correlation value higher than 0.5, are shown in Table 3.3.

Effects of habitat degradation on AMF community

Mycorrhizal colonization data (around 20 % in average) did not show significant differences between the groups of trees (Table 3.3). Comparisons of the AMF community composition between the two groups of yews revealed a similar richness of phylotypes per tree. However, the number of glomeromycotan families detected in the yews growing in the better conserved habitats was significantly higher than that in those with altered habitats (Table 3.3).

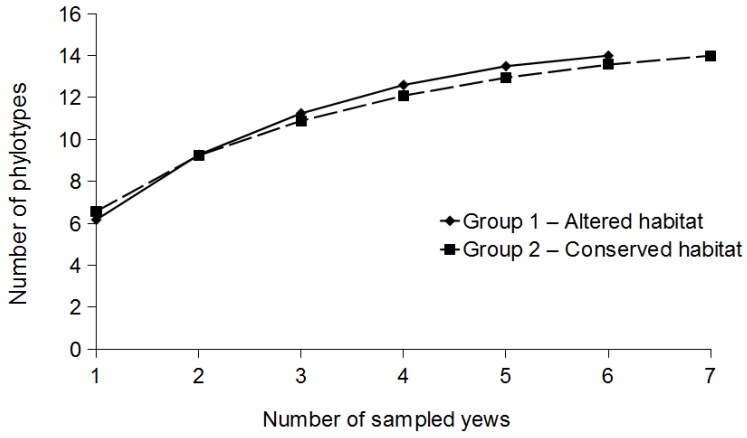

Fig. 3.4 Species-accumulation curves relating the sampling effort(cumulative number of sampled yews) to the cumulative numberof phylotypes detected in each group of yews

The frequency of detection of AMF phylotypes in each group of yews was clearly different (Table 3.4). The most abundant phylotype was Div062, present in every tree studied. Another ten phylotypes were shared between both yew groups, although they showed very different frequencies for group 1 and 2. Two of these

Table 3.4 Frequency of detection of AMF phylotypes in the different group of yews, expressed as the percentage of trees in which they were found

AMF Phylotype	Altered habitat (Group 1)	Better conserved habitat (Group 2)
Aca029	50.0	85.7
Aca285	83.3	57.1
Cla193	33.3	85.7
Div060	100.0	100.0
Gig052	-	42.9
Glo065	66.7	28.6
Glo072	66.7	42.9
Glo143	33.3	28.6
Glo149	33,3	14.3
Glo153	16.7	-
Glo177	33.3	28.6
Glo222	16.7	-
Glo312	-	14.3
Glo319	16.7	-
Pac284	-	14.3
Par336	33.3	85.7
Par281	33.3	28.6

10 phylotypes (Cla193 and Par336) were very common in yew group 2, appearing in 85.7 % of the trees, while in group 1 these phylotypes never had a frequency higher than 33.3%. The other eight shared phylotypes (Aca028, Aca030, Glo065, Glo069, Glo143, Glo149, Glo177 and Par281) had a more similar distribution in both groups. Finally three phylotypes were exclusive to group 1 (Glo153, Glo222 and Glo319) and three exclusive to Group 2 (Gig052, Glo105 and Pac284). However the frequency of these was low, being present only in one or two trees of the group. Despite these differences, PERMANOVA did not show significant differences in the whole community of AMF.

At the family level, some glomeromycotan taxa were absent in the yews living in the more altered habitats. Group 1 showed the presence of members of Glomeraceae, Claroideoglomeraceae, Diversisporaceae, Acaulosporaceae and Paraglomeraceae. In contrast, group 2 was colonized by members of all detected families (Glomeraceae, Claroideoglomeraceae, Diversisporaceae, Acaulosporaceae, Paraglomeraceae, Gigasporaceae and Pacisporaceae). Moreover the frequencies of detection of some families varied widely between groups of yews (Fig. 3.5), e.g. Claroideoglomeraceae and Paraglomeraceae were detected only in 33.3 % of the yews in group 1 while they appeared in more than 80% of the members of group 2.

Discussion

Disturbance effects on plant and AMF assemblages

A relationship between the environmental variables (vegetation, plant and soil characteristics and topological data) and the level of habitat degradation and AMF community composition was evident. In particular, the percent coverage of plants characteristic of altered habitats relative to those of well-conserved habitats surrounding the yews indicated two degradation levels for the studied system: Group 1 includes yews living in a more altered habitat while group 2 contains yews having a better conserved habitat. The physiological state of the yews, as measured by SLA, supports this pattern. Thus, a parallel effect of degradation on both plant and AMF communities was evident. Fitzsimons et al. (2008) also found relationships between the time after disturbance, structure of the plant cover and AMF communities. Soil variables, such as mineral nutrient and organic C contents, were also closely related to the extent of plant cover and AMF community conservation, as found for other ecosystems (Requena et al. 2001; Schnoor et al. 2011). As a general trend, degradation of vegetation cover is concomitant with a reduction of AMF diversity (as reviewed by Barea et al. 2011).

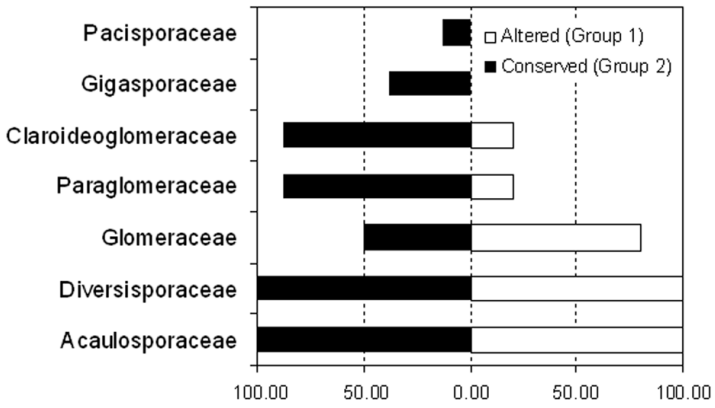

Fig. 3.5 Frequencies of detection for glomeromycotan families,expressed by the percentage of yews in which they are present

The presence of a road track seems to be an important source of disturbances in the study area. In fact, the *distance to the road track* appeared as a very influential variable. Previous work has shown that even a low number of vehicles driving through the forest can affect the surrounding vegetation (Angold 1997; Truscott et al. 2005). Some of these studies have highlighted the increase of ruderal plant species near roads, as also found in the present study. Trampling by visitors also has an impact on

plant communities and soil microbiota (Malmivaara-Lämsä et al. 2008; Lucas-Borja et al. 2011). Additionally, trampling by cattle is probably an important source of disturbance since this road track is regularly used to reach the highest grasslands of the Natural Park. The cattle also contribute to degradation by overgrazing (Blanca & Morales 1991). Other human practices provoking environmental degradation are the fire prevention measures at the edge of the roads, a labour consisting mainly in the clearing of the understory cover, which is necessary due to the dry and hot summers in Mediterranean environments. Clearing the understory is known to affect microbial activities (Mummey et al. 2010).

Altitude and spatial coordinates (longitude) appear to have a similar effect as the distance to the road. This could be due to the position of the road, which runs north-south leaving in the west the top of the mountain. Consequently, it is not possible to separate the effects of both variables. Differences in altitude are not large (about 200 m) which minimize changes in climatic conditions. In turn, the spatial effect can be ruled out as well since small distances separate both yew groups and neither geographical barriers nor changes in topological or geologic features were found between them.

In relation to AMF community composition, the absence of some glomeromycotan families in the roots of yews living in altered habitats is remarkable. The exclusive presence of Gigasporaceae and Pacisporaceae in the better conserved habitat group along with the higher frequencies of Claroideoglomeraceae and Paraglomeraceae, supports that more simple communities in disturbed environments are dominated by species of Glomeraceae, as also found in other studies (Egerton-Warburton & Allen 2000). However, no statistical differences in the AMF community could be found between groups of yew trees. This can be due to the interaction with other variables not clearly related to degradation. For example the available phosphorus, which appears as a determinant of the AMF community, was not different between the two groups of yews. As this variable is typically influencing the effectiveness of the AM symbiosis (Johnson 2010) and probably also the AMF community composition, this variable can thereby lead to blur the sole influence of disturbance factors.

Yew as inoculum source for restoration

An important objective of this study was to assess the possibility that yew trees could act as a refuge for the AMF community, and that these trees could thereby play a role in providing AMF inoculum for the surrounding vegetation. This would represent an important contribution in ecological restoration programs. This hypothesis was based on the importance of woody plants, particularly in Mediterranean ecosystems, acting as resource islands (Allen 1988; Azcón-Aguilar et al. 2003; Caravaca et al. 2005). Yew seemed a good candidate for this role due to its long lifespan (hundreds of years) and because its roots provide an extensive habitat for AMF. If this were the case, yew trees would maintain their original AMF communities while the surrounding environments were affected by degradation. When the disturbance processes were reduced, the progress towards more advanced succession stages could be driven by the presence of this preserved AMF inoculum. However, the hypothesis supporting that yew roots can act as refugia for their original AMF communities should be rejected since our results, showing that AMF phylogenetic diversity was negatively affected by degradation, are in favour of the alternative hypothesis. A similar situation was reported in studies on the effect of habitat fragmentation on different fungal groups (Peay et al. 2007; Grilli et al. 2012) and on AMF in disturbed (agricultural) environments (Jansa et al. 2003; Oehl et al. 2009). This is critical, since a higher phylogenetic diversity can imply a higher functional diversity (Powell et al. 2009), with a parallel increase in AMF life strategies (IJdo et al. 2010). Consequently, a lack of a phylogenetically diverse AMF community can obstruct the development of late-seral plant species, since they can need the association with specific groups of AMF (Davison et al. 2011), and difficult the progress of natural succession.

Conclusion

AMF communities associated with *Taxus baccata* L. are affected by the environmental processes involved in its population decline. The source of disturbance is related, at least in part, to anthropogenic activities as shown by a clear effect of the road track in the study area. Vegetation, plant and soil characteristics and topological data indicated that the studied yews fell into two different degradation levels. The group of yews living in the better conserved habitats had higher AMF phylogenetic diversity. The lack of some families of AMF in the group of yews developing in the more altered

habitats can imply a loss of functional diversity, highlighting the necessity of reintroducing these groups through the appropriate management of AMF in the restoration programs.

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IV. CONCLUSIONES GENERALES

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1ª. Se pone de manifiesto la existencia de estrategias vitales de los HMA claramente diferenciadas entre los que pueden sobrevivir a los estreses ambientales mediante la producción de propágulos capaces de persistir en el suelo y aquellos, más sensibles a perturbaciones, que dependen de su conexión a raíces vivas.

2ª. Los HMA más sensibles a situaciones adversas tienen una estrategia de colonización más rápida y un comportamiento estacional. Se incluyen fundamentalmente en el orden Diversisporales. Los más resistentes presentan mayor diversidad de estrategias de colonización, siendo por lo general más persistentes en la raíz. Pertenecen fundamentalmente al orden Glomerales.

3ª. La variación entre las distintas estrategias vitales encontradas para los HMA tiene una base taxonómica, en la medida en que los hongos que comparten características funcionales similares están próximos filogenéticamente.

4ª. La diferencia en la capacidad de supervivencia entre distintos grupos de HMA condiciona su presencia relativa en el suelo. En los ambientes mediterráneos, las elevadas temperaturas y sequía estival, condicionan una disminución de la presencia de los hongos más sensibles.

5ª. Las características funcionales de los hospedadores vegetales -ciclo de vida y hábito de crecimiento- condicionan la composición de la comunidad de HMA que promueven.

6ª. La época de recolección de los inóculos empleados no produjo cambios significativos en las comunidades de HMA promovidas por plantas herbáceas anuales, pero sí por plantas semi-leñosas perennes. Estos hechos se pueden explicar en base a una distribución diferencial a lo largo del año de HMA con estilos de vida diferentes.

7ª. Los procesos de estrés, ya sean de origen mecánico o inducidos climáticamente, afectan tanto a la composición de las comunidades de HMA, como a sus características funcionales.

8ª. El tejo, planta leñosa de elevada longevidad y relíctica en el ecosistema objeto de estudio, no actúa como refugio para las comunidades de HMA frente a procesos de degradación del medio ambiente.

V. BIBLIOGRAFÍA

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