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Arbuscular mycorrhizal fungi associated with psammophilic vegetation in Mediterranean coastal sand dunes

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Abstract

This study was conducted in order to characterize the natural arbuscular mycorrhizal (AM) biodiversity from Mediterranean sand dune ecosystems and to protect in a collection this biodiversity. The occurrence of AM fungi associated with sand dune plant species in three Mediterranean locations on the north-eastern coast of Spain was examined in one well preserved coastal sand dune and in two embrionary dunes recently protected from public access. Traditional taxonomy and molecular techniques were used to identify the AM fungal species present in these ecosystems. The species identified and isolated were: *Scutellospora persica* (Koske and Walker) Walker & Sanders, *Glomus ambisporum* Smith & Schenck, *Glomus diaphanum* Morton & Walker, *Glomus clarum* Nicolson & Schenck, *Glomus intraradices* Schenck & Smith, *Glomus microaggregatum* Koske, Gemma & Olexia and *Gigaspora margarita* Becker & Hall. Spores of *Glomus* were the most abundant in the direct soil extraction samples. The molecular analysis indicates that the most abundant fungi forming AM in the roots belonged to the Gigasporaceae group followed by fungi of Glomus group A and Glomus group B. The highest diversity of fungi and abundance of the AM fungal spores was found in the well preserved and undisturbed dune systems.

Additional key words: arbuscular mycorrhiza, biodiversity, *Gigaspora*, *Glomus*, *Scutellospora*.

Resumen

Micorrizas arbusculares asociadas a la vegetación psamófila de la costa mediterránea

El objetivo de este estudio fue caracterizar la biodiversidad de los hongos formadores de micorrizas arbusculares en ecosistemas de dunas litorales Mediterráneos. Se ha estudiado la presencia y diversidad de los hongos formadores de micorrizas arbusculares asociados a plantas psamófilas en tres zonas de la costa Mediterránea en el Nordeste de España: una zona de dunas bien conservada y dos zonas de dunas embrionarias protegidas. Para identificar las especies de hongos formadores de micorrizas arbusculares recuperadas se han utilizado métodos tradicionales basados en la morfología de las esporas de resistencia y métodos moleculares. Las principales especies de hongos presentes en este hábitat fueron: *Scutellospora persica* (Koske and Walker) Walker & Sanders, *Glomus ambisporum* Smith & Schenck, *Glomus diaphanum* Morton & Walker, *Glomus clarum* Nicolson & Schenck, *Glomus intraradices* Schenck & Smith, *Glomus microaggregatum* Koske, Gemma & Olexia y *Gigaspora margarita* Becker & Hall. Las esporas del género *Glomus* fueron las más abundantes en las muestras de suelo. El análisis molecular indicó que los hongos formadores de micorrizas arbusculares más abundantes en las raíces de las plantas pertenecían al grupo Gigasporaceae seguido por hongos del grupo A y B de *Glomus*. Tanto en la duna bien conservada como en las dunas embrionarias todas las plantas psammófilas estaban micorrizadas. La mayor diversidad de especies y la mayor abundancia de las esporas de hongos formadores de micorrizas arbusculares se encontraron en los sistemas de dunas bien conservados.

Palabras clave adicionales: biodiversidad, *Gigaspora, Glomus,* micorrizas arbusculares, *Scutellospora*.

Introduction

Coastal sand dunes of Mediterranean geographical areas are exposed to degradation by natural causes and particularly to the human pressure which has a negative impact on the structure and the stability of plant communities. The environmental preservation of coastal dunes depends on the establishment and survival of

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Abbreviations used: AM (arbuscular mycorrhizal), MPN (most probable number), PVLG (polyvinyl-lactoglycerol).

pioneer plants. Coastal sand dunes soils are characterized by low decomposition of organic matter and thus low soil fertility. Plant dunes are subjected to specific stressful conditions that include high temperatures during the day, pervasive strong salty winds and sand accretion. The psammophilic flora is adapted to these harsh abiotic conditions that limit the survival of other plant species. Thus the plants found in sandy dunes are species tolerant to low nutrient sandy soils, to wind and salt influences, and to burial by drifting sand (Rodríguez-Echevarria and Freitas, 2006).

Arbuscular mycorrhizal (AM) fungi play an important role in the uptake of water and nutrients, especially in phosphorus deficient soils, and help plant establishment and growth in harsh environments (Koske and Polson, 1984). In a nutrient-poor environment such as a sand dune, AM fungi contribute not only to plant nutrition but also to the process of dune stabilization by binding sand grains into wind-resistant aggregates, improving soil structure, and protecting plants from root pathogens (Gemma *et al.,* 1989).

Mycorrhizal plants are effective colonizers of disturbed habitats and the lack of mycorrhizal fungi influences plant species composition. Although AM fungi are important to the persistence of sand dune vegetation, little is known about the diversity of this beneficial symbiosis in Mediterranean coastal sand dunes. The prevalence of AM propagules in temperate maritime sand dunes has also been shown to contribute to the effectiveness of mycorrhizal plants as pioneer dune colonizers and it is apparent that the mycorrhizal status of early successional plants is governed by AM fungal species availability, composition and inoculum potential (Koske and Gemma, 1997). The understanding of mycorrhizal associations in sand dunes plants and their distribution in the soil is necessary for the sustainable management of these habitats.

The objective of the study was to assess the presence and diversity of AM fungi associated with the psammophilic flora in three Mediterranean coastal sand dunes located in the Northeast of Spain. The traditional taxonomy methods, based on spore morphology, and molecular analysis were used to identify the AM fungal species. The main species present in these habitats were isolated with the purpose of establishing a collection for future rehabilitation of degraded coastal areas with autochthonous plant species using the benefits of inoculation with the native AM fungi.

Material and methods

Study site and sample collection

The diversity of AM fungal species was measured in sand dunes habitats in three Mediterranean locations in the Northeast of Spain: (1) Les Salines (N40°37' E0°44'), in the Delta of the Ebro river, (2) El Prat (N41°16' E2°05'), in the deltaic plain of the river Llobregat, and (3) Viladecans (N41°16' E2°03'), also in the deltaic plain of the river Llobregat. The sand dunes systems in Les Salines (location 1) are well preserved, meanwhile El Prat (location 2) and Viladecans (location 3) are disturbed beaches that have been recently protected from public access (three years and one year ago respectively). They both can be considered as embryonary sand dunes.

The Delta of the Ebro river forms a natural protected ecosystem in the Mediterranean coast-line in Catalonia and covers an area of about 320 km^2. It is the second largest wetland area in the western Mediterranean, after the French Camargue. It has many natural habitats with kilometres of beaches with sand dunes together with rice fields and salt pans. This coastal diversity of ecosystems with valuable flora and fauna has led to the protection of a large part of the Delta and in 1983 it was declared a Natural Park (www.geographyfieldwork.com). The deltaic plain of the river Llobregat covers almost 100 km^2, in the vicinity of the city of Barcelona, though today only about 600 ha remain of the former extensive system of lagoons and marshes. The locations chosen in this area, el Prat and Viladecans, are sandy beaches backed up by a relic dune system, fixed by *Pinus pinea* mainly planted in the last century, where public access has recently been forbidden, with natural colonization of wild plants. The flora of these beaches includes psammophilic as well as ruderal plants.

Roots and soil from the rhizosphere were collected from the psammophilic plants present in the study sites: *Medicago marina* L.*, Lotus creticus* L.*, Elymus farctus* (Viv.) Runemark*, Pancratium maritimum* L.*, Calystegia soldanella* (L.) R. Br.*,* and *Ammophila arenaria* (L.) Link. Four rhizosphere 5 L samples from the upper 20 cm layer were collected per each plant, when present, in each location.

AM fungal occurrence

In the laboratory, roots of each plant from every location were extracted from the soil samples, washed

free of soil and debris and, after clearing and staining (Phillips and Hayman, 1970; Koske and Gemma, 1989), they were observed under a binocular microscope to evaluate mycorrhizal colonization (Giovannetti and Mosse, 1980).

After root removal, the soil samples from each location were combined to obtain a single sample per location. The resulting rhizosphere soil was used to analyze the chemical characteristics of the soil, to determine the number of infective mycorrhizal propagules and to recover the AM fungi present. The number of infective AM fungal propagules was estimated using the Most Probable Number (MPN) technique, with ten-fold series of soil dilutions with autoclaved sandy soil as a diluent (Porter, 1979; Powell, 1980) and leek (*Allium porrum* L.) as host plant. Spores present in the soil samples were extracted directly using the wet sieving and decanting method (Gerdemann and Nicolson, 1963). Spores with the same morphology were mounted in water, in polyvinyl-lactoglycerol (PVLG) and in PVLG with a drop of Mezler's reagent for microscopic examination. Spores mounted in Mezler's reagent were crushed in order to observe the staining of the different spore wall layers. At least 20 spores of each of the different morphotypes found were mounted in PVLG and 10 spores mounted in PVLG + Melzer's reagent for morphological identification after the original descriptions (Schenck and Pérez, 1990) and also with internet published reference culture data bases (http://invam.caf.wvu.edu).

To also recover the non sporulating AM fungi present in the soil samples, the rhizosphere soil was used to set up trap cultures with leek plantlets as a host plant. Leek seedlings were transplanted into 1-L containers filled with soil from each location and kept in a greenhouse. Once mycorrhizal colonization was confirmed, leek plants were transplanted into sterilized sandy soil to allow fungal development and the formation of chlamydospores. After 6 months growth, AM fungal species colonizing the roots of the trapping plants were identified using the molecular technology available.

Molecular identification of AM fungi in roots

The occurrence of the AM fungi in the roots of the native psamophilic plants from Les Salines, El Prat and Viladecans locations as well as the determination of the AM fungal species colonizing the roots of the trap plants, after the microscopic identification process, were determined by polymerase chain reaction (PCR) analyses of fungal large subunit ribosomal DNA sequences amplified from root fragments using specific sets of primers designed by Van Tuinen *et al.* (1998), Kjoller and Rosendahl (2000), Redecker (2000) and by Gollote *et al.* (2004).

Roots collected from the psammophilic plants of the coastal sand dune locations and roots of the leek plants used as a trap culture were used for DNA extraction. Eight 1-cm root fragments from each isolation soil and from each psammophilic plant species found in each sampling site were analyzed. Each root fragment was washed and crushed with a micro-pestle in the extraction buffer and were used for DNA extraction. The DNA extraction was done using the Power Soil DNA isolation kit (MoBio Laboratories Inc, Carslab, CA, USA) to minimise problems due to PCR inhibitors in the soil. The following steps were done according to the manufacturer's instructions. A primary PCR was performed with the eukaryote specific primers NS5 and ITS4 (Redecker, 2000), with 2 µL of the DNA extracted as template, 2 µL of a 10 µM solution of each primer and 10 µL of Eppenndorf Master Mix 2.5X (Eppendorf AG, Hamburg, Germany) in a total volume of 25 µL. PCR conditions were: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 50 s, extension at 72°C for 1 min 30 s, the last cycle was followed by a final extension period at 72°C for 10 min. Amplicons were then used as templates in three separate PCRs with specific primers for the Glomeromycota: Glomus group A (GLOM1310/ITS4i), Glomus group B (LETC1679/ITS4i) and Gigasporaceae group (NS5/GIGA5.8R). Nested PCRs were carried out in 50 µL volume and the reaction conditions were identical for the three primer combinations and differed from the primary PCR conditions at the annealing phase that was done at 61°C instead of 51°C. PCR products were visualised and separated by electrophoresis in 2% agarose gels stained with ethidium bromide.

Additionally, roots of the leek plants used as trap culture were employed to perform a second PCR analysis using Kjoller and Rosendahl (2000) and Van Tuinen *et al.* (1998) specific primers. PCR products were sequenced directly after DNA purification with the High Pure PCR Product purification kit (Roche Diagnostic GmbH Mannheim, Germany). Sequencing was carried out in both directions by Secogen S.L. (Madrid, Spain) using the corresponding primers. Results were manually aligned using the program

BioEdit alignment editor and compared to existing NCBI data.

In order to evaluate the specific group diversity in the roots of the psammophilic plants the Shannon-Weaver biodiversity index (Magguran, 1988) was used, that takes into account the number of species and the presence of the species. The frequency of occurrence of each AM fungi group in the roots was also calculated using $X_i/X_t \times 100$ (where X_i = the density for an individual group species and X_t = the total population).

Results

Physico-chemical analysis of soil samples indicate that all the soils were slightly alkaline (pH between 8.3 and 8.5) with similar chemical characteristics: no or low organic matter, low P and K contents (Table 1).

All roots of the psammophilic plants recovered from the study site were colonized by AM fungi with a variable percentage depending on the plant species and the location (Table 2). Other not psammophilic plant species were found in the primary successional habitats located in El Prat and Viladecans. Some of the species found in these embrionary sand dunes were ruderal plants belonging to Caryophillaceae, Brassicaceae and Polygonaceae families that are reported as non forming AM fungal associations.

The MPNs of Les Salines, El Prat and Viladecans soils were 34, 27 and 4 propagules/100 mL soil respectively, indicating that infective propagules of AM fungi were present in all the sand dune sites sampled, although their number decreased from the well preserved ecosystem to the more degraded beach.

Table 1. Soil chemical properties of the three sand dune habitats studied

Properties	Les Salines	El Prat	Viladecans
pH	8.3	8.4	8.5
Electric conductivity 25°C (dS m^{-1})	0.67	0.24	0.18
N-NO_3 (mg kg^{-1})	8	4	< 1
P (mg kg^{-1})	9	11	12
K (mg kg^{-1})	45	21	13
$CaCO_3$ (%)	36	21	20
Ca (mg kg^{-1})	2,486	1,845	2,036
Mg (mg kg^{-1})	132	69	55
Na (mg kg^{-1})	208	58	37
Organic matter (%)	ud[1]	ud	ud

[1] ud: undetectable.

Table 2. Percentage of AM root colonization found in plants of the study sites

Plant species	Les Salines	El Prat	Viladecans
Amophila arenaria	30	—	—
Medicago marina	16	41	—
Calystegia soldanella	74	71	61
Lotus creticus	7	—	—
Elymus farctus	5	75	14
Pancratium maritimum	74	—	—

The maximum mean spores density in 100 mL of rhizosphere soil was estimated in location 1, the well preserved and vegetated sand dunes site, followed by location 2, a partly colonized area, and the minimum mean spore density was observed in location 3, the most recently protected beach with a pioneer plant community (Table 3). The diversity of the AM fungal species also decreased from location 1 to location 3. A total of 7 AM fungal species were identified: *Scutellospora persica* (Koske and Walker) Walker & Sanders, *Glomus ambisporum* Smith & Schenck, *Glomus diaphanum* Morton & Walker, *Glomus clarum* Nicolson & Schenck, *Glomus intraradices* Schenck & Smith, *Glomus microaggregatum* Koske, Gemma & Olexia and *Gigaspora margarita* Becker & Hall. The three genera of the Glomeromycota found belong to the Glomeraceae and the Gigasporaceae families.

The use of molecular techniques also confirms that all plants sampled had the AM symbiosis under field conditions. Species belonging to *Glomus* group A, *Glomus* group B and Gigasporaceae group were detected in the colonized roots recovered in all the sites

Table 3. Spore abundance (average mean spore count) in 100 mL soil of AM fungal species present in the sand dune sites studied and Shannon-Weaver biodiversity index in the roots of the psammophylic plants, using eighth 1-cm root fragments from each plant species found in the study sites

Plant species	Les Salines	El Prat	Viladecans
Glomus intraradices	28	10	—
Glomus ambisporum	17	—	—
Glomus diaphanum	4	—	—
Glomus clarum	10	—	—
Glomus microaggregatum	17	—	—
Gigaspora margarita	—	—	1
Scutellospora persica	1	—	1
Total spore number	77	10	2
Shannon-Weaver biodiversity index	1.298	1.159	1.086

sampled. Although spores of the Gigasporaceae group (recovered directly from the rhizosphere of the plants) were low in number in the soil (Table 3) this species group was the most abundant in the roots of plants in the three locations (using the molecular techniques). The highest Shannon-Weaver index of biodiversity was obtained in location 1 followed by location 2 and location 3 (Table 3).

Six months after the establishment of trap cultures, species colonizing leek roots were also identified using molecular techniques. All the species found belonged to the *Glomus* sp. Group A as determined by Redecker (2000) primers. Direct sequencing using Kjoller and Rosendahl (2000) and Van Tuinen *et al*. (1998) specific primers, *G. intraradices* and *G. microaggregatum* were identified in the trap cultures obtained from location 1 whilst in the other locations the sequences could only be ascribed to an undetermined and uncultured *Glomus* sp. The diversity of AM species identified in the trap cultures was lower than the diversity found in the roots directly sampled from the field sites, and was also lower than the diversity associated with the spores recovered from those field locations. When present, spores of *Scutellospora* and *Gigaspora* were detected in the field soil after direct wet sieving and decanting and also detected in roots using molecular techniques but were not found in the roots or in the soil after trapping.

Discussion

This study confirms the existence of a rich diversity of AM fungi in the rhizosphere soils of Spanish Mediterranean coastal dune vegetation. Seven AM fungal species belonging to three genera, *Glomus*, *Scutellospora* and *Gigaspora*, were found in the sandy soil of the coastline dunes. Fungi belonging to the genus *Glomus* were the predominant sporulators in field conditions and also presented the highest number of different species. Our results report the occurrence of *Scutellospora persica* spores in the well preserved Spanish Mediterranean coastal sand dunes. This species has been recently recovered in the northwestern coast of Italy (Turrini *et al*., 2008) and had previously been described from other sand dunes (Koske and Walker, 1985; Blaszkowski and Tadych, 1997; Selvam and Mahadevan, 2002; Rodríguez-Echevarria and Freitas, 2006).

Despite the occurrence of the three groups of Glomeromycota, *Glomus* group A, *Glomus* group B and Gigasporaceae detected in the roots of the plants in the sites analyzed by molecular techniques, only *G. intraradices* (species included in the *Glomus* group A) was recovered in the soil samples of location 2 and only spores of the Gigasporaceae group were found in the soil field samples of location 3. This indicates that species colonizing roots may be different from species recovered from the soil as spores at sampling. Counting and identifying spores recovered from the field is an approach to measure and analyze species diversity of AM fungi. Spores recovered from the field are usually low in numbers. Only those fungi sporulating in the rhizosphere of the plant at the time of sampling are recovered. It is not uncommon to find nonsporulating species colonizing plants in the field based on trap culture results and PCR products from field-collected roots (Stutz and Morton, 1996), because species of AM fungi present different sporulation patterns (Gemma *et al*., 1989).

The differences in root colonization of plants growing in the sand dune ecosystems was intrinsic of the field sampling method and indicates that psammophilic plants are mycorrhizal in the Mediterranean coastal sand dunes studied independently of their disturbance level. All the plant species found in the recently protected sandy dunes were colonized by AM fungi, despite the low mycorrhizal inoculum potential estimated by the MPN bioassay in these areas. However, changes in the Shannon-Weaver biodiversity index along the successional status of the sand dune ecosystem are shown. The index increased in the well preserved and vegetated sand dunes site, while the minimum biodiversity index was found in the most recently protected beach with a pioneer plant community. The soil disturbance seems to affect the reproduction of AM fungi in these sand dunes and thus, the impact of disturbance on spore production seems to be higher than on the root colonization of the host plants.

The reduction in the diversity of AM fungal populations or in the number of spores of a specific fungus observed in the disturbed sand dunes will determine the equilibrium of the natural ecosystem, and some of the pioneer plant species that were established in these sites were neither mycorrhizal nor psammophilic plant species. According to Stukenbrock and Rosendahl (2005) soil disturbance may affect the community composition of AM fungi and the ability of the fungi to form mycelial networks between root systems. It also severely affects the reproduction of AM fungi in the sand dunes systems (Beena *et al.,* 2000). Depen-

ding on the presence of the AM fungal propagules, mycotrophic or non-mycotrophic species can be the dominant colonizers in the earliest stage of primary successional habitats (Koske *et al.,* 1996).

Although trap culture methods led to the detection of non-sporulating AM fungal species and added to those obtained by direct extraction from the field, species of *Glomus* were the predominant root colonizers in trap cultures from all the sites studied. According to Turrini *et al.* (2008) the diversity of the non *Glomus* fungi (like species belonging to the Gigasporaceae group) recovered from the soils is low in ecosystems with high anthropogenic disturbance. In our work, spores from species of *Gigaspora* and *Scutellospora*, when present, were directly extracted in the field but not after a short trap culture cycle. This may be explained by the fact that for *Glomus* species, the spores, the colonized host roots and the hyphae are all capable of initiating a colonization process, meanwhile for the genus *Gigaspora* and *Scutellospora* it has been described that only spores are able to initiate new infections in roots (Biermann and Linderman, 1983). It seems that the trap culture environment, as well as happens in agricultural soils (Giovannetti and Gianinazzi-Pearson, 1994), favors the colonization and sporulation of *Glomus* species. In our study it was especially effective with species of *G. intraradices* and *G. microaggregatum,* both forming spores inside the roots.

Vegetation is an effective means of reducing sand movements on beaches and dunes (Koske and Gemma, 1997) and traditionally planting psamophilic species on the coastal sand dunes has been an option to restore these habitats. In this study it was considered the evolution of two sandy dunes that are now in the process of restoration without human action, only by protecting these areas from public access to allow the natural establishment of plant species. The results confirm that AM fungi are still present in these sandy beaches despite the lack of vegetation for a long period of time.

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