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 embryonic 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 psamófilas 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
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: (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 embryonic 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 km². 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 km², 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 farc tus (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 micros-
cope to evaluate mycorrhizal colonization (Giovannetti
and Mosse, 1980).

After root removal, the soil samples from each loca-
tion were combined to obtain a single sample per lo-
cation. The resulting rhizosphere soil was used to ana-
lyze 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
flushed in order to observe the staining of the different
spore wall layers. At least 20 spores of each of the diffe-
rrent morphotypes found were mounted in PVLG and
10 spores mounted in PVLG + Melzer’s reagent for
morphological identification after the original descrip-
tions (Schenck and Pérez, 1990) and also with inter-
net 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 green-
house. Once mycorrhizal colonization was confirmed,
leek plants were transplanted into sterilized sandy soil
to allow fungal development and the formation of chla-
mydospores. After 6 months growth, AM fungal species
colonizing the roots of the trapping plants were identi-
fied 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 se-
quencies 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 psamphophilic 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 psamphophilic plant species found in each
sampling site were analyzed. Each root fragment was
washed and crushed with a micro-pestle in the extract-
ion buffer and were used for DNA extraction. The
DNA extraction was done using the Power Soil DNA
isolation kit (MoBio Laboratories Inc, Carlsbad, 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 (LET01679/
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 embryonic sand dunes were ruderal plants belonging to Caryophyllaceae, 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

<table>
<thead>
<tr>
<th>Properties</th>
<th>Les Salines</th>
<th>El Prat</th>
<th>Viladecans</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.3</td>
<td>8.4</td>
<td>8.5</td>
</tr>
<tr>
<td>Electric conductivity 25°C</td>
<td>0.67</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>(dS m⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-NO₃ (mg kg⁻¹)</td>
<td>8</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>P (mg kg⁻¹)</td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>K (mg kg⁻¹)</td>
<td>45</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>CaCO₃ (%)</td>
<td>36</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Ca (mg kg⁻¹)</td>
<td>2,486</td>
<td>1,845</td>
<td>2,036</td>
</tr>
<tr>
<td>Mg (mg kg⁻¹)</td>
<td>132</td>
<td>69</td>
<td>55</td>
</tr>
<tr>
<td>Na (mg kg⁻¹)</td>
<td>208</td>
<td>58</td>
<td>37</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>ud¹</td>
<td>ud</td>
<td>ud</td>
</tr>
</tbody>
</table>

¹ ud: undetectable.

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

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Les Salines</th>
<th>El Prat</th>
<th>Viladecans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amophila arenaria</td>
<td>30</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Medicago marina</td>
<td>16</td>
<td>41</td>
<td>—</td>
</tr>
<tr>
<td>Calystegia soldanella</td>
<td>74</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>Lotus creticus</td>
<td>7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Elymus farctus</td>
<td>5</td>
<td>75</td>
<td>14</td>
</tr>
<tr>
<td>Pancratium maritimum</td>
<td>74</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Les Salines</th>
<th>El Prat</th>
<th>Viladecans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomus intraradices</td>
<td>28</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Glomus ambisporum</td>
<td>17</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glomus diaphanum</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glomus clarum</td>
<td>10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glomus microaggregatum</td>
<td>17</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gigaspora margarita</td>
<td>—</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Scutellospora persica</td>
<td>1</td>
<td>—</td>
<td>1</td>
</tr>
</tbody>
</table>

Total spore number 77 10 2

Shannon-Weaver biodiversity index 1.298 1.159 1.086
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-
Arbuscular mycorrhizal fungi associated with psammophilic plants

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References


...ing 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 Gigasperoraceae group) recovered from the soils is low in ecosystems with high anthropogenic disturbance. In our work, spores 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 psammophilic 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.