Original article

Arbuscular mycorrhizal fungi of *Ammophila arenaria* (L.) Link: Spore abundance and root colonisation in six locations of the European coast

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are common organisms in the rhizhosphere of plants in coastal sand dunes where they play a key role in the establishment, growth and survival of plants. This study presents a quantitative analysis of the AMF associated with *Ammophila arenaria*, the most important sand-fixing species in the foredunes of Europe, in six locations along the western European coast. Spore abundance and root colonisation by AMF were estimated in July 2003, October 2003 and April 2004. The number of spores varied significantly with time and location. A clear peak of sporulation in autumn was found for three of the northern sites, but no pattern was detected in the southern sites. Root colonisation showed no seasonal pattern, despite differences between sampling times. Both hyphal coils and arbuscules were observed inside the roots, indicating colonisation by more than one AMF species. No correlation was found between root colonisation and spore number, or between AMF abundance and soil fertility. We conclude that: (a) spore production is driven by climatic conditions in the studied northern sites and by plant phenology in the studied southern sites; and that (b) root colonisation is independent of climate, phenology and soil fertility in the studied locations.

1. Introduction

Coastal sand dunes around the world are important as defences against the sea and for human recreation and water purification. Vegetation establishment in these dunes is often restricted by poor nutrient conditions and large sand deposits. In these conditions, arbuscular mycorrhizal fungi (AMF) play a key role in the establishment and survival of dune plants.

AMF contribute to dune stabilization by binding sand grains [19] and enhance plant nutrient uptake promoting plant growth in sand dunes [20]. Also, AMF can offer protection to dune plants against soil pathogenic fungi and nematodes [16].

Ammophila arenaria (marram grass) is the dominant plant in the mobile foredunes of Europe and North Africa. This plant species is very tolerant to sand burial and promotes dune formation by accumulating wind-blown sand. *Ammophila*

arenaria also develops extensive root systems which are colonized by both mutualistic and parasitic organisms [8,21,29]. Several previous studies have shown the presence of AMF associated with *A. arenaria* [13,14,21,23]. Most studies have been done to verify the presence of AMF, and, in recent years have been focused on the diversity of the AMF associated with *A. arenaria*. Those studies have shown that species of *Glomus*, *Scutellospora*, and *Acaulospora* can be associated with this plant [2–4,21].

We present a quantitative study of the AMF associated with *A. arenaria* in coastal dunes from a range of geographical locations with large differences in environmental and climatic conditions. Previous reports on seasonal variation of AMF often describe a pattern that is closely linked to plant growth [11,13]. Root infection increases during the active growth season and spore abundance peaks just after plant growth finishes. Hence, in locations under temperate climates where plant growth is limited by the low temperatures of winter, root colonisation increases in spring and summer with a decline at the end of the growth season [27,28] and the highest number of spores can be found in autumn and winter [11]. However, under Mediterranean-type climates, plant growth starts in late winter and stops during the hot dry summer, so root infection would be expected to peak in spring and sporulation to occur at the beginning of summer [25]. The patterns referred to above are very general and might change depending on the response of different AMF species to the phenology of the host plant [26]. Thus, we designed a sampling strategy to compare the abundance of AMF in the roots and rhizosphere of *A. arenaria* in locations under different climates. We selected six sites along the European west coast and hypothesised that root colonisation and spore abundance would peak in southern Europe about three months earlier than in northern Europe.

2. Materials and methods

2.1. Site description and sampling

Monoculture stands of *Ammophila arenaria* (L.) Link. were selected in primary dunes in England, Wales, The Netherlands, Belgium, North Portugal and South Portugal (Table 1). Climatic conditions are very different between the sites in northern Europe and Portugal, therefore, the timing for plant growth and flowering differ among both locations. The growing season for *Ammophila arenaria* starts about three months earlier in Portugal than in northern Europe (Fig. 1). Plant growth is mainly limited by the lack of rain during summer in the Portuguese sites and by low temperatures in winter in the northern sites. Three sampling times, July 2003, October 2003 and April

2004, were selected to compare different phenological stages of *A. arenaria*. Rhizosphere samples (including soil and root fragments) were collected at a depth of 20–30 cm from monospecific stands of *A. arenaria*. Samples were collected from the seaward slope of four different foredunes with 50 m intervals between them. In the northern sites (in The Netherlands, Belgium, England and Wales) root samples were also taken from deeper soil layers to compare root colonisation in the same plant between roots that developed this year and older roots from earlier years. Average monthly temperature and precipitation data from May 2003 to April 2004 were collected from the closest meteorological stations to each of the sampling sites (© Crown copyright 2005 Published by the Met. Office, UK; Royal Netherlands Meteorological Institute, INAG Portugal).

2.2. Soil analyses

Soil analyses were performed in the samples taken from each foredune. Available minerals in the soil were extracted in 0.2 N acetic acid [1]. Sodium, potassium, calcium, and magnesium were measured using a Varian ICP atomic emission spectrophotometer. Phosphorus was determined spectrophotometrically using the molybdenum blue method using stannous chloride as the reducing agent [1]. Total soil nitrogen content was estimated using the Kjehldahl method [1].

2.3. Spore assessment

Each soil sample from the 24 sampling points was thoroughly mixed and a volume of 50 cm^3 was used for spore extraction by wet sieving and decanting. The material retained on the 250 μm, 100 μm, and 45 μm sieves was collected in different filter papers (Millipore) and AMF spores were counted under a stereoscopic microscope (Leica MZ 8). The identification of spores up to the genus level was done following the descriptions of INVAM.

2.4. Mycorrhizal root colonisation assessment

The collected roots were stored in 50% ethanol until staining. Roots were cleared in 2% KOH for 1 h at 90 °C. Subsequently they were left to acidify overnight in 1% HCl. Staining was done with blue ink (Parker Quink) for 30 min at 60 °C, followed by destaining in lactoglycerol. The amount of colonisation was estimated using a grid-intersect method with examination of 100 intersects under a compound microscope at 200× magnification [24]. All microscopic examinations were carried out by the same person. Root-intersects that contained vesicles, arbuscules or hyphae were scored as mycorrhizal. The decision to score hyphae as mycorrhizal was based on the

Table 1 – Geographic coordinates of the sampling sites (XX°YY′)						
Site	Oostvoorne, NL	Het Zwin, BE	São Jacinto, PTN	Comporta, PTS	Blakeney Point, UKE	Ynyslas, UKW
Longitude	N5152	N5121	N4041	N4323	N5297	N5231
Latitude	E0404	W0322	W0844	W0848	W0097	W0403

NL: The Netherlands; BE: Belgium; PTN: Portugal North; PTS: Portugal South; UKE: United Kingdom England; UKW: United Kingdom Wales.
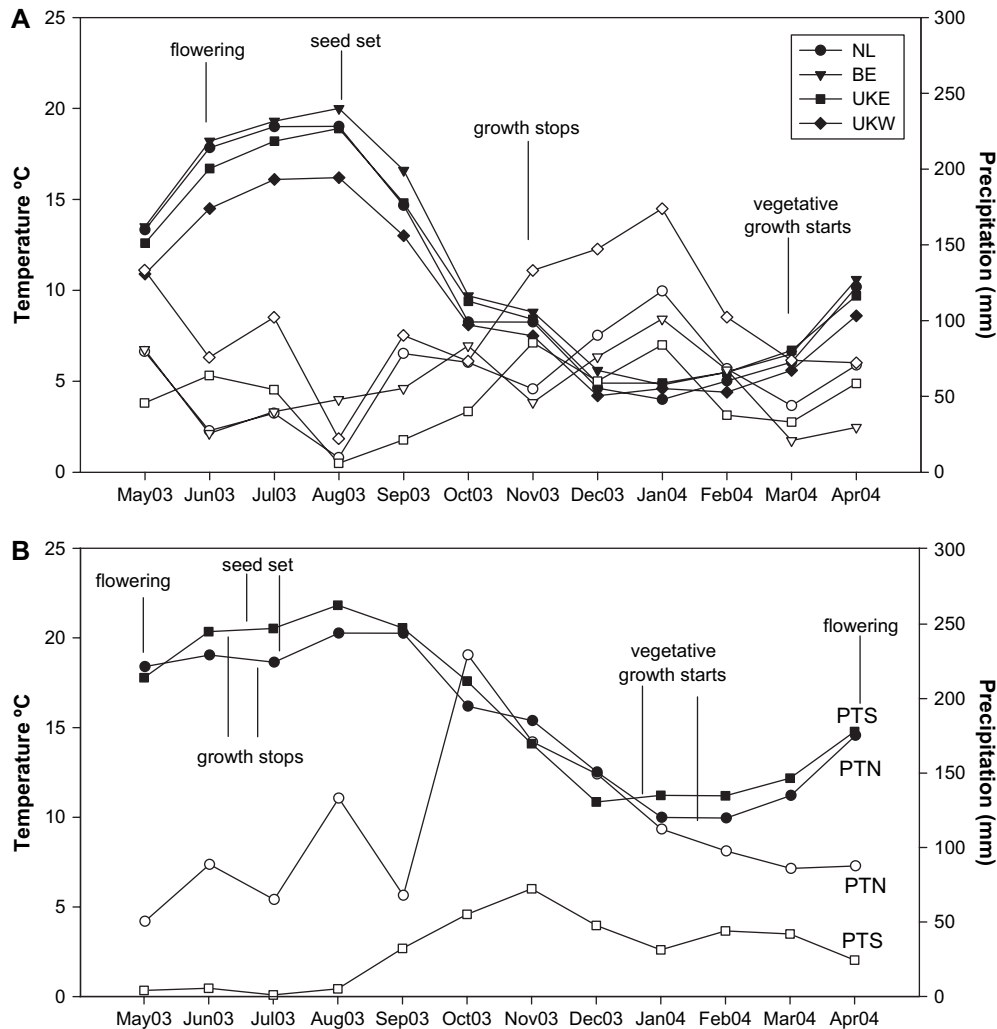
Fig. 1 – Average air temperatures (filled symbols) and total precipitation (open symbols) per month during the study. The main stages of the phenology of A. *arenaria* are also indicated in the graph. A. Northern sites. NL: Oostvoorne, The Netherlands, BE: Het Zwin, Belgium, UKE: Blakeney Point, England, UKW: Ynyslas, Wales. B. Southern sites. PTN: São Jacinto, Portugal North; PTS: Comporta, Portugal South.

associated presence of vesicles, arbuscules, spores, and the morphology of the mycelium. Roots that did not have cortex were excluded from the analysis. In total 84 samples were examined to score 100 intersections in each sample. For four samples from the roots from deeper layers it was not possible to do 100 intersections, but they were included in the analysis as long as more than 50 intersections could be checked. One sample from the deeper root layers, collected in Het Zwin, Belgium in July 2003 was excluded from the analysis because less then 50 intersections were scored.

2.5. Statistical analysis

One way ANOVA was used to compare the soils from the six locations based on the soil analysis data with site as the main factor. Principal component analysis (PCA) was used on the seven measured parameters to summarise the variation present and explore the similarities between soils.

One-way ANOVA was also used to compare the results for spore abundance and root colonisation within each location using time as the main factor. Data on root colonisation and spore abundance were analysed using a two way ANOVA with sampling site and month as main factors. All data were checked for normality and transformed if necessary to meet ANOVA assumptions. Correlation analyses of root colonisation and spore abundance were performed using the Pearson correlation coefficient.

Genstat 7.1, Sigmastat 3.0 and CANOCO 4.5 were used for the statistical analyses.

3. Results

3.1. Soil analysis

The soil from the different sites varied significantly in all the measured chemical properties (Table 2). The PCA showed

Table 2 – Results from the soil analysis of each location (mean ± SEM, N = 4) and least significant difference (LSD) values (P < 0.05) for each analysed element

Site	Oostvoorne, NL	Het Zwin, BE	São Jacinto, PTN	Comporta, PTS	Blakeney Point, UKE	Ynyslas, UKW	LSD
pH	8.5 ± 0.04	8.3 ± 0.05	7.5 ± 0.04	8.2 ± 0.11	7.6 ± 0.18	8.4 ± 0.14	0.43
N (mg kg^{-1})	24 ± 2	55 ± 4	19 ± 1	24 ± 1	26 ± 2	46 ± 4	9
P (mg kg^{-1})	10 ± 1	39 ± 2	7 ± 1	8 ± 1	19 ± 2	19 ± 2	3.9
K (mg kg^{-1})	50 ± 4	47 ± 4	26 ± 1	28 ± 3	27 ± 2	48 ± 4	8
Ca (mg kg^{-1})	9650 ± 565	22370 ± 1902	1998 ± 278	13598 ± 917	1429 ± 89	8288 ± 1159	2770
Mg (mg kg^{-1})	99 ± 11	136 ± 10	24 ± 2	26 ± 5	151 ± 12	279 ± 25	34
Na (mg kg^{-1})	51 ± 4	215 ± 25	57 ± 15	221 ± 23	37 ± 4	68 ± 6	43

NL: The Netherlands; BE: Belgium; PTN: Portugal North; PTS: Portugal South; UKE: United Kingdom England; UKW: United Kingdom Wales.

that Het Zwin (Belgium) and São Jacinto (Portugal North) were the most dissimilar locations (Fig. 2). Blakeney Point (England) and Oostvoorne (The Netherlands) were more similar to each other than São Jacinto (Portugal North) and Comporta (Portugal South) or than Blakeney Point (England) and Ynyslas (Wales). Magnesium, calcium and sodium were the main factors contributing to the variance of the samples. The plot also showed a strong positive relationship between the phosphorus, potassium and nitrogen content. The two axes explained 79% of the variation present in the samples.
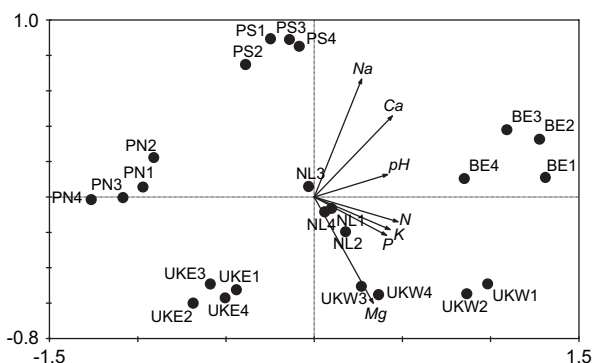
3.2. Spore abundance

Spores from different *Glomus* species were found in all samples. Spores of the genus *Scutellospora* were found in the sites from São Jacinto and Het Zwin. Spores from the genus *Acaulospora* were only found in Ynyslas and Het Zwin. In all sites, the majority of spores corresponded to *Glomus* species. The samples with the greatest numbers of spores were particularly rich in small *Glomus* spores (diameter about 50 μm). Identification of spores up to species was not possible since field spores can have abnormal morphologies because of death or parasitism by other organisms.

The minimum number of spores was found in Oostvoorne in July 2003 and April 2004 with an average of 16 and 21 spores per 50 cm^3 respectively (Fig. 3). This site was also the one in which greater variations between samples were found, since the number of spores in October 2003 was ten times that in

the other two samplings. The same seasonal pattern, with higher spore abundance in October, was observed in the samples from Ynyslas and Blakeney Point. Seasonal variation was not clear in the samples from São Jacinto, where significant differences were not detected between samples. In the remaining two sites, Het Zwin and Comporta, differences were significant (P < 0.05) when comparing April 2004 with the other two samplings. In Het Zwin, spores were more abundant in April 2004 than in July and October 2003. In Comporta, the number of spores found in April 2004 was the smallest of the three samplings (Fig. 3). The two way ANOVA test detected a significant interaction (P = 0.043) between site and time of sampling.
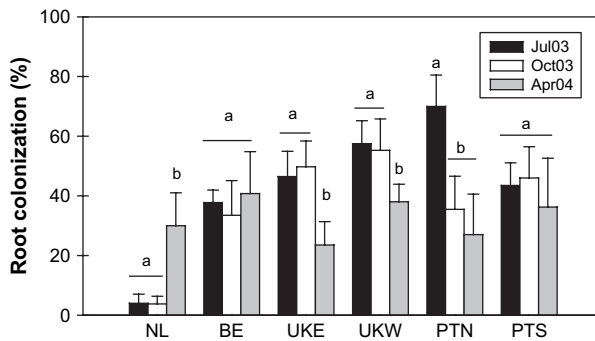
3.3. Root colonisation

AMF root colonisation was found in 95% of the analysed roots, the overall average of colonisation being 38%. Roots without AMF colonisation were found only in Oostvoorne (The Netherlands). The samples from that location had significant less colonisation than the other sites, mainly due to extremely low colonisation levels in the samples from 2003 (Fig. 4). In the last sample, April 2004, the average colonisation in the root samples from Oostvoorne was not different from the other

Fig. 2 – Score plot for the PCA of soil properties for samples from the six studied locations. The two axes explained 79% of the variation present in the samples. Abbreviations as in Fig. 1.
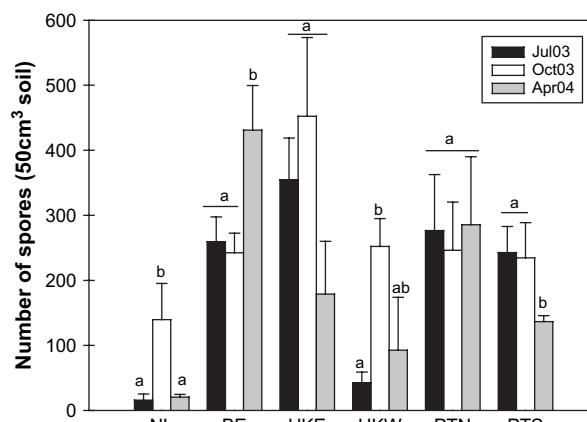
Fig. 3 – Abundance of arbuscular mycorrhiza fungi spores (mean ± SE) in the rhizosphere of *Ammophila arenaria* in July 2003, October 2003 and April 2004. Different letters above the bars show significant differences (P < 0.05) within each site, after one way ANOVA and LSD test for overall comparisons. Abbreviations as in Fig. 1.
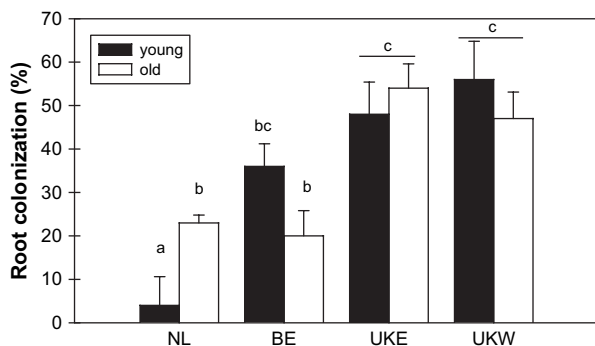
Fig. 4 – Mycorrhizal root colonisation (mean ± SE) in *Ammophila arenaria* **in July 2003, October 2003 and April 2004. Different letters above the bars show significant differences (P < 0.05) within each site, after ANOVA and LSD test for overall comparisons. Abbreviations as in Fig. 1.**

sites. Colonisation in April 2004 was significantly lower than earlier samplings for the sites in the UK. No significant differences were found in root colonisation between the southern and the northern locations (averages 43% and 35% respectively).

The AMF colonisation in the deeper root layers varied from 53% less to 49% more than AMF colonisation in the upper root layer (Fig. 5). Within each site, there were no significant differences between the root layers, except for Oostvoorne.

3.4. Infection type

Hyphal colonisation was the most common form of colonisation found in the roots from all sites. Vesicular colonisation was 18.3 ± 3% of the total colonisation and varied among the different sampling times and sites. Vesicular colonisation was significant lower in October than in April and July (P = 0.026). Both hyphal coils and arbuscules were observed, the first being more common. Average arbuscular root colonisation varied significantly among sites (P < 0.001). Roots from

Fig. 5 – Mycorrhizal root colonisation (mean ± SE) in *Ammophila arenaria* **in roots from different soil layers. Data were pooled for July and October 2003. Different letters above the bars show significant differences (P < 0.05) after ANOVA and LSD test for overall comparisons. NL: Oostvoorne, The Netherlands, BE: Het Zwin, Belgium, UKE: Blakeney Point, England, UKW: Ynyslas, Wales.**

Ynyslas had an average of 10% arbuscular colonisation, while roots from all other sites had less than 5% arbuscular colonisation.

3.5. Correlation analysis

The Pearson coefficient was calculated for all sampling times and sites and also within each sampling time. A significant correlation (P < 0.05) between spore abundance and root colonisation was only found for the data of Het Zwin and Oostvoorne in July 2003.

4. Discussion

AMF proved to be a common feature of *A. arenaria* roots and rhizosphere and were quantitatively quite constant both spatially and temporally. The results do not support our original hypothesis of differential seasonal changes in root colonisation for the northern and southern sites. Our results suggest that *A. arenaria* might have a fairly constant mycorrhizal colonisation independent of climatic conditions. In spite of the lack of a seasonal pattern in root colonisation, there was a significant variation in the content of vesicles and arbuscules which probably indicates physiological differences in the symbiotic interaction between the plant and the fungi through the year and between locations.

The differences in root colonisation between October 2003 and April 2004 observed in the samples from Oostvoorne (The Netherlands) agree with the work done by Ernst [10], who reported colonisation of 4% in winter and 30% in summer in Dutch sand dunes. Low levels of root colonisation were also found in this site in July 2003, but this could be related to the abnormal climatic conditions registered at that time: June 2003 was one of the three hottest June-months since 1901 (Royal Netherlands Meteorological Institute).

Other studies in sand dunes have also found significant seasonal variation in both mycorrhizal root colonisation and spore abundance. Gonzalez et al. [15] measured the highest frequency in spring and the lowest in autumn in Chilean dunes and Giovannetti et al. [13] found increasing colonisation from January until June in Italian dunes. Although we might have missed peaks of root colonisation, greater differences were expected between the winter and summer samples.

There was a clear seasonal pattern of sporulation in the samples from Oostvoorne, Blakeney Point and Ynyslas. For these three sites, the spore number was greater in October 2003 than in the other two samplings, which is in agreement with the predicted pattern. Clearly, in these locations the dominant species of AMF produce spores before the winter in order to survive the period in which there is no active plant growth [20]. The sampling site in Belgium presented a different trend that could have an explanation in the deposition of fresh sand to rebuild the dune system in this site some weeks before the sampling in April 2004. Sand deposition could have stimulated root growth and sporulation at that time of the year.

No differences were found between the number of spores in summer and autumn in the sites in Portugal, suggesting that sporulation can occur all along the year in these sites. Since winters are milder in Portugal, we proposed that AMF

sporulation is more controlled by the phenology of *A. arenaria* than by seasonality [12]. This hypothesis is supported by the differences found in April 2004 in the two Portuguese sites. There was a strong decline in the number of spores in Comporta (Portugal South) in April 2004, whereas no differences were found in São Jacinto (Portugal North). In April the plants in Comporta were already in flower, but in São Jacinto flowering only happened three weeks after our sampling. The exchange of nutrients between AMF and the plant is particularly important during flowering, therefore, it is likely that AMF sporulation could be inhibited during this process.

We did not find any relationship among AMF colonisation and/or sporulation and soil fertility. Although the sites in The Netherlands and England were very similar in nutrient content, the number of spores differed greatly. Large differences in nutrients between other sites did not reflect either in spore abundance or root colonisation. For example, root colonisation by AMF was not inhibited by the higher phosphorus content of the soil from Het Zwin or the high levels of magnesium found in Ynyslas. High levels of magnesium can reduce calcium uptake, causing premature root senescence with consequently negative effects on AMF sporulation and colonisation [18].

The presence of both hyphal coils and arbuscules in the same root sample suggests that more than one fungal species was colonising the roots of *A. arenaria*. The type of colonisation is determined by the identity of both the plant host and the fungi [6,22]. Although *Arum*- and *Paris*-type colonisation have been observed in *Gigaspora margarita* on barley, it usually means that more than one species of fungus is colonising the roots [9]. However, most of the morphological studies on AMF have been done with crop plants. Our finding of both hyphal coils and arbuscules in a non-crop plant requires more research to link structure to fungal identity in natural communities.

The fact that only 5% of the samples contained no AMF shows that these are commonly in association with *A. arenaria* and probably offer some benefits under the harsh conditions of the foredunes. Since differences in soil fertility were not related to differences in AMF abundance, it can be assumed that nutrient uptake is not the only benefit that *A. arenaria* obtains from the mycorrhizal association. AMF could have an important role in increasing plant resistance or tolerance to root pathogens, which accumulate in the rhizosphere of *A. arenaria* [5,17]. Greenhouse experiments have shown that AMF can indeed ameliorate the stress caused by pathogenic fungi and root-feeding nematodes in *A. arenaria* [7,16] Therefore, the uniformity of colonisation through the year could be related to plant protection against harmful soil organisms. Further research is needed to unravel the interactions between AMF and other soil organisms in sand dunes.

Acknowledgements

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