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Arbuscular mycorrhizal fungal propagules in a salt marsh

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Abstract The tolerance of indigenous arbuscular mycorrhizal fungi (AMF) to stressful soil conditions and the relative contribution of spores of these fungi to plant colonization were examined in a Portuguese salt marsh. *Glomus geosporum* is dominant in this salt marsh. Using tetrazolium as a vital stain, a high proportion of field-collected spores were found to be metabolically active at all sampling dates. Spore germination tests showed that salt marsh spores were not affected by increasing levels of salinity, in contrast to two non-marsh spore isolates, and had a significantly higher ability to germinate under increased levels of salinity (20‰) than in the absence of or at low salinity (10‰). Germination of salt marsh spores was not affected by soil water levels above field capacity, in contrast to one of the two non-marsh spore isolates. For the evaluation of infectivity, a bioassay was established with undisturbed soil cores (containing all types of AM fungal propagules) and soil cores containing only spores as AM fungal propagules. Different types of propagules were able to initiate and to expand the root colonization of a native plant species, but spores were slower than mycelium and/or root fragments in colonizing host roots. The AM fungal adaptation shown by this study may explain the maintenance of AMF in salt marshes.

Keywords Arbuscular mycorrhizal fungi · Infectivity · Propagules · Salt marsh · Spores

Introduction

Salt marshes are stressful environments regularly flooded with salt water, which leads to high soil salinity and soil anoxia (Armstrong et al. 1985). Although mycorrhizal colonization has been found in salt marsh plants (e.g. Hoefnagels et al. 1993; Carvalho et al. 2001; Hildebrandt et al. 2001), little is known about the survival and infectivity of arbuscular mycorrhizal fungi (AMF) in these harsh ecosystems. One potential mechanism maintaining AMF in salt marshes is fungal tolerance to salt and flooded conditions, which may be species or origin specific. Identification of salt marsh spores has revealed that apparently few AM fungal species occur and that one species, *Glomus geosporum*, is usually dominant in European salt marshes (Carvalho et al. 2001; Hildebrandt et al. 2001; Landwehr et al. 2002). These findings suggest fungal adaptation to salt marsh conditions.

The maintenance of AMF in ecosystems is dependent on the persistence of an inoculum potential in soils (Brundrett 1991). The sources of inoculum of AMF contributing to the infectivity of a soil are spores, infected root fragments and extraradical mycelium. The relative contribution of each type of propagule to plant root colonization is difficult to determine (Smith and Read 1997). There is evidence that this differs among taxa of AMF (Klironomos and Hart 2002) or among habitats, linked generally to differences in their behavior in response to environmental conditions (Braunberger et al. 1996; Requena et al. 1996; McGee et al. 1997). The tolerance strategies of AMF to adverse salt marsh conditions may include the existence of tolerant propagules able to maintain an infective inoculum. To our knowledge, no studies have assessed the contribution of the different types of propagules in salt marshes.

There is growing evidence that high spore number can be reached in salt marshes (e.g. Brown and Bledsoe 1996; Hildebrandt et al. 2001; Landwehr et al. 2002). In a salt marsh of the Tagus estuary (Portugal), a high number of spores were metabolically active, regardless of the marsh zones, the levels of salinity or the tidal flooding regimes. In this marsh, spore abundance and distribution seemed to be more related to plant distribution rather than to soil properties (Carvalho et al. 2001, 2003b). These data led us to suspect that spores have a relevant contribution to

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plant colonization and to the persistence of AMF in salt marshes of the Tagus estuary.

In this present work, we studied the propagules of indigenous AMF from an undisturbed salt marsh by evaluating the metabolic activity of spores at different times and the germination of spores at a range of salinity and water levels, and compared the infectivity of different types of propagules. Specifically, we aimed to evaluate the tolerance of indigenous AMF to salt marsh conditions and to estimate the relative contribution of AMF indigenous spores to plant colonization.

Materials and methods

Study site

Soil samples were collected from the Pancas salt marsh located within the Tagus estuary, Portugal (Carvalho et al. 2001). Some of the plant species present, such as *Aster tripolium* L., *Inula crithmoides* L. and, at a very low level, *Puccinellia maritima* (Huds.) Parl., are mycorrhizal. Of these, only *A. tripolium* occurs in both the lower and higher marsh zones (Carvalho et al. 2001). Rhizosphere soil samples for each experiment were collected in the lower marsh zone within *A. tripolium* stands.

Field spore vital staining

Soils were collected in July 1997, April 1998, November 1999, and April and November 2000 and stored at 4°C for 4 months. Spores of AMF were isolated by wet sieving followed by sucrose gradient centrifugation (Daniels and Skipper 1982). From each core sample (7 cm in diameter and 18 cm long), 100 g of soil was sieved and the fraction collected in the last sieve (53 μm) was centrifuged in a 60% (w/v) sucrose solution for 2 min at 3,000 rpm. Spores were collected from the water-sucrose interface, poured through a sieve, rinsed with distilled water and transferred to a Petri dish. Isolated spores were examined under a dissecting microscope at ×45 magnification. The predominant spores from this salt marsh were identified as *Glomus geosporum* (Nicol. and Gerd.) Walker, accounting for 84% of the total spore population, and *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe; two further unidentified *Glomus* species were also present (Carvalho et al. 2001). Forty spores from the two predominant species were collected in proportions representative of those found in the field, placed in iodonitrotetrazolium (INT) solution (1 mg ml^{-1}) and left at room temperature for 48 h (Walley and Germida 1995). Spores were checked for viability and the results expressed as percentage of stained spores. Five samples from each sampling date were collected and processed.

Spore germination tests

The AM fungal spores used in the germination tests originated from collected salt marsh field soils and from cultures of reference non-marsh species. The field soil was collected in April 1998 and stored at 4°C for 4 months. Two *Glomus* isolates provided by J. Klironomos, originating from the Long-Term Mycorrhizal Research Station at the University of Guelph, Canada and maintained in pot cultures, were used as reference non-marsh spore isolates, namely *Glomus geosporum* and *Glomus mosseae*. These isolates were maintained in our lab as pot cultures with sorghum seedlings in a 1:1 (v/v) mixture of Turface and sand. After 1 year of growth cycling, the pot culture substrates were stored at 4°C for 4 months.

Spores were tested for germination following the procedure of Weissenhorn et al. (1993). Spores from each isolate type (salt marsh soil-borne, *Glomus geosporum* reference and *Glomus mosseae* reference) were isolated by the method described above. Forty healthy spores were collected from each isolate. The sample of salt marsh soil-borne spores represented the two predominant species (*Glomus geosporum* and *Glomus mosseae*) in the proportions found in the field. The spores were placed between two sterilized filter membranes (Gelman, 0.45 μm pore size, 47 mm diameter, grid) held together by a photographic slide frame. This experimental unit with the membranes was buried to half the depth of a glass Petri dish (9 cm diameter) filled with 100 g of sterilized washed river sand (particle size <1 mm, autoclaved at 110°C for 1 h on two consecutive days and allowed to stabilize for 2 weeks before use). The sand was watered with the test solution according to each treatment. In the salinity test, the sand was watered to field capacity (25 ml) with an NaCl solution at levels of 0‰ (0 mM), 10‰ (171 mM), 20‰ (342 mM), 30‰ (513 mM) and 40‰ (684 mM). In the water-level test, distilled water was added to oven-dry (80°C for 48 h) sand to produce gravimetric water contents of 0 (no water added), 10, 17.5, 25 (soil field capacity level), 37.5 and 50%. The last two levels simulated salt marsh saturated conditions due to flooding. All Petri dishes were sealed with Parafilm to prevent water loss and incubated at 25°C in the dark. The temperature and time of incubation were based on the results of previous experiments (unpublished data). Each treatment was replicated three times. After 4 weeks, the experimental units were removed from the sand, rinsed with tap water and stained with glycerol-trypan blue solution (0.05%) for 1 h. The two membranes were carefully separated and examined under a dissecting microscope at ×45 magnification. The percentage of germinated spores was assessed.

Assessment of propagule infectivity

Undisturbed soil cores were collected in November 2000 from 12 randomly selected points. At each sampling point, four intact soil cores were taken immediately adjacent to each other with PVC tubes, 9 cm in diameter and 15 cm long. Each soil core remained in the PVC tube. The amount of soil in each core was 945 g (± 38 SE) fresh weight or 481 g (± 19 SE) dry weight.

For the propagule infectivity tests, one core from each one of the 12 sets of four cores remained intact, thus containing all types of AM fungal propagules ('all-propagule' treatment). From each set, two cores were used for the 'spore treatment'. The soil from one core was removed from the PVC tube and AM fungal spores were extracted from the entire soil volume and stored for 24 h in distilled water at 4°C. The soil from the other core was removed from the PVC tube, autoclaved at 110°C for 1 h on two consecutive days and allowed to stabilize for 2 weeks before use. The stored spore solution was mixed with the autoclaved soil and placed into a new PVC core. Thus, each of the two treatments had twelve cores. Of the remaining cores, the soil of six cores was removed, autoclaved and repacked into new PVC tubes (control cores). The other five cores were analyzed for spore density and extraradical hyphal length of AMF. In both propagule treatments (all propagule and spores only), three seedlings of *A. tripolium* germinated on Petri dishes in the dark at 25°C for 7–10 days were transplanted into each core of soil. The cores with the seedlings were placed randomly in a growth chamber at temperatures between 15 and 24°C and a photoperiod of 16 h at a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Plants were watered to field capacity with a 50% solution of artificial seawater (adapted from Epstein 1972).

Six replicate cores were harvested from each treatment at 3 or 6 weeks after transplanting. Three of the control cores were also harvested on both dates. At each harvest, the roots were extracted, cleaned and the total length determined by digital image analysis with WinRhizo software (Regent Instruments Image Analysis Systems, Canada). The roots were cleared and stained by a modified Phillips and Hayman (1970) procedure in which roots were cleared for 50 min in a 10% KOH solution at 90°C, rinsed, placed in 10% HCl solution for 10 min and then stained with glycerol-trypan blue solution (0.05%) at 90°C for 20 min. Infection units originating from entry points were counted at ×200 magni-

fication (Franson and Bethlenfalvay 1989). The results were expressed as infection units per root length. Root colonization by AM fungi was estimated by the gridline intersection method at ×45–100 magnification (Giovannetti and Mosse 1980) and expressed as percentage of root length colonized and colonized root length.

In each of the five field soil cores from different sets, spores were isolated from 30 g of soil and expressed as spore number per soil dry weight. Extraradical hyphae were extracted from two soil samples (5 g fresh weight each) by a modified Miller et al. (1995) procedure. Individual soil samples were suspended in 495 ml of water and homogenized in a blender for 1 min. The suspension was decanted and then stirred with an electronic stir bar. One 20-ml aliquot was removed from halfway between the beaker edge and the vortex and diluted in 80 ml of water. This mixture was stirred again and one 10-ml aliquot was transferred to a filter holder with a nitrocellulose membrane filter (47 mm diameter, 1.2 µm pore size). Trypan blue stain (0.05%) was added and the stained suspension was drawn through the filter using vacuum suction after 5 min. The filter was cut in half and placed on microscope slides. After drying, the filters were covered with low viscosity immersion oil. Extraradical mycelium was estimated by the gridline intersect method recording 140 fields of view using a 10×10 squared grid eyepiece reticule and viewed at ×200 magnification. Only aseptate hyphae with a characteristic 'knobby' appearance and dichotomous branching were considered as AM fungal hyphae. Total hyphal length was calculated based on Tennant (1975) and expressed as meters per g dry soil on the basis of soil moisture content measurements.

Statistical analysis

All data from the three experiments were analyzed by ANOVA and significant results (P<0.05) were analyzed by Duncan's test. Field spore vital staining data were analyzed by one-way ANOVA to test for differences between sampling dates. Spore germination data were analyzed by two-way ANOVA to test for differences between spore isolates and salinity or water level. Prior to analyses, germination data were arcsin square root-transformed (Zar 1984). Infectivity assessment data were analyzed by two-way ANOVA to test for differences between propagule type and harvest date variables. Prior to analyses, percentage of root length colonized data were arcsin square root-transformed and infection units data were log-transformed (Zar 1984). Statistica software (StatSoft, Tulsa, USA) was used for all statistical analysis.

Results

Field spore vital staining

The percentage of stained field spores was generally high, ranging from a mean of 61 to 80% (Fig. 1). A significant sampling date variation was found ($F_{4,18}$=7.5, P<0.01): the percentage of stained spores was higher in spring and summer than in autumn.

Spore germination tests

In the soil salinity germination tests (Fig. 2), there was a significant spore isolate × NaCl concentration interaction ($F_{8,30}$=6.9, P<0.001) along with significant main effects of the origin of the isolates ($F_{2,30}$=89.3, P<0.001) and NaCl concentration ($F_{4,30}$=15.8, P<0.001). This supports the hypothesis that the isolates exhibit different responses

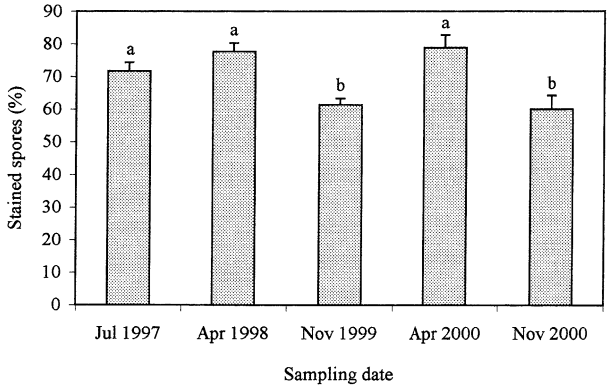

Fig. 1 Vital staining of field spores collected from Pancas salt marsh. Values (means ± SE of 5 replicates) followed by the same letter are not significantly different at P<0.05 (Duncan's test)

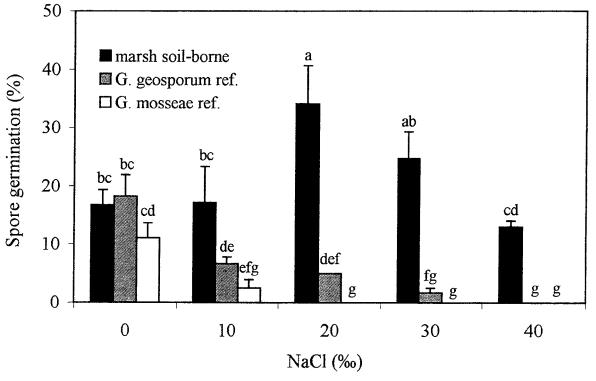

Fig. 2 Germination of spores collected from Pancas salt marsh and reference non-marsh spores of *Glomus geosporum* and *Glomus mosseae* under different soil salinity levels (NaCl concentrations). At each NaCl concentration, values (means ± SE of 3 replicates) followed by the same letter are not significantly different at P<0.05 (Duncan's test)

to NaCl concentrations. Germination of *Glomus geosporum* and *Glomus mosseae* reference spores decreased with increasing NaCl concentrations and spores of these isolates failed to germinate at concentrations higher than 10‰ (*Glomus mosseae*) or 30‰ (*Glomus geosporum*). Spores originating in the salt marsh had significantly higher germination at 20‰ NaCl than at lower concentrations, and germination decreased slightly with higher concentrations. However, at the highest concentration tested, germination of spores was not significantly different from that at the lowest concentrations (0 and 10‰). Germination of salt marsh spores was significantly higher than reference isolates at NaCl concentrations higher than 0‰.

In the soil water level germination tests (Fig. 3), besides the significant main effects of spore origin ($F_{2,34}$=3.6, P<0.05) and water level ($F_{5,34}$=34.5, P<0.001), there was a significant spore isolate × water level interaction ($F_{10,34}$=3.5, P<0.01), indicating differences in the responses of the different isolates to water levels. All spores failed to germinate without water in the

Table 1 Frequency of soil cores with colonized roots, percentage of root length colonized, colonized root length and number of infection units per meter of root in the intact soil cores (all types of propagules) and in the spore soil cores, after 3 and 6 weeks of *Aster tripolium* growth. *F*-values with significance levels are given for a two-way ANOVA. Degrees of freedom was 1 for each source of variation (propagules, harvest period and their interaction). Values (means ± SE, $n = 6$) within each column followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

Type of propagules and harvest time	Colonized cores (%)	Root length colonized (%)	Colonized root length (cm)	Infection units
3 weeks				
All	100	24 ± 7a	15 ± 4b	283 ± 93a
Spores	50	1 ± 1b	1 ± 0c	3 ± 1c
6 weeks				
All	100	22 ± 12a	41 ± 28ab	82 ± 44b
Spores	100	21 ± 7a	50 ± 17a	62 ± 26b
Source of variation				
Propagules	–	5.5*	3.3	17.3**
Harvest period	–	4.1	26.0***	1.3
Propagules × harvest	–	7.3*	15.0**	21.6***

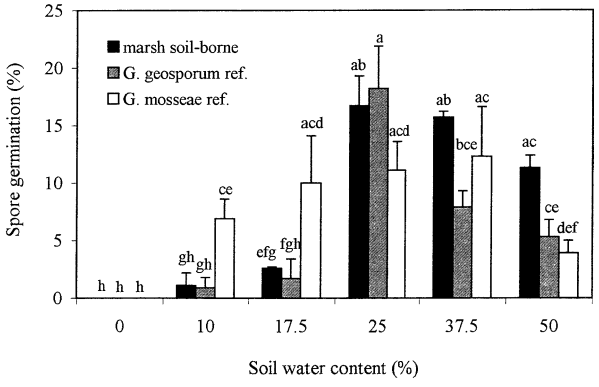

Fig. 3 Germination of spores collected from Pancas salt marsh and reference non-marsh spores of *Glomus geosporum* and *Glomus mosseae* under different gravimetric soil water contents (g H_2O per 100 g dry soil). At each water level, values (means ± SE of 3 replicates) followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

incubation soil. Soil water content below field capacity (<25%) significantly reduced germination of salt marsh spores and *Glomus geosporum* reference spores, but not *Glomus mosseae* spores. Germination of salt marsh spores was not significantly affected by increased soil water contents (above field capacity) as compared to non-marsh spores, in particular to the *Glomus geosporum* reference isolate. At the highest soil water level tested, salt marsh spores had higher germination than non-marsh spores, although this was only significant for the *Glomus mosseae* reference isolate.

Assessment of propagule infectivity

An average of 4.7 m g^{-1} soil dry weight (± 0.8 SE) extraradical AM fungal mycelium and 3.8 spores g^{-1} soil dry weight (±0.6 SE) were found in salt marsh soil.

The significant type of propagules (all propagules vs. spores only) × harvest period interactions for percentage of root length colonized, colonized root length and number of infection units, showed that the differences in colonization between propagules treatments were not consistent in the two harvest periods (Table 1). Colonization started earlier in the all-propagule treatment than in the spore treatment. Roots were colonized in all cores of the all-propagule treatment at 3 weeks, while only half of the cores displayed root colonization in the spore treatment. After 3 weeks of plant growth, root length colonization and infection units were significantly higher in the all-propagule treatment than in the spore treatment, where those parameters were extremely low. In the spore treatment, infection units and colonization of root system increased significantly with time, in contrast to the all-propagule treatment. In the latter, a significant decrease in the number of infection units occurred with time, probably due to the coalescence of many entry points. After 6weeks of plant growth, colonized plant frequency and root length colonization were similar in the two treatments. An increase in the number of infection units was detected over time in the spore treatment. However, the infection units recorded at 6 weeks in the spore treatment appeared to be lower, but not significantly different, than those detected in the all-propagule treatment at 3 weeks. The significant difference in the percentage of root length colonization between propagule treatments at each harvest was reflected in the colonized root length, indicating that the dissimilarity was not due to differences in the total root length of host plants. Shoot dry weight of *A. tripolium* plants was not significantly influenced by propagule type treatment (data not shown). At each harvest date, neither root colonization nor infection units were observed in plants of the control cores.

Discussion

In this study, we found evidence for potential adaptation of indigenous AMF to salt marsh conditions and for the ability of different propagules of these fungi to infect new plants and spread the infection through the roots.

In the Pancas salt marsh, the results of the vital staining tests showed a high proportion of metabolically active spores at different time periods and also when compared to other types of ecosystems (e.g. McGee et al. 1997; An et al. 1998). The high abundance (Carvalho et al. 2001) and metabolic activity of spores indicates a substantial spore pool throughout the year in salt marsh soils, with a high proportion of spores likely to germinate. However, the proportion of spores that germinated in the tests was considerably lower than the proportion of field spores, from the same collection date, identified as viable by the vital staining test. If the experimental conditions of the germination tests were conducive to maximal germination, then possible explanations for the discrepancy include the staining of dormant spores and/or the cytoplasm of immature spores that do not germinate. Although tetrazolium staining can be a useful and fast procedure to assess spore metabolic activity, we suggest precaution in the interpretation of results when it is used to estimate the potential of spores to germinate, in agreement with other reports (McGee et al. 1997).

Spore germination tests showed that spores from the salt marsh were more tolerant to salinity than spores of non-marsh reference isolates and required NaCl levels of 20 or 30‰ for maximal germination. This corresponds to similar or higher salinity levels found in the Pancas salt marsh (Carvalho et al. 2001). Koske et al. (1996) also observed salt tolerance of sand dune spores of *Gigaspora gigantea*, while other studies showed a strong inhibition by sodium and chloride salts of spore germination in *Gigaspora margarita* (Hirrel 1981) and *Glomus mosseae* (Estaun 1989). We also observed a strong reduction in germination by these salts in the reference isolate of *Glomus mosseae*.

Germination of salt marsh spores was not affected by water level at field soil capacity or above but decreased below it, probably due to water stress. Similar observations were reported by Daniels and Trappe (1980) for *Glomus epigaeum* and by Koske (1981) for *Gigaspora gigantea*. However, reduced spore germination in soils with water levels above field capacity, as we observed for the reference isolate of *Glomus geosporum*, has been observed for some species of *Glomus* (Sylvia and Schenck 1983). The reduced germination at high water levels may be related to the low tolerance of some fungal species of hypoxic conditions.

Since AMF are obligate symbionts, spore germination and subsequent early hyphal growth of hyphae are the only stages of the AM fungal cycle in which the fungi can be studied in the absence of plants. Therefore, spore germination is a good predictor to test the influence of certain factors on AMF. The ability of salt marsh spores to germinate under high salinity and above soil field capacity suggests that the AMF present in the salt marsh soil are probably AM fungal ecotypes adapted to the stressful conditions present in this ecosystem. Similarly, Landwehr et al. (2002) suggested the occurrence of *Glomus geosporum* ecotypes in Central European salt marshes.

The results of the infectivity tests showed that different types of propagules of salt marsh AMF are able to initiate new colonization and to spread the infection through the root system of a native plant. Mycelium and/or root fragments were relatively more important for the initiation of plant colonization than spores, at least in the experimental conditions used. Since spores required 3–6 weeks to initiate colonization in the spore treatment, we conclude that the infection units and colonization of roots harvested at 3 weeks in the all-propagule treatment were due to non-spore propagules. We do not know the relative proportion of infection units derived from extraradical mycelium or root fragments. But it is reasonable to suggest that mycelium was an important propagule for the initiation of colonization. In spite of the stressful environment, salt marsh soil contained a considerable hyphal network. Previous reports have indicated that hyphal networks are a very important source for the rapid initiation of colonization (McGee et al. 1997; Smith and Read 1997). Low abundance and viability of spores may sometimes explain the failure of spores to rapidly initiate colonization (McGee 1989; Requena et al. 1996), but this was not evident in the present study. The inherent time required for the transition from dormancy to germination, which is a highly variable character in the Glomales (Tommerup 1983), may have accounted for the slower initiation of infection by spores than by the hyphal network.

Salt marshes are subjected to large temporal and spatial variation of soil properties (Armstrong et al. 1985). High levels of salinity and flooding in soils were observed to significantly reduce the extraradical mycelium length of AMF from the Pancas salt marsh (Carvalho et al. 2003a). In the present investigation, colonization levels due to spores were similar to those found for field plants (Carvalho et al. 2001). This result and the large proportion of active spores indicate that the pool of spores is likely sufficient to maintain the usual levels of AM colonization in salt marsh plants. Although our data do not indicate that spores make a large contribution to the initiation of colonization, we can not completely exclude that spores play a significant role in salt marshes. Further studies testing the effects of season, salinity and flooding on the infectivity of each type of propagule are necessary to determine whether spores, as resistant structures, function as survival units of AMF in salt marshes.

The results of this study show that fungal adaptation is one potential mechanism to explain the maintenance of AMF in the stressful salt marsh soils. The specific adaptation and tolerance to salinity and water levels of the AMF from the studied salt marsh may provide an explanation for the low fungal diversity and wide occurrence of AMF in marsh zones, regardless of the

tidal flooding regime (Carvalho et al. 2001). These salt marsh AMF may have the potential to confer salt and flooding tolerance to plants and, therefore, may influence plant distribution in salt marshes.

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