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ORIGINAL PAPER

Impact of a natural soil salinity gradient on fungal endophytes in wild barley (*Hordeum maritimum* With.)

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Abstract Occurrence and distribution pattern of fungal endophytes in different tissues of halophytic plants across saline depressions are poorly studied. We investigated the endophytic fungal communities inhabiting roots, stems and leaves of *Hordeum maritimum* collected in a soil salinity gradient, i.e. non-saline, slightly saline and saline, using a culture-dependent approach. A total of 20 taxa belonging to *Ascomycota* phylum were identified by ITS rRNA gene sequence. *Pyronema domesticum* and *Alternaria* spp. were the most frequently isolated. Roots host higher diversity and were more frequently colonized by endophytes than aboveground organs. Endophytic composition of all organs surveyed differed according to salinity gradient. Contrary to expectations, the colonization rate of roots increased with soil salinity, indicating that under salt stress the endophyte-plant association is promoted. All the isolates exhibited in vitro saline tolerance, especially those belonging to genera *Xylaria*, *Chalastospora*, *Alternaria* and *Pyronema*. Fungal tolerance to NaCl under in vitro conditions appears to be more dependent on the isolates than on the sites of their isolation, suggesting that under natural conditions other factors, beyond soil salinity, should be taken into account. These findings highlight the importance of fungal endophytes in the protection and/or adaptation of both interacting species (plant-fungus) to salt stress under natural conditions.

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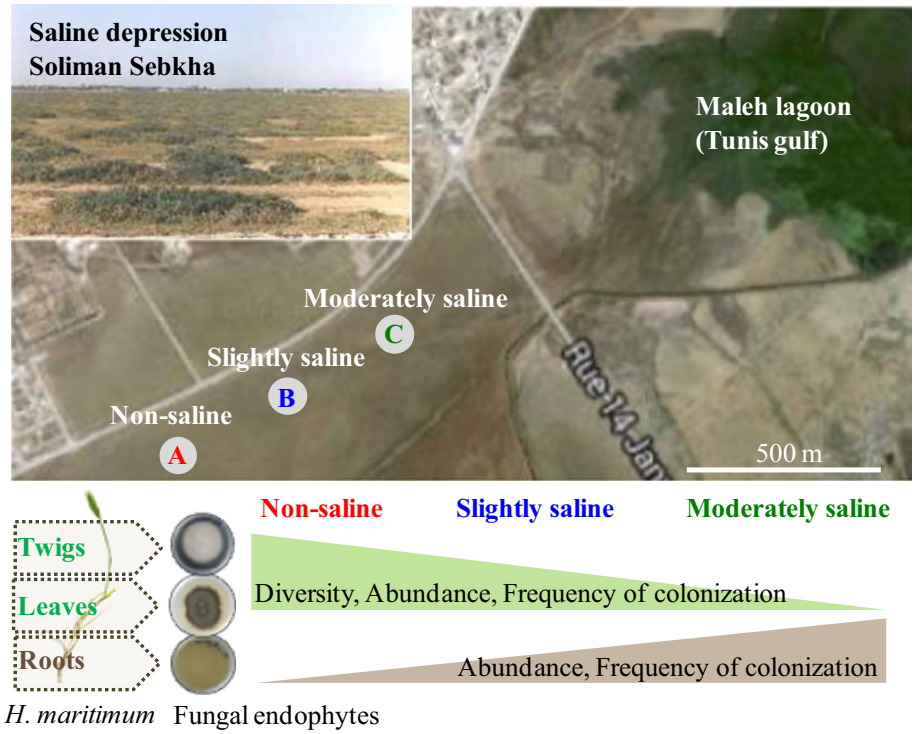
Graphical Abstract



Keywords Endophytic communities · Halophytes · Saline depression · Similarity · Tolerance index

Introduction

Soil salinity is one of the main physiological threats to ecosystems and is responsible for substantial economic loss in agricultural production throughout the world (Shrivastava and Kumar 2015). In fact, almost 1 billion ha worldwide (about 7 %) is affected by salinity (Yensen 2008) and the predicted global climate changes associated to the accelerated growth of irrigated agriculture threatens to increase this area (Yensen 2008; Shrivastava and Kumar 2015). In recent years, considerable attention has been given to the ability of fungal endophytes to alleviate plant stress caused by salinity (e.g. Waqas et al. 2012; Khan et al. 2011, 2013). Such beneficial effect has been mainly assessed in plants inoculated with a single fungal species or a mixed inoculum of few species, grown under salt stress conditions (e.g. Waqas et al. 2012; Khan et al. 2011, 2013). By contrast, the function of fungal endophytic assemblages, as a whole, in conferring benefits to plants growing in saline soils, has received little attention by researchers (Maciá-Vicente et al. 2012). Similarly, fewer studies address the ecological and environmental settings which determine the nature of relationships between communities of endophytic fungi and host plants. Those studies performed in saline and extreme environments, can generate information about the survival strategies of plants that may contribute to the development of biotechnological solutions. In fact, over eons of evolution, plants have been faced with environmental changes, including increased soil salinization, forcing them to adapt or succumb to selective pressures (Munns and Tester 2008). Beyond plant genome (Smallwood et al. 1999), fungal endophytes are thought to play a critical role in this adaptive process of plants to environmental stresses (Rodriguez et al. 2012). For example, the endophytic fungus *Fusarium culmorum* colonizes the tissues of the coastal dunegrass *Leymus mollis*. Yet, when grown separately the host plant does not survive and the endophyte's growth is delayed when exposed to levels of salinity experienced in their native habitat (Rodriguez et al. 2008). But, when grown symbiotically, both partners tolerate sea water salinity levels. When comparing the effect of plant inoculation with *F. culmorum* isolated from *L. mollis* and from non-coastal plants, the same authors verified that only the isolates from the coastal plants had the ability to confer salt tolerance (Rodriguez et al. 2008). This ability was suggested to be a habitat-specific symbiotically adapted phenomenon (Rodriguez et al. 2008). Further research, involving other plant-endophyte symbiotic relationships within natural settings, are needed to reveal the potential role of fungal endophytes in the adaptation or tolerance of plants to salinity stress.

Environmental gradients, such as saline depressions, provide an excellent system to analyze such mutualistic interactions (Maciá-Vicente et al. 2012). These saline depressions display abrupt edaphic changes at a relative small spatial scale, from low salinity to extremely high soil salinity, and thus provide a scene for unique case studies along salt stress gradients. In Tunisia, the Soliman Sebkha (El Maleh lagoon), located on the gulf of Tunis, shows high spatial variation of soil salt content (Rabhi et al. 2009), making it an excellent ecosystem to study such habitat-specific symbiotically adapted phenomena. It maintains a multitude of plant species, including the perennial facultative halophyte *Hordeum maritimum* With. (syn. *Hordeum marinum* Huds.; Family Poaceae). This is a Mediterranean wild grass typical of saline coastal or inland meadows (Lombardi and Lupi 2006), making it potentially useful for fodder production in saline zones (Hafsi et al. 2007).

Therefore, the aim of this study was to compare fungal endophytic communities inhabiting roots, leaves and twigs of *H. maritimum* growing along a salt gradient in Soliman Sebkha, to assess how spatial patterns of distribution are influenced by salt soil contents. The salt tolerance of the fungal isolates was also tested in synthetic media supplemented with NaCl. This characterization will improve our knowledge concerning the ecology and evolution of mutualistic plant-endophytic interactions.

Materials and methods

Plant sampling

Plants of *H. maritimum* were collected in Soliman Sebkha in North Tunisia, an area at the edge of a saline depression, with the geographical coordinates: 36°41′47″N; 10°29′30″E. In this area, three sites, distributed perpendicularly to Maleh lagoon at about 1100, 900 and 700 m away from the coastline and named respectively as A, B and C, were selected for plant samplings. Each site was divided into three non-contiguous square plots (100 m^2 each), and in each plot three *H. maritimum* plants were randomly selected for endophyte isolation on September 2012. All samples were stored at 4 °C and processed within 2 days. Simultaneously to plant sampling, three soil cores from each plot were collected to be chemically analyzed.

Soil characterization

Soil samples were chemically characterized in terms of electric conductivity (EC), pH, sodium and potassium contents using common research facilities from the Center of Biotechnology of Borj Cédria (CBBC).

Isolation of endophytic fungi

The plants were first rinsed in distilled water, and the root system, leaves and twigs were separated and further selected for assessment. Five segments (ca. 4 cm long each) of twigs, roots and leaves per plant were randomly selected, and surface sterilized through sequential immersion in 70 % (v/v) ethanol for 2 min, 3–5 % (v/v) sodium hypochlorite for 3 min (for leaves and twigs) or 5 min (for roots), 70 % (v/v) ethanol for 1 min and rinsed three times (1 min each) with sterile distillate water. After being dried in sterile filter paper, each root and twig segments was cut in five segments of approximately 4–5 mm, and each leaf in four segments of ca. 5 × 5 mm from the lamina and one from the petiole of approximately 4–5 mm. These tissue segments were immediately transferred to 9 cm diameter Petri plates, containing 10 mL of sterile Difco potato-dextrose agar (PDA) medium supplemented with 0.01 % (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK) and incubated at 25 ± 2 °C in the dark. In total, 1215 segments (9 plots × 3 plants × 3 plant organs × 15 tissue segments) were used in this study. Efficiency of the surface sterilization procedure was ascertained by imprinting randomly selected surface sterilized leaves, roots and twigs segments onto PDA Petri plates. The fungi growing out of the tissue segments were recorded as endophytic fungi and were sub-cultured on individual PDA plates to obtain pure isolates for subsequent identification. Pure cultures of each isolate were deposited in the culture collection of the Center of Biotechnology of Borj Cédria (CBBC).

Identification of fungal isolates

A combination of morphological and molecular approach was used to identify fungal isolates. At first, isolates from pure cultures were grown on PDA medium and maintained at 25 ± 2 °C in the dark for 1–3 weeks, depending on their growth rate. These fungal cultures were examined periodically, and groups of strains were formed according to their morphological similarity. Isolates were divided based on the morphology of the colony (e.g. color, shape and texture) or hyphae, the characteristics of the spores (e.g. size and shape) and reproductive structures. One representative strain for each morphotype was selected for further molecular identification using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA).

Genomic DNA was extract from spores or mycelial mat following the protocol of Oliveira et al. (2012). The ITS region (ITS1, 5.8S, ITS2) was amplified using the universal ITS1 and ITS4 primers (White et al. 1990) and the PCR protocol described by Oliveira et al. (2012). Amplified products (~600 pb) were purified using JETQUICK PCR

Product Purification Spin Kit (Genomed) following the manufacturer's instructions. Clean PCR products were sequenced with the ITS1 and ITS4 primers using STAB-Vida services (Oeiras, Portugal). The DNA sequences were analyzed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and the BLAST algorithm. Blast results were sorted according to the higher identity score and the lowest E-value. For sequence identities >98 %, the genus and species were accepted; for sequence identities between 95 and 97 %, only the genus was accepted; and for sequence identities <95 %, isolates were labeled as 'unknown' fungi. The operational taxonomic units (OTUs) identified were taxonomically classified according to the Index Fungorum Database (www.indexfungorum.org).

Screening of salt-tolerant endophytic fungi

In order to study in vitro fungal tolerance to NaCl, the endophyte strains were cultivated on PDA medium amended with three different concentrations of NaCl (50, 100 and 200 mM). The control treatment was performed in the same medium without NaCl (0 mM). Ten mL of each medium was poured onto 9.0-cm-diameter Petri plates and each one was centrally inoculated with one hyphal plug (5 mm diameter) of 2-week-old mycelia, sealed with Parafilm and incubated in the dark at 25 ± 2 °C. Three replicates of each combination (NaCl concentration *vs.* fungus) were performed. The radial growth of the developing colony was measured every 4 days, using two cardinal diameters previously drawn on the bottom of the plate, for 12 days. The salt tolerance index (Ti) was calculated from the following relation: Ti = radius (cm) of fungi in NaCl-medium/radius (cm) of fungi in NaCl-free medium (control).

Data analysis

The diversity of fungal endophytes within the several plant organs of *H. maritimum* collected from sites A, B and C was evaluated at the level of their richness (number of different taxa), their abundance (number of isolates per taxa), and also by computing the most widely used indexes of species diversity, such as Simpson's Reciprocal Index (1/D) and Shannon–Wiener (H). Both indexes combine species richness and abundance in different ways (Magurran 2004), and were computed in Species Diversity and Richness v. 4.0 (Seaby and Henderson 2006).

The frequency of colonization (FC, %) of an endophyte taxon was calculated as the total number of plant tissue segments colonized by each endophyte divided by the total number of plant segments. The relative abundance (RA, %) of an endophyte taxon was determined as the total number of isolates of a taxon divided by the total number of isolates of all taxa.

Non-metric multidimensional scaling (NMDS) was carried out to explore the similarity of microorganisms' community among *H. maritimum* organs and among *H. maritimum* plants collected at various salinity gradients. The correspondence of the ordination diagram to the distances is described by a stress value (Kruskal's stress), with values less than 0.2 representing good ordination plots and greater than 0.3 provides a poor representation (Clarke 1993). NMDS was performed by using Jaccard's and Bray-Curtis similarities matrices. Jaccard's index compares presence or absence of taxa among samples, disregarding species abundance (Magurran 2004). Bray-Curtis index compares taxa presence or absence and abundance among samples. This coefficient ignores cases in which the species is absent in both community samples, and is strongly influenced by the abundant species so rare species add very little influence (Bray and Curtis 1957). The ordination analyses were conducted on presence/absence data (Jaccard's index) and raw data (Bray-Curtis coefficient).

Analysis of similarities (ANOSIM) was used to test significant differences on endophytic fungal species composition between *H. maritimum* collected at various gradients of salinity, with a significance level of 0.05. This analysis compares species composition between-groups (sites A, B and C) and generates an R-value ranging from 0 (completely similar) to 1 (completely dissimilar) (Clarke and Gorley 2015). This analysis was conducted using Bray–Curtis distance matrices (obtained from raw abundance data), with 1000 permutations. When a significant difference was observed, similarity percentage analyses (SIMPER) were performed to reveal which taxa contributed to dissimilarity between *H. maritimum* collected at various gradients of salinity (with 0 being completely similar and 100 being completely dissimilar). All multivariate analyses were done using the Community Analysis Package v. 4.0 (Henderson and Seaby 2007).

Significant differences among samples were determined by analysis of variance (ANOVA), using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.) and averages were compared using Tukey's ($p < 0.05$). The same software package was used to determine all the Pearson correlation coefficients and to perform principal component analysis (PCA). The PCA was performed with Ti values obtained for each endophytic fungi after 4, 8 and 12 days of growing in 50, 100 and 300 mM NaCl, and chemical soil variables (pH, EC, Na and K contents) of the sites sampled (A, B and C), with an attempt to describe the relationship between these two parameters (Soil *vs.* Ti).

Results

Soil chemical characterization

Table 1 shows the soil chemical analysis where *H. maritimum* samples were collected. Soil from site C had the highest EC and sodium content, followed by soil from sites B and A. The soil pH was very similar among sites whereas potassium was significantly higher in soil from site A than from sites B and C.

Endophytic fungal community associated to *H. maritimum*

The results indicate a great diversity and abundance of fungal endophytes associated to *H. maritimum.* A total of 264 isolates belonging to 20 OTUs were isolated from the 27 plants, collected from the three sites (Table 2). Among these, one strain showed <95 % sequence identity with the ones deposited in GenBank and were labeled as "Fungal endophyte sp. 1″ (Table S1). The ITS sequence of Endophyte sp. 2 was not identified to the species level or even to the genus level in the GenBank sequence database. The OTUs identified belonged to 10 genera (Table S1). The genera comprising more diversity were *Alternaria* (6 species) and *Fusarium* (4 species). In terms of richness, *Alternaria* sp.1, *Alternaria* sp.2, *Pyronema domesticum*, Fungal endophyte sp.2 and *Alternaria infectoria* were the dominant, representing together 54.5 % of the total isolates (data not shown). The high diversity of fungal endophytes associated with *H. maritimum* was corroborated by the Simpson and Shannon-Wiener indices estimate (Table 2).

Fungal community comparison between *H. maritimum* organs

The *H. maritimum* organs differed markedly in the percentage of colonization and in the endophytic fungal community. Regardless of the site, was verified that many of the roots (96 %) harboured fungi compared to the twigs (69 %) and leaves (4 %) (Table 2). Similarly, the diversity of fungal endophytes was higher on roots (18 taxa) than on twigs (13 taxa) and leaves (6 taxa) (Table 2). This result was corroborated by the species diversity indices calculate (1/D and H). The roots presented the greatest number of unique species (5 OTUs out of 20, namely *Alternaria clamydospora*, *Aspergillus* sp., *Fusarium* sp.1, *Embellisia* sp. and *Monosporascus* sp.); whereas only one OTU (*Chalastospora ellipsoidea*) was found to be restricted to aboveground *H. maritimum* organs (Fig. 1). Only *P. domesticum*, *Fusarium* sp.2 and *Alternaria* sp.3 were isolated from all the organs surveyed.

The fungal endophyte community of the roots was dominated by Fungal endophyte sp.2 and *P. domesticum* (11.6 % each), *A. infectoria* (10.3 %) and *Alternaria tenuissima* (8.9 %), which occurred on more than 45.9 % (data not shown) of the root segments analysed (Fig. 1). *Alternaria* sp.1 (22.3 %), *Alternaria* sp.2 and (19.6 %) and *Alternaria* sp.3 (10.7 %) were the most dominant OTUs in twigs, infecting more than 43.7 % of this organ segments.

Table 1 Chemical analysis of the soils (A, B and C) with different gradients of salinity (median ± SD, n = 3)

Soils	EC (mS/cm)	pH	Na (mg/g)	K (mg/g)
A	1.3 ± 0.18^{a}	8.2 ± 0.14^{a}	0.8 ± 0.09^{a}	0.13 ± 0.083^{b}
B	3.2 ± 1.47^{b}	7.7 ± 0.16^{a}	1.9 ± 0.79^{b}	0.03 ± 0.018^{a}
C	5.8 ± 1.28^{c}	7.8 ± 0.54^{a}	3.0 ± 0.33^{c}	0.03 ± 0.020^{a}

Different superscript lower case letters denote statistically significant differences ($p < 0.05$) between soils

EC Electric conductivity

Table 2 Abundance and diversity of fungal endophytes isolated from roots, leaves and twigs of *H. maritimum* collected from soils with different gradients of salinity (non-saline –A; slightly saline—B; and moderately saline—C)

Plant organ	Parameters	A	B	C	Total
Roots	S	14	10	13	18
	N	48	45	53	146
	FC (%)	94.1	95.7	98.1	95.9
	H	2.41	1.90	2.17	2.54
	1/D	11.28	5.56	7.87	11.15
Leaves	S	3	2	1	6
	N	3	2	1	6
	FC (%)	6.6	4.4	2.2	4.4
	H	1.10	0.69	–	1.79
	1/D	3.00	2.00	1.00	2.25
Twigs	S	10	9	8	13
	N	45	33	34	112
	FC (%)	90.0	63.5	54.8	69.5
	H	1.69	1.92	1.56	2.16
	1/D	4.57	3.61	3.36	6.84
Total	S	17	13	15	20
	N	96	80	88	264
	FC (%)	63.6	54.6	52.4	56.9
	H	2.40	2.10	2.32	2.72
	1/D	8.72	6.93	8.51	13.25

Frequencies of colonization of fungal endophytes are also shown

S Total number of taxa; *N* total number of isolates; *FC* frequency of colonization; *H* Shannon–wiener; 1/*D* Simpson's reciprocal index

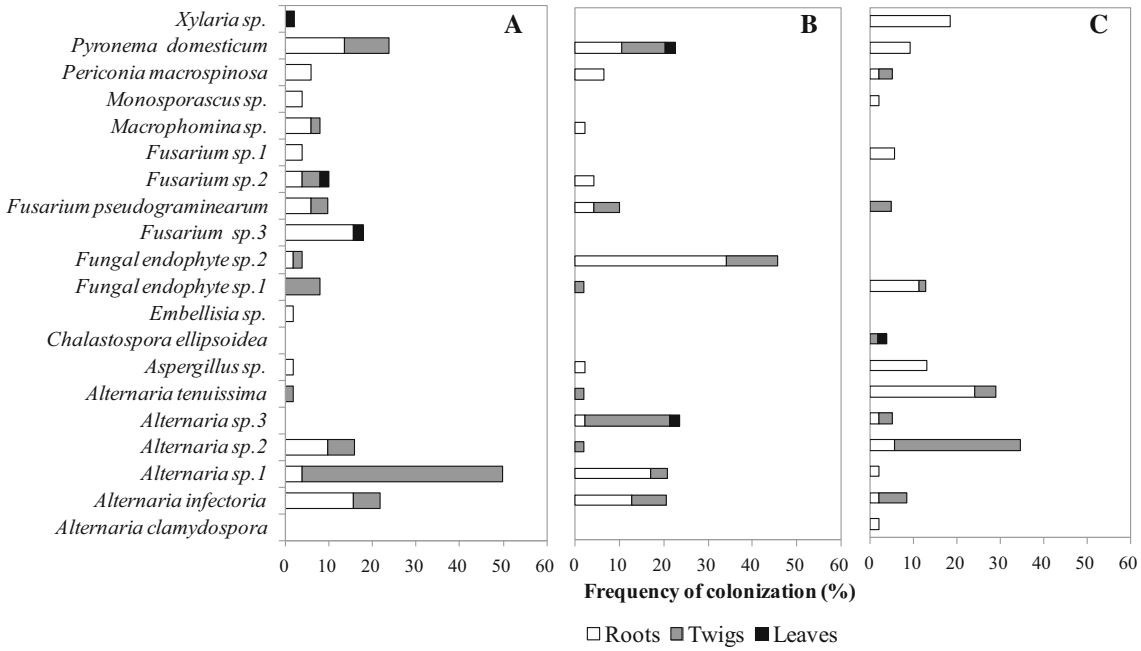


Fig. 1 Frequency of colonization (%) of each fungal endophyte isolated from roots, leaves and twigs of *H. maritimum* collected from soils with different gradient salinity: non-saline (*A*), slightly saline (*B*) and moderately saline (*C*)

The endophytic species inhabiting leaves were represented by one isolate each.

Fungal community comparison between *H. maritimum* collected from soils with different gradient salinity

The diversity and abundance of endophytes differed between *H. maritimum* plants collected from soils with different gradient salinity (Table 2). Endophytic fungi were more diverse and abundant in plants collected in site A (17 OTUs, 96 isolates) when compared to sites B (13 OTUs, 80 isolates) and C (15 OTUs, 88 isolates). However, these differences were not statistically significant ($p = 0.17$–0.52). Overall colonization rate of fungi was highest in plants of site A (64 %), followed by site B (55 %) and C (52 %) (Table 2). The plants collected in sites A, B and C were colonized mostly by *Alternaria* sp.1, Fungal endophyte sp.2 and *Alternaria* sp.2, respectively (Fig. 1). Two OTUs were exclusively found in each of sites A (*Fusarium* sp.3 and *Embellisia* sp.) and C (*C. ellipsoidea* and *A. clamydospora*), whereas no taxa was found to be restricted to site B. Among the 20 OTUs identified, eight were isolated from plants collected in the three sampling sites (A, B and C).

Cluster analyses of endophyte community based on Jaccard's and Bray-Curtis similarity present in two-dimensional NMDS plots showed differences on endophytic assemblage between *H. maritimum* collected from soils with different gradient salinity (Fig. 2). In general, the endophytes clustered according to the sampled sites, being

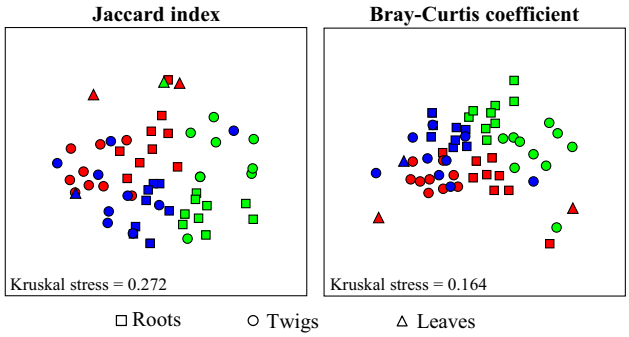


Fig. 2 Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of fungal endophytic community isolated from roots (*square*), twigs (*balls*) and leaves (*triangles*) of *H. maritimum* per site location indicated by different colours (*red* for non-saline—*A*; *blue* for slightly saline—*B*; and *green* for moderately saline—*C*). Clustering analysis was performed with two different community similarity measures, namely Jaccard's index and Bray-Curtis coefficient. Kruskal's stress values inferior to 0.2 represent good ordination plots. (Color figure online)

however endophytes from site C the most clearly separated from sites A and B. This was particularly noticed when the ordination was based on Bray-Curtis, which considered both presence/absence and abundance of total fungal taxa. The confidence of this pattern is tolerable due to the Kruskal's stress value obtained (<0.2). The ANOSIM shows that the overall differences on fungal endophytic composition among sites is statistically significant (Global $R = 0.76$, $p < 0.001$; Table S2). Pairwise comparisons indicated that site A and B were the most similar ($R = 0.50$, $p < 0.001$), whereas site A and C were the most

dissimilar ($R = 0.95$, $p < 0.001$). SIMPER analyses revealed that *Alternaria* sp.3, Fungal endophyte sp.2, *A. tenuissima* and *Aspergillus* sp. account for more than 59.9 % of the average dissimilarity found between the three sites. ANOSIM test confirmed that endophytic composition of roots and twigs of wild barley plants collected in the three sites were different with statistical significance (Table S2). SIMPER analyses, based on mean abundance estimates, showed that Fungal endophyte sp.2, *Alternaria* sp.3 and *A. tenuissima* were the species that contributed more to the difference found between sites A and B, A and C, and B and C, respectively, for both roots and twigs.

Effect of salt concentration on fungal growth

The tolerance index of endophytic fungi on the NaCl amended PDA media, estimated over the incubation period of axenic cultures, is represented in Fig. 3. This analysis was performed for seventeen fungal species, among the twenty isolates, because the remaining fungi failed to grow after several subcultures on synthetic media. This analysis was done with five isolates from soil A (*Embellisia* sp., *Monosporascus* sp., *P. domesticum*, *Fusarium* sp.2 and *Xylaria* sp.), two from soil B (*Alternaria* sp.2 and *Macrophomina* sp.), and ten from soil C (*A. clamydospora*, *A. infectoria, Alternaria* sp.1, *A. tenuissima*, *Aspergillus* sp., *C. ellipsoidea*, *Fusarium pseudograminearum, Fusarium* sp.1, *Periconia macrospinosa* and Fungal endophyte sp.1). There was significant ($F = 36.85$, $p < 0.001$) variation between fungal species for NaCl tolerance. Although all the seventeen fungal species were able to grow at all NaCl concentrations tested. *Xylaria* sp., *C. ellipsoidea*, *Alternaria* sp.1 and *P. domesticum* were found to be the most salt-tolerant, reaching Ti values higher to 1.5 at 200 mM NaCl. By contrast, *Alternaria* sp.2 exhibited the lowest tolerance ($Ti \leq 0.9$).

Tolerance indexes were also found to vary among NaCl concentrations, although no significant differences were detected. For the majority of the endophytes tested, Ti values increased with the higher NaCl concentration up to 100 mM, and afterwards Ti values decreased or remained practically the same. *Xylaria* sp., *A. tenuissima*, *Fusarium* sp.1 and *Fusarium* sp.2 showed, by contrast, a much higher tolerance to high NaCl levels (i.e. 200 mM) than to low NaCl levels (i.e. 50 and 100 mM). This relationship between tolerance and NaCl levels was corroborated by the results obtained from Pearson correlations. A significant positive correlation was found between Ti and NaCl concentrations either when considering all the endophytes ($r = 0.129$, $p < 0.01$) or for most of the species when considered alone (range of coefficients 0.175–0.795, $p < 0.01$ or $p < 0.05$; Table S3).

It was also noticed that Ti values changed significantly ($F = 33.13$, $p < 0.001$) over the incubation period. In general, Ti values decreased as days progress after incubation, which was further confirmed by the significant negative correlation found between these two parameters ($r = -0.148$, $p < 0.01$). In fact, at 4 days of incubation, Ti values were higher (reaching values up to 10.0) than at the end of 8 and 12 days of incubation (reaching values up to 1.5). The most salt-tolerant fungi after 4 and 8 days of incubation were *Xylaria* sp. (Ti ranging from 0.9 to 10.0) and *C.*

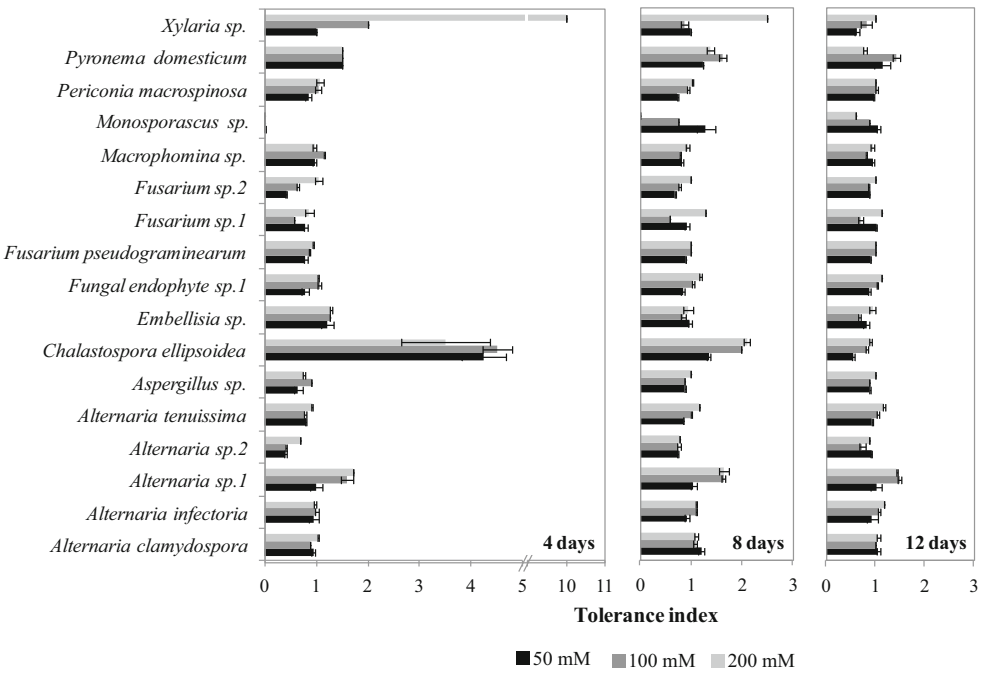


Fig. 3 Tolerance index (median ± SE, n = 3) of endophytic fungi strains grown on PDA medium amended with three different concentrations of NaCl (50, 100 and 200 mM), recorded after 4, 8 and 12 days of incubation

ellipsoidea (Ti ranging from 1.4 to 4.5); while at 12 days of incubation were *Alternaria* sp.1 and *P. domesticum* (Ti ranging from 1.0 to 1.5 and from 0.8 to 1.4, respectively). When the relationship between tolerance and incubation period was assessed for each fungal species, a positive significant correlation was found for 10 taxa (coefficients ranging from 0.220 to 0.847, $p < 0.01$ or $p < 0.05$) and a negative significant correlation for 5 taxa (coefficients ranging from −0.280 to −0.868, $p < 0.01$ or $p < 0.05$; Table S3).

The relationship between Ti values obtained from the tested endophytes and soil chemical characteristics of sampled sites (A, B and C), were investigated by means of a PCA (Fig. 4). The PCA showed a clear separation of the three sampling sites surveyed. However, it was noticed that chemical soil variables (pH, EC, N and P contents) of these three sites and tolerance indices of all the tested fungal isolates were, in general, not related to each other. In fact, among the seventeen fungal endophytes tested only ten were clustered with their original sites (one from site A, two from site B and seven from site C).

Discussion

This study is the first reporting the presence of fungal endophytes in *H. maritimum* growing in soils with different levels of salinity. Based on EC results, the surveyed soils

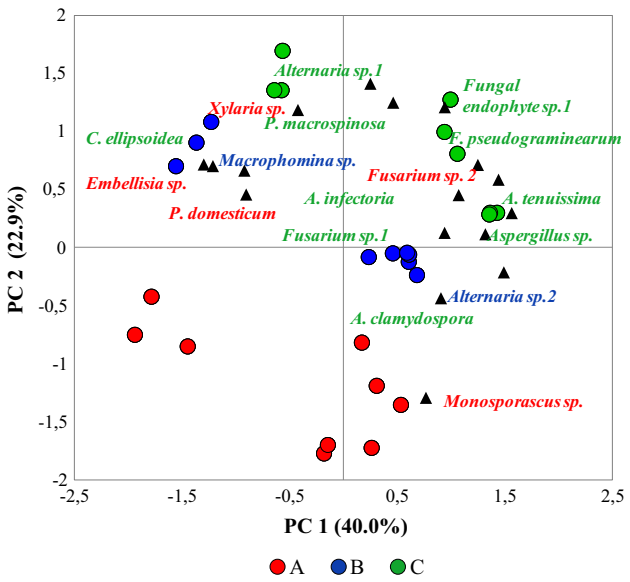


Fig. 4 Principal component analysis of soil chemical variables (pH, EC, N and P contents) and tolerance index of endophytic fungi recorded after 4, 8 and 12 days of incubation time under 50, 100 and 200 mM of NaCl. Points indicate sites of soil sampling: non-saline (*A*), slightly saline (*B*) and moderately saline (*C*). Endophytic fungi indicated in red, blue and green are isolated from *H. maritimum* collected in sites *A*, *B* and *C*, respectively. The PCA factors explain 62.9 % of the total variance

are classified as non-saline (soil A; EC <2), slightly saline (soil B; 2.1< EC <4) and moderately saline (soil C, 4.1< EC <8) (FAO 1988). All plants collected from these soils were colonized by fungal endophytes at high frequency (>52 %). The identified OTUs belong to Ascomycota. *Alternaria* spp. and *Fusarium* spp. were the dominant taxa observed. These species were also reported to be dominant endophytes in other halophytes growing in different geographical locations, such as Egypt, India, China and South Korea (El-Morsy 2000; Sun et al. 2011; You et al. 2012; Kannan et al. 2014). The isolation of these genera from multiple host halophytes growing in distant continents suggests that they are host-generalists. Their occurrence in halophytes also indicated that, at least, some species of these genera are well adapted to saline environments and may have a role in plant adaptation. However, other endophytes, also found in the present study, show some degree of host specificity. One example is *C. ellipsoidea,* which to our knowledge has not been previously recorded as a plant endophyte. Other species recovered in the present study such as *P. macrospinosa*, *P. domesticum*, *Monosporascus* sp. and *Macrophomina* sp. have similarly not been commonly isolated from halophytes (Suryanarayanan and Kumaresan 2000; El-Morsy 2000; Sun et al. 2011; Maciá-Vicente et al. 2012; You et al. 2012; Kannan et al. 2014).

The composition of the endophytic community of *H. maritimum* varied substantially between above- and belowground organs, suggesting an influence of the environment where the organs grew (soil *vs*. air) as well as the host tissue type (roots *vs*. leaves/twigs) on fungal assemblages. To our knowledge, only Kannan et al. (2014) have recently studied fungal endophytes simultaneously in leaf, stem and roots of the halophytes *Spinifex littoreus* (Poaceae), *Salicornia* sp. (Amaranthaceae), *Portulaca portulacastrum* (Aizoaceae) and *Bauhinia* sp. (Fabaceae), and they found little similarity between these plant organs. Wearn et al. (2012) also reported low similarity on endophytic fungal assemblages between roots and leaves of three co-occurring non-halophytes grassland forbs (*Cirsium arvense*, *Plantago lanceolata* and *Rumex acetosa*).

In the present study, the observed exclusive occurrence of some fungal OTUs to either roots (*A. clamydospora*, *Aspergillus* sp., *Fusarium* sp.1, *Embellisia* sp. and *Monosporascus* sp.) or aboveground (*C. ellipsoidea*) *H. maritimum* organs, suggested a lack of systemic growth by these endophytes from one organ to another. This result also suggested that air and soil could be an important source of endophytic fungi in above- and belowground organs, respectively. In fact, the endophytic fungal community present on *H. maritimum* comprise several genera (e.g. *Fusarium* spp., *Alternaria* spp.) that are reported to be the most abundant in the atmosphere (Frohlich-Nowoisky

et al. 2012) and soil (Tedersoo et al. 2014). It is also noteworthy that, in the present study, roots host higher diversity and were more frequently colonized by fungal endophytes than aboveground organs. Similar result has been obtained from studies on other halophytic (Kannan et al. 2014) and non-halophytic (Wearn et al. 2012) plants. The aboveground plant organs are exposed to stress factors such as UV radiation, desiccation and lack of nutrients (Lindow and Brandl 2003) that could account for the lower fungal diversity and colonization of leaves and twigs comparatively to roots. Airborne microorganism concentration were typical lower than those of soilborne microorganisms (Bulgarelli et al. 2013), which presumably may also contribute to the differences found on fungal diversity and abundance between above- and belowground *H. maritimum* organs.

The composition of fungal endophytic community associated to *H. maritimum* varied significantly among plants collected from non-saline (A), slightly saline (B) and moderately saline (C) soils. For example, *Alternaria* sp.3 and Fungal endophyte sp.2 attained higher average abundances within plants from soil B and were virtually absent in plants from soil A and C, respectively. In the moderately saline soils, *H. maritimum* plant assemblages were strongly dominated by *Alternaria* sp.2, *A. tenuissima* and *Xylaria* sp., which were not the most prevalent in plants from soils A and B. This difference on fungal endophytic communities in plants collected from soils with different levels of salinity was observed for above- as well as for belowground *H. maritimum* organs. Therefore, *H. maritimum* appeared to harbor an endophytic fungal community that varied with its position along the salinity gradient on Soliman Sebkha. This variation on fungal composition, at a relative space restricted scale, suggests that soil salinity levels had a large influence on the structure of fungal endophytic communities of *H. maritimum*. It is possible that, in each of the soils surveyed, specific adaptations by certain fungi either to live in high salt conditions or to enter and colonize *H. maritimum* tissues may take place. These endophytes seem to have an important ecological role by contributing to host plant adaptation to salt stress, as reported earlier for the endophyte *Fusarium culmorum* (Redman et al. 2011). Future research should be performed in order to clarify the possible contribution of the endophytes obtained in our study in the survival of *H. maritimum* under high salt conditions. Previous studies of fungal endophytes associated with the roots of the halophyte *Inula crithmoides* grown along a space restricted salinity gradient have also shown distinct fungal communities (Maciá-Vicente et al. 2012). According to the same authors this difference in fungal assemblage is largely due to the soil salinity levels.

Despite the overall trend for reduced diversity, abundance and frequency of endophytic fungal colonization of *H. maritimum* as the soil salinity increased, the differences on these parameters between the three surveyed soils (A, B and C) were not statistically significant. Interestingly, this decrease in fungal richness and colonization from soil A to C was most notorious in aboveground organs of *H. maritimum* than in its roots. In the roots a slightly increase (up to 1.1-fold) in fungal abundance and frequency of colonization occurred as the soil salinity increased. A similar pattern has been previously observed in roots of the halophyte *I. crithmoides* collected between a salt marsh and a sand dune (Maciá-Vicente et al. 2012). We hypothesize that under non-stressful salt conditions *H. maritimum* may not be willing to spend energy for the development of the association with fungal endophytes. However, at high soil salt levels, colonization of host plant roots by fungal endophytes increased to allow plants to grow in such harsh conditions. Rice plants inoculated with the endophyte *F. culmorum* also showed an increase in the percentage of colonized roots when exposed to salt stress conditions compared to the same plant in the absence of stress (Redman et al. 2011).

Several susceptibilities to the NaCl have been noticed among the genera and even among isolates of the same genus. For example, the most salt-tolerant endophytes identified belonged to different genera such as *Xylaria*, *Chalastospora*, *Alternaria* and *Pyronema*. As far as we known, among these genera only *Alternaria* was previously identified in natural hypersaline environments (Gunde-Cimerman et al. 2009; Wang et al. 2013; Gunde-Cimerman and Zalar 2014). These variations in the salt tolerance may be attributed to the different mechanisms of tolerance exhibited by different isolates (Plemenitaš et al. 2014). Tolerance to NaCl also decreased with incubation time, which suggested that toxicity of NaCl increased with incubation time. This was presumably due to the increase with time of the levels of toxicant ions (Na^+ and/or Cl^-) and/or the occurrence of osmotic stress, which have a detrimental effect on microbial growth (Yancey 2005). For the majority of the endophytes tested, increased Ti values occurred as NaCl concentration increased up to 100 mM. It is possible that these isolates required a certain level of NaCl in the culture medium, because of their origin from salt-affected soils. Similarly, diverse findings reported that some microorganisms prefer or require a high salt (NaCl) medium to grow (Kralj Kuncic et al. 2010; Zajc et al. 2014). For example, *Wallemia ichthyophaga* is a fungus that grows only on solid or liquid medium supplemented with NaCl at concentrations between 15 and 20 % (wt/vol) (Zajc et al. 2014). At 10 % NaCl the growth rate of this fungus was the lowest and the colonies were the smallest. A similar behavior was also described for many other fungal species (e.g. *Aspergillus* spp., *Penicillium* spp. and related teleomorphic genera *Emericella* and *Eurotium*) that

were isolated from hypersaline environments (reviewed by Gunde-Cimerman and Zalar 2014).

Findings of the present study also indicate that only a few fungal endophytes (e.g. *Alternaria* sp. 1 and *C. ellipsoidea*) isolated from *H. maritimum* collected in moderately saline soils exhibited high tolerance to NaCl. Among the most salt-tolerant endophytes identified, two were isolated from *H. maritimum* plants collected in non-saline soil (*Xylaria* sp. and *P. domesticum*). This suggested that the tolerance of the isolates exhibited under in vitro conditions depended much more on the endophyte tested than on the sites of its isolation. Similarly, Maciá-Vicente et al. (2012) have reported the high sensitivity to NaCl under in vitro conditions of an endophytic fungus of the halophyte *I. crithmoides* collected from lower salt marsh; whereas other fungal isolates which were rare or absent in the lower salt marsh were unaffected by NaCl. In our study, there are two main reasons for the differences found on fungi behavior between natural and in vitro conditions: (1) under natural conditions, the distribution of endophytes along the salinity gradient on Soliman Sebkha, may be influenced by other factors, beyond soil salinity, and in the in vitro experiments such factors are absent; (2) and/or in natural conditions, the salt tolerance exhibited by the endophytes could be a result of its association with the host plant. We hypothesized that the establishment of plant-endophyte association may probably aid fungi to escape or mitigate the impacts of salt stress, as previously suggested (Maciá-Vicente et al. 2012).

In conclusion, under natural conditions the colonization of *H. maritimum* tissues, especially roots, by fungal endophytes is promoted in saline soils for adaptation and/or survival of both interacting species to hostile environmental conditions. The endophytes *Alternaria* sp. 1 and *C. ellipsoidea*, seems to have specific relationships with local *H. maritimum* growing in saline soils and their ecological and agronomic role (e.g. adaptation to salinity), is worthy for further investigation.

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References

Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 27:325–349

Bulgarelli D, Schlaeppi K, Spaepen S, van Themaat EVL, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838

Clarke J (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143

Clarke KR, Gorley RN (2015) PRIMER v7: User Manual/Tutorial. PRIMER-E, Plymouth

El-Morsy EM (2000) Fungi isolated from the endorhizosphere of halophytic plants from the Red Sea Coast of Egypt. Fungal Divers 5:43–54

FAO (1988) Salt-affected soils and their management. FAO Soils Bull. 39. FAO, Rome, Italy

Frohlich-Nowoisky J, Burrows SM, Xie Z et al (2012) Biogeography in the air: fungal diversity over land and oceans. Biogeosciences 9:1125–1136

Gunde-Cimerman N, Zalar P (2014) Extremely Halotolerant and Halophilic Fungi Inhabit Brine in Solar Salterns Around the Globe. Food Technol Biotech 52:170–179

Gunde-Cimerman N, Ramosb J, Plemenitas A (2009) Halotolerant and halophilic fungi. Mycol Res 113:1231–1241

Hafsi C, Lakhdhar A, Rabhi M, Debez A, Abdelly C, Ouerghi Z (2007) Interactive effects of salinity and potassium availability on growth, water status, and ionic composition of *Hordeum maritimum*. J Plant Nutr Soil Sci 170:469–473

Henderson PA, Seaby RMH (2007) Community Analysis Package 4.0. Pisces Conservation Ltd, Lymington, UK

Kannan KP, Kumar DM, Ramya PR, Nika SM, Meenatchi G, Sowmya AN, Bhuvaneswari S (2014) Diversity of endophytic fungi from salt tolerant plants. Int J ChemTech Res 6:4084–4088

Khan AL, Hamayun M, Ahmad N, Hussain J, Kang SM, Kim YH, Adnan M, Tang DS, Waqas M, Radhakrishnan R, Hwang YH, Lee IJ (2011) Salinity stress resistance offered by endophytic fungal interaction between *Penicillium minioluteum* LHL09 and glycine max. L J Microbiol Biotechnol 21:893–902

Khan AL, Waqas M, Khan AR, Hussain J, Kang SM, Gilani SA, Hamayun M, Shin JH, Kamran M, Al-Harrasi A, Yun BW, Adnan M, Lee IJ (2013) Fungal endophyte *Penicillium janthinellum* LK5 improves growth of ABA-deficient tomato under salinity. World J Microbiol Biotechnol 29:2133–2144

Kralj Kuncic M, Kogej T, Drobne D, Gunde-Cimerman N (2010) Morphological response of the halophilic fungal genus *Wallemia* to high salinity. Appl Environ Microbiol 76:329–337

Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883

Lombardi T, Lupi B (2006) Salt-tolerance in wild *Hordeum* (poaceae) species: differences between *H. maritimum* with. and *H. hystrix* roth. Atti Soc tosc Sci nat, Mem, Serie B 113:31–35

Maciá-Vicente JG, Ferraro V, Burruano S, Lopez-Llorca LV (2012) Fungal assemblages associated with roots of halophytic and non-halophytic plant species vary differentially along a salinity gradient. Microb Ecol 64:668–679

Magurran AE (2004) Measuring Biological Diversity. Blackwell, Hoboken

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Oliveira I, Pereira JA, Lino-Neto T, Bento A, Baptista P (2012) Fungal diversity associated to the olive moth, *Prays oleae* Bernard: a survey for potential entomopathogenic fungi. Microb Ecol 63:964–974

Plemenitaš A, Lenassi M, Konte T, Kejžar A, Zajc J, Gostinčar C, Gunde-Cimerman N (2014) Adaptation to high salt concentrations in halotolerant/halophilic fungi: a molecular perspective. Front Microbiol 5:199

Rabhi M, Hafsi C, Lakhdar A, Hajji S, Barhoumi Z, Hamrouni MH, Abdelly C, Smaoui A (2009) Evaluation of the capacity of three halophytes to desalinize their rhizosphere as grown on saline soils under nonleaching conditions. Afr J Ecol 47:463–468

Redman RS, Kim YO, Woodward CJDA, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to

abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS ONE 6:e14823

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim YO, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. ISME J 2:404–416

Rodriguez RJ, Woodward CS, Redman RS (2012) Fungal Influence on Plant Tolerance to stress. In: Southworth D, Wiley J & Sons (ed) Biocomplexity of Plant-Fungal Interactions. Wiley-Blackwell, Hoboken, pp 155-164

Seaby RM, Henderson PA (2006) Species Diversity and Richness Version 4

Shrivastava P, Kumar R (2015) Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci 22:123–131

Smallwood MF, Calvert CM, Bowles DJ (1999) Plant Responses to Environmental Stress. BIOS Scientific Publishers Limited, Oxford

Sun Y, Wang Q, Lu XD, Okane I, Kakishima M (2011) Endophytic fungi associated with two *Suaeda* species growing in alkaline soil in China. Mycosphere 2:239–248

Suryanarayanan TS, Kumaresan V (2000) Endophytic fungi of some halophytes from an estuarine mangrove forest. Mycol Res 104:1465–1467

Tedersoo L, Bahram M, Toots M et al (2014) Global diversity and geography of soil fungi. Science 346:1256688

Wang F, Li A, Dai D, Xu X, Li D (2013) New halotolerant species of *Alternaria* from Qinghai-Tibet Plateau, China. Mycotaxon 123:251–253

Waqas M, Khan AL, Kamran M, Hamayun M, Kang SM, Kim YH, Lee IJ (2012) Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. Molecules 17:10754–10773

Wearn JA, Sutton BC, Morley NJ, Gange AC (2012) Species and organ specificity of fungal endophytes in herbaceous grassland plants. J Ecol 100:1085–1092

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A guide to Methods and Applications. Academic Press, San Diego, pp 315–322

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208:2819–2830

Yensen NP (2008) Halophyte uses for the twenty-first century. In: Khan MA, Weber DJ (eds) Ecophysiology of High Salinity Tolerant Plants. Springer, Dordrecht, pp 367–396

You YH, Yoon H, Kang SM, Shin JH, Choo YS, Lee IJ, Lee JM, Kim JG (2012) Fungal diversity and plant growth promotion of endophytic fungi from Six halophytes in Suncheon bay. J Microbiol Biotechnol 22:1549–1556

Zajc J, Kogej T, Galinski EA, Ramos J, Gunde-Cimerman N (2014) The osmoadaptation strategy of the most halophilic fungus, *Wallemia ichthyophaga*, growing optimally at salinities above 15% NaCl. Appl Environ Microbiol 80:247–256