A window into fungal endophytism in *Salicornia europaea*: deciphering fungal characteristics as plant growth promoting agents

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Abstract
Aim Plant-endophytic associations exist only when equilibrium is maintained between both partners. This study analyses the properties of endophytic fungi inhabiting a halophyte growing in high soil salinity and tests whether these fungi are beneficial or detrimental when non-host plants are inoculated.

Method Fungi were isolated from *Salicornia europaea* collected from two sites differing in salinization history (anthropogenic and naturally saline) and analyzed for plant growth promoting abilities and non-host plant interactions.

Results Most isolated fungi belonged to Ascomycota (96%) including dematiaceous fungi and commonly known plant pathogens and saprobes. The strains were metabolically active for siderophores, polyamines and indole-3-acetic acid (mainly *Aureobasidium* sp.) with very low activity for phosphatases. Many showed proteolytic, lipolytic, chitinolytic, cellulolytic and amylolytic activities but low pectolytic activity. Different activities between similar fungal species found in both sites were particularly seen for *Epicoccum* sp., *Arthrinium* sp. and *Trichoderma* sp. Inoculating the non-host * Lolium perenne* with selected fungi increased plant growth, mainly in the symbiont (*Epichloë*)-free variety. *Arthrinium gamsii* CR1-9 and *Stereum gausapatum* ISK3-11 were most effective for plant growth promotion.

Conclusions This research suggests that host lifestyle and soil characteristics have a strong effect on endophytic fungi, and environmental stress could disturb the plant-fungi relations. In favourable conditions, these fungi may be effective in facilitating crop production in non-cultivable saline lands.

Keywords Salinity · Halophyte · Grass · Endophyte · Fungi · Plant growth promotion

Introduction
Endophytic fungi are the functionally vital members of the plant microbiome colonising varied plant species (Porras-Alfaro and Bayman 2011; U’Ren et al. 2012) causing no visible damage or disease symptoms in their host (Rodriguez et al. 2008). Abiotic factors, e.g. seasonal fluctuations and changes in soil chemical properties disturb the microbiological balance, eventually favouring the survival and increase of the more robust and competitive microbes, which commonly include the fungal pathogens or saprophytes (Pritchard 2011).
Although some endophytes evolve from plant pathogenic fungi and may cause disease of plants under stress conditions (Redman et al. 2005), the true endophyte state (non-pathogenic) is established during their long-term association in the host plant.

Fungal enzymes play an important role in the mineralisation and degradation of organic materials and these enzymes may affect other microorganisms, by competing for plant host entry and nutrients inside the plant. Most plants contain secondary metabolites that protect them from pathogens, and some metabolites probably affect the entry of endophytic fungi (Jia et al. 2016). The fungi secrete detoxification enzymes, such as cellulases, lipase, xylanase, and protease, which can decompose these secondary metabolites before they penetrate the defence systems of their host (Sieber 2007). The plant—endophytic microbial interaction only exists when equilibrium is achieved between the colonising fungi and the chemical reactions of the host plant and maintained over time (Kogel et al. 2006).

Once the endophytic niche is established, the fungi may benefit and enhance plant fitness. Plants inhabited by fungal endophytes show increased vegetative vigour (Gropppe et al. 1999); higher production of generative tillers (Clay 1990); better defences against invasive fungi (Arnold et al. 2003) and various phytopathogens (Arnold et al. 2003; Mendoza and Sikora 2009); deterrence against herbivores (Herre et al. 2007), insects (Wilkinson et al. 2000) and nematodes (Kimmons et al. 1999); and abiotic stress tolerance (Redman et al. 2002; Rodriguez et al. 2004). The endophytic fungi may produce vital bioactive metabolites and secondary compounds, exclusive of those produced by their host plants that may contribute to plant fitness and that can stimulate the production of novel metabolites for pharmaceutical use (Zhang et al. 2006). In return, the residing endophytes gain a specific ecological niche, access to nutrients, protection from abiotic stress and desiccation, and dissemination to the next generation of hosts (vertical transmission via seeds) (Faeth and Fagan 2002).

The functional role of endophytic fungi may be determined by the type of environment they are adapted to. For instance, endophytic fungi isolated from plants in geothermal soils demonstrate thermotolerance (Rodriguez et al. 2004), those from heavy metal contaminated areas revealed significant mechanisms of metal tolerance (An et al. 2015) and strains obtained from medicinal plants produced bioactive compounds (Ganley et al. 2004). Endophytic fungi isolated from the halophyte *Suaeda salsa* conferred salt stress tolerance in rice seedlings (Qin et al. 2017). Thus it is clear that the traits of endophytic fungi are imposed by the type and conditions of their host plant (e.g. Arnold et al. 2003; Redman et al. 2002; Waller et al. 2005).

Despite extensive work on fungal endophyte—halophyte systems (e.g. Bilal et al. 2018; Gopi and Jayaparakashvel 2017; Indira et al. 2015; Kalmuratova et al. 2015; Macia-Vicente et al. 2012; Sun et al. 2011a, b), there is limited information on the properties of endophytic fungi from saline habitats and how they contribute to improving the plant performance. Consequently, in this study we investigated a salt-accumulating halophyte, *Salicornia europaea* L. (family Amaranthaceae), which is one of the most salt tolerant halophytes in the world (Nikalje et al. 2017). This halophyte can develop in very high salinity conditions (1 M NaCl in soil) (Ushakova et al. 2005). *S. europaea* is extensively established in the two saline sites selected in this study. It is generally identified to comprise varied physiological and biological adaptive mechanisms, which allow it to survive under extreme saline conditions (Nikalje et al. 2017). Since *Salicornia* endophytes establish and stabilise in their host tissues, it is interesting to speculate whether these fungi inherit traits related to alleviating salt stress conditions (e.g. different source of salinity present at two test sites: anthropogenic and natural) or whether they produce some beneficial compounds. Endophytic bacterial strains isolated from other halophytes show plant growth promoting capabilities when *Salicornia brachiata* (Jha et al. 2012), *S. europaea* (Zhao et al. 2016), *Limonium sinense* (Qin et al. 2014), *Beta vulgaris* (Piernik et al. 2017), *Cucumis sativus* and *Oryza sativa* (Yuan et al. 2016) were inoculated. Similarly, some studies on the plant growth promoting traits of endophytic fungi isolated from halophytes were observed in *Carex kobomugi* (Hwang et al. 2011), Waite rice seedlings (Young-Hyun et al. 2012) and Chinese white poplar (Pan et al. 2018). To our knowledge, this is the first report on the potential role of endophytic fungi from *S. europaea*.

In our previous report, we performed metagenomic analysis to reveal the *Salicornia* endophytic bacterial and fungal community in the same selected saline sites in two seasons (Furtado et al. 2019). Hence, in this study we aim to reveal the culturable endophytic fungal diversity as a consequence of differing soil salinity at the two sites and the potential activity of endophytic fungi in promoting non-host plant growth. We selected the perennial ryegrass *Lolium perenne* as a non-host model plant as it is particularly popular for its high-quality turf and valuable forage (Wilkins 1991). This grass is
associated with Epichloë sp. (known as Neotyphodium sp.) which is a symbiont of cool-season grasses of the family Pooidae that is vertically transmitted through seeds (Leuchtmann et al. 2014). Species of Epichloë are known to produce bioactive alkaloids that protect the grasses from herbivores and insect damage (Saikkonen et al. 2016). We hypothesised that: (i) fungal endophytes of the obligatory halophyte S. europaea consist of a unique group of isolates with plant growth promoting metabolic activity and tolerance to high salt concentrations, analogous to their host, (ii) the resilience and compatibility of fungal endophytes can be tested by inoculating them in non-host plants, which can prove to be a beneficial association.

Materials and methods

Sample collection and isolation of endophytic fungi

The plant samples of S. europaea were collected from two saline sites. The two saline sites are located in the Kujawy region in Central Poland: site 1 (S1) is situated in the vicinity of three brine graduation towers in the Spa Park in Ciechocinek (natural source of salinity) and site 2 (S2) is a meadow next to the waste ponds of a soda factory in Inowroclaw (anthropogenic source of salinity). The characteristics of the soils at the two sites are previously described in Furtado et al. 2019. S1 sometimes has significantly higher salinity than S2, with the electrical conductivity (ECe) of soil samples collected in autumn 2015 being 100.5 and 76.0 dS m⁻¹ respectively. However, the ECe of the soil samples collected in the spring 2016 were quite similar at 51.1 and 59.7 dS m⁻¹ respectively. Soil samples from S1 had significantly higher Na⁺ levels, while S2 had significantly higher Ca²⁺ levels in both sampling seasons (Furtado et al. 2019).

The plant samples (each sample consisting of at least 10 plants) were collected from three plots in each of the saline sites during the two seasons (autumn 2015 and spring 2016). Samples from each plot were collected in triplicate (36 samples in total). The plants were thoroughly washed in sterile distilled water to remove adhering soil followed by the surface sterilization technique described by Hryniewicz et al. (2010) with modifications. The shoots and roots were separately washed in sterile 2% NaCl (6 times), and subsequently using sterile hydrogen peroxide (H₂O₂) solution for 5 min (2% H₂O₂ for shoots and 5% H₂O₂ for roots). The plant samples were finally rinsed with sterile 2% NaCl (3 times) and the last solution was plated to check for optimization of sterilization technique. The shoots and roots were dried using sterile filter paper and ground to a fine paste in a mortar. A series of serial dilutions were performed. Using the pour plate technique 1 ml suspension was mixed with potato dextrose agar (PDA) (Difco, BD Biosciences) supplemented with tetracyclin (30 μg/ml). To increase the probability of obtaining most of the culturable endophytic fungi, PDA was amended with different salt concentrations (0 mM, 100 mM, and 200 mM NaCl). All the dilutions and plating were prepared in three replicates. The plates were incubated at 24 ± 2 °C and observed regularly for fungal growth and isolates were selected (selection based on the colony morphological features such as colour, margin, mycelium form and microscopic slide preparations as described in Germain and Summerbell (2010) and transferred to fresh PDA plates during this period.

Molecular identification of endophytic fungi

DNA isolation for 320 endophytic fungi was performed using GeneMATRIX environmental DNA extraction kit (EurX, Poland) following the manufacturer’s protocol. Universal primers ITS1 (5-CTTGGTCTATTTAGAAAGTAGGTAATTGATATGC-3) and ITS4 (5-TCCCTCGGTATTGATATGC-3) (Manter and Vivanco 2007) were used to amplify the internal transcribed sequence (ITS) region using thermal cycler (Qiagen). Amplification products were resolved by agarose gel electrophoresis (1%) and visualized using a gel documentation system (UVP, MultiDoc-It™ System). The PCR products were purified using the PCR purification kit (GenoPlast Biochemicals), quantified at 260 nm using Nanodrop (Thermo Scientific™ NanoDrop 2000) and confirmed on agarose gel electrophoresis (1%). The samples were commercially sequenced in the sequencing facility of the Institute of Biochemistry and Biophysics Polish Academy of Sciences (IBB, Warsaw, Poland). The sequences were analyzed using the Sequencher version 5.1 and were compared with available sequences from the NCBI database. The sequences showing 99% similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLASTn) program available at the National Center for Biotechnology Information (NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST). Evolutionary analyses were conducted in MEGA 7 (Tamura et al. 2013).
Plant growth promoting (PGP) traits of endophytic fungi

Thirty-nine endophytic fungal strains (Fig. 3) were selected from a collection of 320 fungal isolates. Selection criteria were based on information provided in the literature search for all strains, selecting only non-pathogenic strains for further screening. The fungal strains were cultivated on PDA agar at 24 ± 2 °C and the 7-day old mycelium was used as inoculum for screening all activities. All the tests were conducted in triplicate. Positive and negative controls using bacterial strains were also maintained for all experiments (strains previously reported by Szymańska et al. (2016) and strain collection retrieved from the Department of Microbiology, Nicolaus Copernicus University, Poland).

Synthesis of siderophores

Secretion of siderophores by the strains was determined according to the modified method of Alexander and Zuberer 1991 using Chrome azurol S (CAS) agar media. The strains were inoculated on CAS agar and incubated at 26 ± 2 °C for 7 days.

Phosphate solubilizing capacity

The efficiency of strains to solubilize organic phosphates was determined according to Pikovskaya (1948). The strains were inoculated on the Pikovskaya agar containing two different sources of phosphates. One set contained Pikovskaya agar with tricalcium phosphate (TCP) while the other set contained Pikovskaya agar with dicalcium phosphate (DCP). The plates were incubated at 24 ± 2 °C for 7 days.

Indole-3-acetic-acid (IAA) synthesis

The ability of endophytic fungi to produce IAA was assessed based on the colorimetric methods described by Bose et al. (2013) and Gordon and Weber (1951) with slight modifications. Fungal strains from PDA plates were inoculated in 100 ml of Malt Extract medium (Difco) supplemented with 500 μg/ml L-tryptophan and incubated at 24 ± 2 °C on a shaker at 120 rpm for 7 days. The fermented broth was centrifuged at 8000 rpm for 15 mins to separate mycelium growth. The above supernatant (2 ml) was used to estimate the amount of IAA produced by the fungal strains.

Polyamine production

Polyamine production was detected by inoculating strains on Long Ashton Decarboxylase (LAD) agar (protocol according to Amprayn et al. 2012) and pH was adjusted to 5.5 (Cloete et al. 2009). Inoculated LAD agar plates were incubated in the dark at 24 ± 2 °C for 7 days. Red halos on a yellow background indicated arginine decarboxylation by the strain.

Enzyme production by endophytic fungi

Enzyme assays for the selected 39 endophytic fungal strains were assessed for the following activities: proteolytic activity for hydrolysis of gelatin (Smibert and Krieg 1994), cellulolytic activity for hydrolysis of carboxymethylcellulose (CMC) (Berg and Pettersson 1977), pectinolytic activity for pectin hydrolysis (Strzelczyk and Szpotański 1989), chitinolytic activity for chitin hydrolysis (Agrawal and Kotasthane 2012), amylolytic activity for starch hydrolysis and lipolytic activity for hydrolysis of tributyrin (Gibson and Gordon 1974). The endophytic fungal strains were cultivated in fresh Potato dextrose agar (PDA) plates and used for the analysis of different enzyme activities. The test plates were incubated at 24 ± 2 °C. The plates were stained after 8 days and the presence of a halo zone or zone of clearance around the fungal mycelia confirmed the positive enzyme activity. Positive and negative bacterial strains for all the activities were used as controls (these strains previously reported by Szymańska et al. (2016) and strain collection retrieved from the Department of Microbiology, Nicolaus Copernicus University, Poland).

Salt tolerance tests

The salt tolerance of endophytic fungal strains was evaluated on different concentrations of NaCl. The test was performed by transferring 7-day old fungal mycelium plugs onto PDA agar plates supplemented with 0 mM, 200 mM, 500 mM and 700 mM NaCl concentrations. All tests were performed in triplicate. The plates were incubated at 24 ± 2 °C and the diameter of the mycelium was measured at 2, 5 and 7 days during the incubation period.
Plant growth promotion experiment

We selected *Lolium perenne* as a model plant in this study, to test the effects and endophytes’ capability in promoting plant growth. The seeds of the two ryegrass (*L. perenne*) varieties: variety 1 (*Epichloë* free: E) and variety 2 (*Epichloë* infected: E) were provided by DLF Seeds A/S, Denmark. The two varieties were sown in trays (54 pots of 6 × 6 cm) filled with sterile sand: vermiculite (1:1). The plants were grown in a sterile growth chamber at ~22-24 °C and a 16 h light period under a sodium lighting system. Simultaneously, the six endophytic fungal strains were selected (based on their metabolic activity: positive for IAA, siderophores, polyamines and enzyme activity: positive for cellulase, protease). These strains were cultivated in malt extract medium (Difco, BD) for 7 days, then the fungal mycelium was collected and washed in sterile distilled water and crushed using sterile glass beads to obtain dispersed inoculum. This fungal mycelium (500 μl) was inoculated at the shoot-root junction of the 1 week old germinated ryegrass seedlings and maintained under controlled conditions. The plants were watered using sterile distilled water twice a week and Hoagland’s medium was added once a week during the 7 week incubation period. The non-inoculated plants of the two varieties served as controls and 27 plant replicates were maintained for all treatments. Twelve plants were randomly selected from each treatment and the remaining plants were stored for further analysis. Observations on the shoot and root length, wet and dry weight of roots and shoots were evaluated.

Data analysis

The coefficient of activity (W<sub>act</sub>) was calculated using the formula proposed by Hryniewicz et al. 2010: where S<sub>h</sub> indicates the diameter of the hydrolysis zone, S<sub>c</sub> indicates the colony diameter and t indicates the time of incubation, W<sub>act</sub> = S<sub>h</sub><sup>2</sup> / (S<sub>c</sub> × t). Statistical analysis for the data showing the effect of fungal inoculation on plant growth was conducted using Statistica software package (version 10.0, StatSoft 2006). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2008). Reference sequences (100 sequences) were obtained from the NCBI blast search. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is depicted next to the branches (Felsenstein 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The phylogenetic tree was redesigned using Interactive tree of life (iTOL) v3 (Letunic and Bork 2016).

Results

Taxonomic classification of endophytic fungal strains of *S. europaea*

In total, 320 endophytic fungal strains were isolated from the roots and shoots of *S. europaea* collected from the two salt-affected sites during the two seasons. All DNA sequences obtained were submitted to GenBank SUB5097742 and accession numbers were assigned: MK460774 - MK461093 (see Table 1. Supporting information). We obtained 165 endophytic fungal strains (85 in autumn and 80 in spring) from S1 (natural source of salinity) and 155 endophytic fungal strains (72 in autumn and 83 in spring) from S2 (anthropogenic source of salinity). The growth rate of many isolated fungal strains revealed strong effects of salinity based on the analysis in PDA media, which decreased in the following order 0 mM >100 mM >200 mM NaCl salt concentrations. The collection of identified endophytic fungal strains from *S. europaea* mainly consisted of the phylum Ascomycota (96%) (Fig. 1). The strains were represented by nine orders: Pleosporales (representing 67.9% dominated by the genera *Alternaria* sp. and *Stemphylium* sp.), Eurotiales (representing 16.1%, and mainly including the genera *Aspergillus* sp. and *Penicillium* sp.), and Hypocreales (representing 5.7% and consisting of only *Fusarium* sp. and *Trichoderma* sp.). The remaining genera represented the order Dothideales, Incertae sedis, Capnodiales, Sordariales, Botryosphaerales and Chaetothyriales.

Most of the endophytic fungal strains represented the order Pleosporales and were found in all sites, seasons and plant organs. Among the strains classified to Ascomycota, *Alternaria* sp. (67.9%) was the most dominant genus found in both investigated sites (Fig. 2). *Alternaria* species were more prevalent at S2. Other species belonging to *Aspergillus*, *Stemphylium* and *Phoma* were found frequently at both sites.
Some fungal strains belonged to the Phylum Basidiomycota (4%) (Fig. 1), which was categorised into four orders, Polyporales (45.4% with the most representative genus being *Peniophora*) and the other strains corresponded to Russulales, Agaricales and Cantharellales.

Selection of non-pathogenic strains

On the basis of information available in previously published articles, we selected 39 fungal strains (Fig. 3). As described in Fig. 3 the strains belonged to the genera: *Aureobasidium* (6 strains), *Coprinellus* (2), *Epicoccum* (6), *Stereum* (1), *Podospora* (1), *Coriolopsis* (1), *Trichoderma* (5), *Arthrinium* (6), *Acremonium* (1), *Porostereum* (1), *Stemphylium* (1), *Emericellopsis* (2), *Neocamarosporium* (1), *Ascochyta* (2), *Sarocladium* (2), and *Ascomycota* (1). These strains were used to screen for the plant growth promoting activities of fungi.

Plant growth promoting characteristics of the 39 endophytic fungal strains

The results presented in Fig. 3 (see Table 2. Supporting information provides detailed numerical values) showed that most of the endophytic fungal strains from both the S1 and S2 produced siderophores, polyamines, and IAA but showed very low or no activity for DCP, and TCP solubilization. Nearly all of the fungal strains from S2 possessed the ability to produce polyamines (90%...
isolated strains from shoots and 83% from roots) and siderophores ($W_{act}$ value ranging from 1 to 10; 70% from shoots and 83% from roots) compared to the strains from S1. Ascomycota sp. ISK3-7, Arthrinium arundinis IS3-2, A. arundinis CS2-15, Coprinellus ellisii CS1-21, and A. arundinis CS1-14 were only positive for polyamine production. Similarly, Trichoderma sp. CR3-5, Trichoderma harzianum CR2-3, Stemphylium sp. IS3-12 and Aureobasidium pullulans ISR2-10 were only active for IAA synthesis. The ability to biosynthesise IAA was detected at similar frequencies ($W_{act} < 1$) in fungi isolated from sites S1 and S2. However, most strains located in the plant roots synthesised IAA (87% from S1 and 83% from S2), whereas polyamine producers (66% from S1 and 90% from S2) were found mainly in the shoots. The strains A. pullulans CSK1-9, A. pullulans CSK3-1, Aureobasidium sp. CSK3-6 and Emericellopsis maritima IR3-5 were active producers of siderophores ($W_{act} > 1$), polyamine, DCP ($W_{act} = 0.5$) and IAA synthesis.

Extracellular enzymatic activities of the 39 endophytic fungal strains

Most of the endophytic fungal strains isolated from both S1 and S2 displayed proteolytic, lipolytic and chitinolytic activity (Fig. 3). On comparing the two sites, the fungal strains from S1 possessed higher cellulolytic ($W_{act}$ ranging from 1 to $<25$, 66% isolated strains from shoot and 50% from roots), proteolytic (100% isolated strains from both organs)
and amylolytic activities ($W_{act}$ ranging from 1 to 10; 60% isolated strains from shoot and 62% from root). Whereas the strains from S2 possessed proteolytic (90% isolated strains from shoot and 100% from root), lipolytic ($W_{act} < 10$, 100% isolated strains from both organs), and chitinolytic activities ($W_{act} < 10$; 90% from shoot and 83% from root). The strains isolated from the *S. europaea* shoots from S2 ($W_{act}$ value >25) and the roots from S1 ($W_{act}$ value ranging from 10 to >25) exhibited very high proteolytic activity. The pectolytic activity was comparatively lower ($W_{act}$ value <1) in all cases.

The strains *A. pullulans* CSK1-11, *A. pullulans* CSK1-9, *A. pullulans* ISK1-14 and *Sarocladium* sp. CR3-2 displayed $W_{act}$ value >25 for cellulolytic activity. Similarly, very high proteolytic activity ($W_{act} > 20$) was exhibited by a few strains e.g. *Sarocladium* sp. CR3-2, *Ascomycota* ISK3-7, *Stereum gausapatum* ISK3-11, *Epicoccum nigrum* ISK2-5, *Epicoccum* sp. ISK1-6, *Porostereum spadiceum* IS2-6, and *Stemphylium* sp.

![Fig. 3 Plant growth promoting properties of 39 endophytic fungal strains associated with *S. europaea*. The color key determines the level of activity expressed by the strains and the measures ($W_{act}$ "*" and μg/ml "**") used to calculate their activity. Abbreviations used: Ascomycota “A” and Basidiomycota “B”](image-url)
Effect of salt on the growth of endophytic fungi

The 39 endophytic fungal strains were cultivated in PDA enriched with different NaCl salt concentrations and the average mycelium diameter was measured as shown in Fig. 3. All the fungal strains exhibited varying salt tolerance capabilities. The fungal strains in S1 (63%) displayed higher salt tolerance (>700 mM NaCl) than the strains from S2 (17%). Many strains tolerated salt concentrations of more than 700 mM NaCl (A. arundinis: CS1-14, CS2-15, CR1-10, CR2-4 and IS3-2; Trichoderma atroviride CSK3-13, Trichoderma citrinoviride CR1-1, T. harzianum CR2-3, Trichoderma sp. CR3-5, Porostereum spadiceum IS2-6, Stemphylium sp. IS3-12, C. ellisii CS1-21 and C. domesticus IR3-9) and the diameter of their mycelia measured on agar was similar to their control without salt. Meanwhile, a few strains proliferated at 500 mM NaCl (A. pullulans CSK1-11, Podospora sp. CSK2-13, Emericellopsis sp. IS3-7 and Acremonium sp. IR2-6) when compared to their no-salt controls. Only the fungal strain E. nigrum ISR3-14 found in the roots of S2 showed decreasing growth of mycelium in the presence of NaCl salt.

Growth of L. perenne inoculated with endophytic fungi

Based on the results obtained from the metabolic and enzyme activities of the strains, we selected six representative strains: S. gausapatum ISK3-11, E. nigrum ISR3-14, A. pullulans CSK1-11, C. ellisii CS1-21, Sarocladium strictum CSR1-13 and Arthrinium gansii CR1-9 for inoculating two varieties of L. perenne. Seven weeks after inoculation with six different fungal isolates, positive effects on the growth of the two L. perenne seed varieties were observed. Two-way ANOVA analysis (Fig. 4) revealed that the six fungal isolates positively influenced the overall growth of Variety 1 (Epichloë free- E−) including a significant increase in length, fresh and dry weights of the shoots and roots when compared to their non-inoculated control. However, in the case of Variety 2 (Epichloë infected- E+), significant positive differences were consistently seen in fungal strains S. gausapatum ISK3-11 (fresh and dry weights) and A. gansii CR1-9 (for all growth parameters) in the shoots and roots in contrast to their non-inoculated control.

Discussion

Culturable diversity of endophytic fungi in S. europaea

Most of the endophytic fungi obtained from this halophyte are known plant pathogens and saprobes in other plants. For example, of the dominant and abundantly distributed genera in our study, Alternaria causes leaf blight or leaf spot (Thomma 2003), Phoma causes disease in citrus and oilseed rape (Aveskamp et al. 2008), while Aspergillus causes blights in most plants (Pryor and Michailides 2002). Some saprobes found in this study include Epicoccum, Alternaria, Fusarium, Cladosporium, Penicillium, Acremonium and Aspergillus (Fisher and Petrini 1992; Thongkantha et al. 2008). However, we suppose that these endophytic fungi in halophytes may have “co-evolved” in their host. A high colonization frequency of these endophytes in healthy halophyte plant tissue indicates they are not pathogenic. A few studies have discussed the abilities of fungi to switch lifestyles between the endophyte-pathogen (Fisher and Petrini 1992; Hyde and Soytong 2008) and endophyte-saprotroph (Hyde and Soytong 2008; Promputtha et al. 2010). Many endophytes and pathogenic fungi can persist as saprobes (as a mode of nutrition) once their host plant reaches senescence or leaves abscise (Hyde and Soytong 2008; Promputtha et al. 2010). The entry of pathogens and saprotrophs in halophytes could also be an “escape strategy” to avoid the high salt concentrations in the soil.

The majority of the culturable fungal diversity was classified as Ascomycota and a fraction as Basidiomycota. This finding is comparable to our previous study where sequencing techniques indicated that Ascomycota with Pleosporaceae were the dominant family in all samples from the same sites and seasons in this study (Furtado et al. 2019). The metagenomic study concluded that Paradendryphiella sp. was the more frequently
present endophyte of *S. europaea* in both sites, followed by the occurrence of *Alternaria* sp. (Furtado et al. 2019) while this study using culturable techniques showed 67.9% of the isolates comprised the genus *Alternaria* sp. The dominance of *Alternaria* sp. (Ascomycota) in *S. europaea* was previously reported in Canada (Muhsin and Booth 1987), Japan (Okane and Nakagiri 2015), South Korea (You et al. 2014) and India (Kannan et al. 2014). However, *Alternaria* may not be a dominating species in all halophytes: a few examples include *Artemisia fukudo*, *Carex scabrifolia*, *Kochia scoparia*, *Phragmites communis*, *Suaeda australis* and *S. japonica* found in the Suncheon bay in South Korea (Young-Hyun et al. 2012); *Acanthus ilicifolius*, *Arthrocnemum indicum*, *Kochia scoparia*, *Phragmites communis* and *S. europaea* from mangroves in India (Suryanarayan and Kumaresan 2000); and *S. maritima*, *Limonium tetragonum*, *Suaeda australis*, *Phragmites australis*, and *Suaeda glauca* from Muan salt marsh of South Korea (Khalmuratova et al. 2015). Fungal endophyte colonisation of the plant may depend on the plant host (*Salicornia* accumulates salts in its tissue) and the soil characteristics (level of salinity) at the location (Maciá-Vicente et al. 2012). Indeed, the highest frequency of fungal colonisation occurred in *Aster tripolium* roots, followed by *Limonium angustifolium* and *S. europaea*, which are the most abundant halophytic plant species from Sečovlje salters (Slovenia) (Sonjak et al. 2009). Notably, some species e.g. *Trichoderma* sp., *Phoma betae*, *Fusarium* sp., *Emericellopsis* sp., *Epicoccum nigrum*, *Alternaria* sp., *Lewia* sp., *Pleosporales* sp., *Cladosporium* sp. and *Stemphylium* sp. were previously reported endophytes of *S. europaea* found near a lakeside in Japan (Okane and Nakagiri 2015). Most of these reports on *S. europaea* endophytes present in roots do not indicate the plant growth stage at which the diversity analysis was carried out. Conversely, our study analyzes the whole plant (shoot and root) for fungal endophyte diversity and considers the plant growth stage (*S. europaea* samples were collected in both spring and autumn, in young and mature plants).

Besides the influence of the host plant, environmental effects were considered by selecting two sites with different sources of salinity. Most endophytes originate from the soil and enter the plant through cracks, wounds, root hairs or stomata in leaves.
The occurrences of certain endophytic and rhizospheric fungal communities in roots of a halophyte and non-halophyte plant were positively correlated to the differing soil salinities (Maciá-Vicente et al. 2012). Site S1 of our study was characterised as a highly saline site (high electrical conductivity, EC_e) with more Na^+ ions in the soil and a longer duration of salinisation than Site S2 (lower EC_e and more Ca^+ ions found in soil) (Furtado et al. 2019; Szymańska et al. 2014). Some genera were detected at one site only. Plants at site S1 hosted Fusarium sp., Phaeosphaeriaceae sp., Trichoderma sp., Ascochyta sp., Peniophora sp., Sarocladium sp., Ceriporia sp., Cladophialophora sp., Coriolopsis sp., Fusicolla sp., Phanerochaete sp., Podospora sp., and Thanatephorus sp. Conversely, Eurotium sp., Emericellipsoides sp., Dematiopleospora sp., Nectria sp., Porostereum sp. and Stereum sp. were only isolated from S2. Most of these fungal isolates were halophilic and/or halotolerant to NaCl salt, and demonstrated high salt tolerance ability to concentrations up to 500 mM NaCl while a few isolates showed tolerance at 700 mM NaCl. Thus the type and concentration of salinity in the soil can influence the microbial niche at the site and its cellular metabolic rate to tolerate salts (Collado et al. 1999; Lembicz and Olejniczak 2009). Some strains detected in our study can be categorised as black fungi or dematiaceous fungi, such as the genera Alternaria, Phoma, Cladosporium, Lewia, Pleospora, Epicoccum, Stemphylium, Ascochyta, Peniophora, Plenodomus, Neocamarosporium, Dematiopleospora, Aspergillus, Penicillium, Eurotium, Talaromyces, Fusarium, and Aureobasidium. Species of dematiaceous fungi are morphologically plastic. Their cell walls contain melanin, they possess some protective substances or antimicrobial compounds; or synthesising phytohormones such as auxins (Bose et al. 2014); synthesising fungal siderophores (Bartholdy et al. 2001); solubilising phosphates (Barrow and Osuna 2002); producing inhibitory substances or antimicrobial compounds; or synthesising degrading enzymes (Rajesh and Ravishankar Rai 2013). Plant growth promotion by endophytic fungi occurs in several crops including wheat (Dingle and Mcgee 2003), barley (Waller et al. 2005). Although the metabolic and enzyme activities measured in this study were from experiments conducted under ideal conditions, production of metabolites or enzymes may differ in soils with a high EC_e and other environmental stresses.

Abiotic stresses (e.g. salinity) limit plant growth and at times this damage is irreversible. In such circumstances, the endophytic fungi can facilitate plant growth and development in multiple ways, by producing polyamines (Fan et al. 2014), or phytohormones such as auxins (Bose et al. 2014); synthesising fungal siderophores (Bartholdy et al. 2001); solubilising phosphates (Barrow and Osuna 2002); producing inhibitory substances or antimicrobial compounds; or synthesising degrading enzymes (Rajesh and Ravishankar Rai 2013).
produced polyamines. This activity can be correlated with shoot hyper-accumulation of salts in *Salicornia*, which is more stressful for fungal colonisation than in the roots. Furthermore, the fungal polyamines are involved in many cellular maintenance processes in the host plant as well as functioning in plant growth promotion (Amprayn et al. 2012). Additionally, siderophores production by fungi is one of the biocontrol mechanisms observed in most of the *Salicornia* endophytic strains. The ability of the fungi to chelate iron makes them better competitors thus preventing the growth of pathogenic microorganisms in plant hosts (Johnson 2008).

Siderophores also play a role in stimulating induced systemic resistance (ISR) while fungal siderophores modulate iron homeostasis in *Epichloë festucae* infected ryegrass plants (Hardoim et al. 2015; Johnson et al. 2013). Furthermore, fungi enhance the growth of host plants by producing phytohormones, mainly IAA (Shi et al. 2009; Waqas et al. 2012). IAA increases the colonisation efficiency of endophytic bacteria and may have a similar function in fungi (Suzuki et al. 2003). In the host *Artemis annua*, the endophytic fungus *Colletotrichum* sp. produced substances like IAA to regulate plant processes (Lu et al. 2000). In another study, *Trichoderma virens* displayed characteristic auxin-related phenotypes such as increased plant biomass and stimulated lateral root development in Arabidopsis, but mutations in auxin biosynthesis genes reduced the effects of *T. virens* inoculation (Contreras-Cornejo et al. 2009). Interestingly, the relatively low capacity of endophytic fungi to produce auxins in our study suggests that this is not the main mechanism by which it affects halophyte growth. Most IAA synthesising fungi were mainly isolated from the roots of the host plant in our study. Conversely, no fungal strains could solubilise phosphate. Phosphate solubilisation ability could be adversely influenced by environmental factors, especially under stress conditions. For instance, during the summer when the soil was simultaneously exposed to high salt, high pH and high-temperature stress, bacteria growing in alkaline soils in India demonstrated diverse levels of phosphate solubilisation ability and poor growth of phosphate solubilising bacteria (Nautiyal et al. 2000). There is no subsequent evidence on the effects of fungal phosphate solubilisation from salt stress environments.

Our study supposed that the fungal endophytes potentially regulated halophyte growth by improving plant nutrition and catalyzing different biochemical processes by the action of fungal enzymes such as cellulase, pectinase, chitinase, amylase, protease, and lipase (Choi et al. 2005). The enzymes cellulase, chitinase, and amylase, produced by most of the strains, are important for endophyte colonisation (Caldwell et al. 2000). Therefore, the capability of endophytic fungi to produce enzymes to degrade cellulose and lignin is also a possible strategy to allow some endophytes to decay host tissue and persist as saprobes after host senescence (Lumyong et al. 2002; Oses et al. 2008). Endophytic fungal strains producing chitinase and protease may enhance toxicity against plant pathogens including insects, pests and nematodes (Kredics et al. 2005; Viterbo et al. 2002). For example, *Trichoderma* is among the well-studied species known to produce these enzymes that parasitise cell walls or hyphae of many plant pathogens (Kredics et al. 2005; Viterbo et al. 2002). A similar role may be played by the enzymes cellulases, pectinases, amylases, and protease to suppress plant pathogen activities directly, and these can degrade the cell walls of pathogenic fungi and oomycetes (Gao et al. 2010; Yarullina et al. 2016). In this study, the multifunctional lifestyle of all the strains of genus *Aureobasidium* was evidenced by a broad-spectrum of enzyme activities, unlike the genus *Coprinellus*.

Beneficial associations between plants and endophytic microbial communities play an important role in both natural and agricultural ecosystems (Cheplick 2004; Rodriguez et al. 2004, Redman et al. 2005). Our data did, however, support the hypothesis of variations in the properties of endophytes between different genera, sites and plant organs. The activity of endophytes from S2 (lower salinity) was higher than observed at S1 (higher salinity). Moreover, we suggest that duration of soil salinisation at the two sites (salinity has existed at S1 much longer than at S2) could disturb the metabolic function of microorganisms. Variations in the metabolic and enzyme activities among similar endophytic fungal species isolated from different conditions were seen in *Epicoccum* sp., *Arthrinium* sp. and *Trichoderma* sp. The information on the contribution of environmental factors in the fungal endophyte distribution and composition in *S. europaea* was previously discussed in Furtado et al. 2019.

*Salicornia* endophytic fungi promote growth in non-host plants

Advantageously, *Salicornia* endophytes are well adapted to extreme conditions, so they can be applied...
to salt-stressed plants as growth promoting agents. In our collection of characterised fungal isolates, we selected six endophytes that exhibited most of the tested activities for inoculation: *S. gausapatum* ISK3-11, *E. nigrum* ISR3-14, *A. pullulans* CSK1-11, *C. ellisi* CS1-21, *S. strictum* CSR1-13 and *A. gamsii* CR1-9. These fungal strains are regarded as plant promoting fungi e.g. the strain *Aureobasidium pullulans* has been effective in the control of sour rot of citrus, by producing chitinase enzyme against the phytopathogen (Ferraz et al. 2016). *A. pullulans* has several mechanisms against plant pathogens (Benchqroun et al. 2007) including the production of antifungal compounds (Zhang et al. 2012), volatile and cell-free compounds (Di Francesco et al. 2015), killer toxins (Ferraz et al. 2016) and the antibiotic aureobasidin A (AbA) (Liu et al. 2007), and the induction of plant defence responses (Ippolito et al. 2000). Endophyte *Epicoccum* sp. isolated from *Taxus fauna* possessed novel antimicrobial compounds against *Staphylococcus aureus* (ATCC6538) and *Candida albicans* (CI.1 4043) (Jadoon et al. 2016). Another in vitro experiment revealed increased enzymatic activity of *Epicoccum* sp. in rice leaf blast suppression against rice pathogens (Sena et al. 2013). *Sarocladium strictum* (also named *Acremonium strictum*) and *Acremonium gamsii* were the other two strains used in our study. *Acremonium* species are common symbionts in tall fescue grass and confer drought tolerance in grasses (White et al. 1992). The association of *Acremonium* endophytes can ameliorate biotic stress in grass species e.g. *Lolium perenne* and *Festuca longifolia* against the larvae of autumn armyworm, green bug and yellow sugarcane aphids (Breen 1993a, b). The *Coprinellus* species isolated from the Brazilian medicinal plant *Solanae cernuum* exhibited antibacterial activities (Vieira et al. 2012). It is a basidiomycetous genus and some of its species were obtained as endophytes of coastal grasses and forest trees *Theobroma gileri* (Márquez et al. 2008; Thomas et al. 2008). Lastly, *S. gausapatum* collected from oak forest and orchards in southern Serbia displayed high lignin-degrading abilities and the efficiency of lignin removal from beech wood sawdust (Jović et al. 2018). Not much information was reported on the plant growth promoting activities of this strain.

In our study, the six representative endophytic fungal strains from *S. europaea* displayed positive compatible associations and confirmed plant growth promoting traits in the non-host ryegrass. Similar results demonstrated the positive effects of bacterial endophytes from *S. europaea* in *Beta vulgaris* grown under different salt concentrations (Piernik et al. 2017), thus confirming them as inoculants for plant growth promotion. Under controlled growth conditions, Variety 1 (E−) inoculated with *Salicornia* endophytes significantly increased shoot and root length, and wet and dry weights compared to non-inoculated controls. Conversely, in Variety 2 (E+) only strains *S. gausapatum* ISK3-11 and *A. gamsii* CR1-9 were significantly consistent in promoting plant growth. The variety 2 was symbiotically associated with endophytic fungi *Epichloë* (known as *Neotyphodium* sp.), that may have disturbed the colonization of some of the inoculated fungal strains in this experiment, with the exception of *S. gausapatum* ISK3-11 and *A. gamsii* CR1-9 strains that were not affected (Scharl et al. 2004). Similar results were obtained where inoculation and colonization of fungi *Ascochyta leptospora* in perennial ryegrass was not inhibited in the presence of symbiotic fungi *Epichloë festucae* (Tian et al. 2008).

**Conclusion**

Colonisation of fungi in *S. europaea* depends on host plant lifestyle (*Salicornia* is known to accumulate salts in its shoot) and the type of soil at the site (natural and anthropogenic salinity). Most of the identified endophytic fungi (94%) were known plant pathogens and saprobes in other plants, which highlights the ability of fungi to switch lifestyles and co-evolve in their hosts. *Salicornia* endophytic fungi were confirmed as mainly halotolerant and possess enzymes and metabolic traits that might improve growth and development of the host-plant. Endophytes of *S. europaea* actively produce siderophores, and polyamines, and have cellulolytic, proteolytic, lipolytic and chitinolytic activities. Among the selected strains, *Aureobasidium* spp. displayed broad-spectrum activities, proving its multifunctional lifestyle. Enzyme and metabolic activities of fungal endophytes isolated from the anthropogenically saline site were higher than those from the site with naturally high soil salinity, highlighting that salinity levels affect microbial activity. Only two strains out of the six fungal strains inoculated in non-host *L. perenne* exhibited compatible and beneficial associations in the two grass varieties. We can infer that the presence of native microbiota in plants affects the inoculated strains which may
be due to some inhibitory response or competition from the native endophyte, which needs further investigation. Two fungal strains, *S. gausapatum* ISK3-11 and *A. gamsii* CR1-9, significantly improved plant growth.

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**Author contribution**  BF did all analyses and wrote the first version of the manuscript. SSz prepared the sequencing data and the statistical data. KH designed and managed field and lab experiments as well as participated in the preparation of the manuscript. All authors revised the manuscript and approved the publication.

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**Compliance with ethical standards**

**Competing interests**  The authors declare that they have no competing interests.

**Data deposition**  The DNA sequences files were submitted to GenBank SUB5097742 and accession numbers were assigned: MK460774 - MK461093.

**Ethics statements**  Not applicable.

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