Effects of fungal inoculation on the growth of *Salicornia* (Amaranthaceae) under different salinity conditions

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Abstract

Endophytic fungi are known to be present in roots of salt marsh plants, but their ecological role in this symbiosis is still largely unknown. Generally considered parasitic or saprophytic, they may still be mutualistic, at least under certain circumstances. Among salt marsh plants, *Salicornia* spp. are recognized as particularly salt-tolerant and their frequent colonization by root endophytes has also been reported. This study aimed to investigate whether the inoculation of *Salicornia* with different root endophytes isolated from field-collected *Salicornia* affects biomass production, nutrient uptake and photosynthesis (assessed via chlorophyll fluorescence). In addition, we investigated whether fungal inoculation confers tolerance to salt stress given that endophytes are suggested to increase salt tolerance and improve plant fitness in other less salt-tolerant plants. The inoculation of *Salicornia* with an isolate of the genus *Stemphylium* positively influenced total biomass production and nitrogen concentration in roots at optimum salinity condition (150 mM NaCl). However, under salt stress (650 mM NaCl), no significant effects of fungal inoculation on biomass production and photosynthesis were observed. Further, positive and negative effects of fungal inoculation on nutrient concentrations were observed in roots and shoots, respectively. Our results indicate that different endophytic fungi and their interaction result in distinct fungal species-specific plant growth responses of *Salicornia* under different growth conditions.

Keywords Root endophytic fungi · *Stemphylium* · *Alternaria* · Salt marsh · *Salicornia* · Salt stress

1 Introduction

Fungal endophytes are well-known for their ubiquitous occurrence in roots of almost all plant species. Based on their potential to enhance plant growth, this association is one of the most investigated types of symbiosis (Hardoim et al. 2015). Apart from the well-studied mycorrhizal association, many other fungi are associated with the root microbiome. However, in most cases the nature of the interaction is unknown.

The relationship between root endophytes and their hosts can range from parasitism to mutualism (Mandyam and Jumpponen 2015). One group of these endophytes are the dark septate endophytes (DSE), a diverse group of

Ascomycetes, which have been frequently highlighted as mutualists (Jumpponen 2001; Mandyam and Jumpponen 2005; Andrade-Linares et al. 2011; Newsham 2011; He et al. 2020; Mateu et al. 2020), although some species have also been identified as plant pathogens. DSE commonly occur in stressful environments including highly contaminated areas, arid ecosystems or salt marshes (Knapp et al. 2012; Calado and Barata 2012; Wang et al. 2016). Among the DSE, species of the order Pleosporales are the most frequent root colonizers occurring in various plant species, including halophytes (Kandalepas et al. 2010; Li et al. 2020).

Studies supporting DSE as mutualists demonstrated improved protection of plants against herbivores (Yu et al. 2001) and pathogens (Arnold et al. 2003), remediation of abiotic stresses such as drought (Li et al. 2019), heat (Redman et al. 2002; Pennisi 2003), heavy metal toxicity (Wang et al. 2016) and salinity (Santos et al. 2017; Gupta et al. 2020; Mateu et al. 2020). Plants, in return, provide photosynthates and disseminate the fungi through decaying plant parts to the next generation of hosts (Faeth and Fagan 2002).

Halophytes share different mechanisms to adapt to saline environments such as ion extrusion and compartmentalization

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and osmotic adjustments (Flowers and Colmer 2015). In addition, their association with microorganisms, such as fungal endophytes, has been linked to an improvement in growth and fitness under salt stress (Ruppel et al. 2013; Mateu et al. 2020). Some of the physiological and biochemical benefits provided by fungal endophytes include an increase in biomass and improvement of nutrient uptake, photosynthesis and proline concentration (Gupta et al. 2020). However, Rodriguez et al. (2008) emphasized that tolerance in plants occurs via habitat-adapted symbiosis. They showed that root endophytic fungi from coastal species are able to confer salt tolerance under high salinity but not heat or disease resistance to their host plants.

Among halophytes, *Salicornia* (Amaranthaceae) is a genus containing 53 species (POWO 2019) with the potential to grow in highly saline conditions (up to 1 M NaCl in soil) (Ushakova et al. 2005). In salt marshes in northern Germany, the genus is distributed from the mudflat to the lower salt marsh and two different cytotypes occur: the tetraploid *Salicornia procumbens*, occurring from the mudflat to the lower salt marsh, and the diploid *Salicornia europaea*, which occurs mainly in the lower salt marsh (Kadereit et al. 2007; Teege et al. 2011). Similar to other halophytes, *Salicornia* developed multiple strategies to survive high salinity, for example the compartmentalization of Na⁺ into vacuoles (Flowers and Colmer 2015). Similarly, fungal endophytes inhabiting *Salicornia* tissues have strategies to cope with high salt concentrations, such as specific substances in the membranes or cell wall constructions, exclusion of ions from the cells, and the adaptation of proteins and enzymes to high concentrations of soluble ions (Ruppel et al. 2013).

Previous research on *Salicornia* fungal associates has shown the ubiquitous occurrence of root endophytes, especially the genus *Alternaria* (Okane and Nakagiri 2015; Maciá-Vicente et al. 2016; Aletaha et al. 2018; Furtado et al. 2019a). However, there is still a research gap in the understanding of the ecological function of this association. While endophytic bacteria do positively affect *Salicornia* growth under high salt stress conditions (Bashan et al. 2000; Zhao et al. 2016; Mesa-Marín et al. 2020), it has previously not been studied whether the mycobiome provides beneficial effects to *Salicornia* under high salinity.

In one of the few studies addressing this aspect, Mateu et al. (2020) assessed the role of fungal endophytes in promoting salt tolerance in the facultative halophyte *Phragmites australis* showing that inoculated seedlings growing under salt stress had higher aboveground biomass compared to controls. In another study, Furtado et al. (2019b) tested the potential of root endophytes isolated from *S. europaea* to promote growth of *Lolium perenne* showing that two DSE fungal isolates significantly increased plant growth. Nevertheless, no study investigated the reaction of *Salicornia* towards fungal

inoculation with strains isolated from the same species and under varying salinity.

Given the ecological importance of *Salicornia*, we aimed to investigate whether DSE fungi successfully colonize plants under greenhouse conditions promoting salt tolerance by improving biomass production, nutrient uptake and photosynthesis. Firstly, we isolated root-associated fungi from field-collected *Salicornia* and subsequently conducted an experiment to evaluate the host response to fungal inoculation. We addressed the following questions: (i) Do the different fungal endophytes affect growth, nitrogen and phosphorus concentrations, and photosynthesis (assessed via chlorophyll fluorescence) of *Salicornia*? and (ii) do inoculated plants respond better to salt stress compared to non-inoculated ones?

2 Material and methods

2.1 Fungal isolation and molecular identification of fungal isolates

In August 2019, 15 individuals of *Salicornia* were collected in the pioneer zone and lower salt marsh of a salt marsh located within the limits of the Nationalpark Niedersächsisches Wattenmeer at the island of Spiekeroog, northern Germany (53°45'44.3"N 7°43'23.3"E). In the field, plants were carefully removed from soil, placed in plastic bags, and brought back to the laboratory for further processing.

In the laboratory, roots were separated from the shoots, washed off the remaining soil with tap water and sectioned in fragments of about 1 cm. Subsequently, the root surface sterilization was carried out following the protocol of Crous et al. (2009). In short, roots were washed by placing them in sterile water for 1 min, transferred to 0.53% sodium hypochlorite for 4 min, and ethanol 70% for 1 min followed by a washing step in sterilized water for 5 min. After sterilization, roots were dried for 1 min on sterilized filter paper and subsequently five root fragments per individual plant were placed in petri dishes on commercial PDA (potato-dextrose-agar, 39 g L⁻¹) medium (Sigma-Aldrich, St. Louis, MO, USA). The plates were sealed and incubated in darkness at room temperature. Plates were regularly observed, and as soon as fungal colonies started to emerge after about 5 days, they were selectively transferred to fresh PDA plates. Initially, there were 91 isolates recovered. From those, 12 representative isolates were selected for molecular identification based on their frequent occurrence.

The selected isolates were identified using DNA barcoding. Aerial mycelium was scraped from the PDA medium surface and placed into a 2 mL extraction tube containing two steel beads. Mycelium was milled using a Mixer Mill (Retsch, Germany) for two cycles of 1 min each with 30 oscillations per second, in order break the cell walls and release

the intracellular components. Genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytic Jena AG, Jena, Germany), following the manufacturer's instructions. The ITS region of rDNA was amplified using the universal primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) following the PCR amplification conditions described in Bonfim et al. (2016). The PCR products were checked on 1.2% agarose gel stained with ethidium bromide (Sigma-Aldrich). Subsequently, they were sequenced at LGC Berlin. Finally, sequences were used in a BLAST search to assign taxonomic identity.

We used three isolates (DRG94, DRG96 and DRG97) of Pleosporales (Ascomycota) of which the closest matches were *Phoma* sp. (accession number MK460837.1, 99.84% identity match) for isolate DRG94, *Alternaria conjuncta* (accession number MK461066.1, 99.68% identity match) for isolate DRG96 and *Stemphylium* sp. (accession number MK460817.1, 99.19% identity match) for DRG97. Strengthening our choice to choose these isolates, the GenBank sequences mentioned were generated from endophytic fungi isolated from *Salicornia europaea* collected in saline habitats in Poland (Furtado et al. 2019a). In addition, the three genera (*Alternaria*, *Stemphylium* and *Phoma*) were abundantly detected in roots of *Salicornia* in metabarcoding studies previously performed in Spiekeroog (Buhk 2020) and elsewhere (Maciá-Vicente et al. 2012; Okane and Nakagiri 2015; Furtado et al. 2019a).

2.2 Preliminary test for fungal growth in different salt concentrations

The ability of the selected isolates to grow under salt stress was tested in culture. The isolates were cultivated on PDA medium amended with salt concentrations (0, 200, 400, 600 and 800 mM NaCl). For each treatment, a fungal mycelium disk of 5 mm of an actively growing isolate was cut and placed in the middle of a petri dish containing PDA medium. Five replicates of each isolate were used for each salt concentration. The colony diameter was measured after 10 days of incubation in darkness at 26 °C.

3 Greenhouse experiment

3.1 Soil treatment

The soil used in the greenhouse experiment was collected from a lower salt marsh located at Neuharlingersiel, Germany (53°70'11.6"N, 7°70'9.57"E) in May 2020. The soil was taken up to a depth of 20 cm at three different points selected in areas where *Salicornia* spp. occur. The vegetation was dominated by *Atriplex portulacoides*, *Limonium vulgare*, *Salicornia* spp. and *Suaeda maritima*. The soil consisted

mostly of sand. In the laboratory, root fragments and other residues were removed from the soil. After that, the soil was autoclaved following a two-step procedure described by Bonkowski (2019) with minor modifications: soil was placed in autoclavable bags and autoclaved at 120 °C for 20 min. Then, a washing step was conducted to leach the larger amounts of nutrients from the killed soil microbiota, followed by drying for 48 h. Finally, another autoclaving step at 120 °C for 20 min was applied. The sterilized soil was used 1 day after the sterilization process. The elemental chemical composition of a composite soil sample (collected at three different points) was analysed. The elemental analysis was conducted at the Institute for Soil and Environment, Lufa Nord-West (Oldenburg, Germany) (Table S1).

3.2 Experimental design

The experiment had a factorial design including two factors: fungal isolate and salinity level. Due to the difficult differentiation of *Salicornia* species, especially at seed harvest time, we did not distinguish between the two cytotypes. At the end of the experiment, the ploidy level of a subset of individuals was analysed by flow cytometry following the protocol of Baranyi and Greilhuber (1996). The fungal factor consisted of five different treatments: three single isolate inoculations (*Alternaria conjuncta* DRG96, *Phoma* sp. DRG94 and *Stemphylium* sp. DRG97), a mixed inoculation of the three isolates, and a mock inoculation as control (non-inoculated PDA plugs). Two levels of water salinity were applied: 150 mM NaCl (approximately 5.8 ppt), which is around the optimum water salinity concentration for *Salicornia* growth under greenhouse conditions (Singh et al. 2014) and 650 mM NaCl (approximately 38 ppt), which exceeds the porewater salinity from the lower salt marsh where *Salicornia* spp. occur (approximately 34 ppt).

The experiment was set up in May of 2020 in the greenhouse of the University of Oldenburg. The seeds of *Salicornia* were washed with distilled water and surface-sterilized for 1 min in ethanol 70%, 2 min in a 3% sodium hypochlorite solution and then washed three times in distilled water. Approximately 20 surface-sterilized seeds were sown in each pot (9 cm × 9 cm × 9 cm). The substrate consisted of the sterilised salt marsh-collected soil covered with a fine layer of sterilized potting soil (Spezial Substrat Hawita Professional, Hawita Gruppe, Vechta, Germany). After sowing, seeds were covered with a fine layer of sterilized sand. One month after germination, in each pot, five individuals were maintained while the others were cut off. Each tray contained ten pots and was watered twice a week with distilled water. Trays were constantly kept with water and electrical conductivity of the filled water was measured every third day using a WTW Cond 330i conductivity controller (WTW GmbH, Weilheim, Germany), and salt concentrations were

adjusted when necessary. Each treatment was replicated 20 times (pots) resulting in a total of 200 pots, each of them with five plants. Pots were maintained for 90 days (June to August 2020) and no additional nutrients were given during the experiment.

3.2.1 Fungal inoculation

The soil was inoculated twice following the protocol of Singleton et al. (1992) with some modifications. Prior to sowing the seeds, 12 inoculated agar plugs (5 mm × 5 mm) of an actively growing colony of each isolate were combined and mixed with the first layer (2 cm) of the soil. For the treatment consisting of a mixture of the three different isolates, four PDA plugs of each fungal isolate were used. One month after germination, a second inoculation was performed. For that, a small hole was opened near the root zone of each seedling and three PDA plugs of each isolate were placed around the root and covered with soil. For the treatment consisting of a fungal mixture, one PDA plug of each fungal isolate was used.

4 Harvesting and determination of plant growth parameters

4.1 Biomass measurements

In each treatment, 50 plants were harvested including the entire root system. For biomass determination, the plants were separated in shoots and roots. The roots were carefully washed and together with shoots were placed in a paper envelope and dried at 70 °C for 72 h. Then, dried mass of shoots and roots was determined with an analytical balance (Precisa LS-220 A SCS, Switzerland, resolution 0.1 mg).

4.2 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed using a portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany), equipped with a leaf clip holder. A subset ($n = 20$) of plants from each treatment were selected for measuring maximum quantum efficiency of photosystem II (Fv/fm). The measurements were conducted at the same hour (7:00 to 7:30 am) to minimize differences in sunlight and temperature. First measurement was recorded before the fungal inoculation (measurement = t_0) followed by weekly measurements until the end of the experiment (measurement = t_7).

4.3 Nitrogen and phosphorus analysis

After dry mass determination, subsamples of shoot and root tissues were ball milled in Stainless steel tubes (Retsch

MM400, Haan, Germany) to pass a 280 µm DIN ISO-3310-1 screen (Retch, Haan, Germany). Aliquots of about 1.5 mg were further used for measuring nitrogen concentration. The samples were weighed using a microscale balance with a resolution of 0.001 mg (M2P, Sartorius, Göttingen, Germany). Shoot and root N concentrations were determined with an organic elemental analyser (Flash EA 1112, Thermo Fisher Scientific, Milano, Italy). To determine total P in the plant material, aliquots of about 5 mg were weighed using an analytical scale with a resolution of 0.01 mg (Quintix 125D, Sartorius, Göttingen, Germany). Plant material was wet-washed prior to measurements (Chao-Yong and Schulte 1985). Total P concentrations in shoots and roots were determined colorimetrically (Hoffmann and Ohnesorge 1966) in a 100 µL aliquot of the digesting solution using a UV-VIS spectrophotometer (Specord 50, Analytik Jena, Jena, Germany).

4.4 Detection of fungi in the roots and re-isolation

In order to confirm root colonization by the different fungal isolates, a subset of seedlings from each treatment was collected. Root fungal colonization was assessed following the root staining protocol described in Vierheilig et al. (1998). Roots were bleached in a 2.5% potassium hydroxide (KOH, Merck, Darmstadt, Germany) solution for 5 min at 90 °C with subsequently washing them three times with distilled water. After that, a solution of ink (Royal Blue, Pelikan PBS-Produktionsgesellschaft, Peine, Germany) and 5% acetic acid (Merck, Darmstadt, Germany) was added into 50 mL tubes containing the root fragments and boiled at 90 °C for 3 min. After staining, roots were washed under tap water, acidified with a few drops of 5% acetic acid and subsequently, stored in 50% glycerol. Root fragments were mounted on microscope slides.

A subset of plants was used for re-isolation of fungal strains. The roots were washed under tap water to remove adhering soil particles. After that, samples were surface sterilized for 1 min in 70% ethanol, 2 min in a 3% sodium hypochlorite solution and finally washed 3 times in distilled water to eliminate fungal epiphytes growing on root's surface (Crous et al. 2009). After sterilization, roots were plated on PDA medium (potato-dextrose-agar) and were kept at 25 °C for colonies observation.

4.5 Data analyses

All the data analyses were performed in R (version R-3.6.3, R Development Core Team 2011). Data normality was checked through a Shapiro-Wilk test followed by the observation of the residual's distribution and homoscedasticity of variances. For variables without a normal distribution, data were log-transformed before analysis. The fungal growth at different

NaCl concentrations was analysed with a two-way ANOVA with fungal isolates and salinity as factors, followed by a post hoc Tukey's test to identify differences among treatments. For the chlorophyll fluorescence data, a repeated-measures ANOVA was applied in order to analyse the effect of fungi and salinity on chlorophyll fluorescence over a period of 7 weeks. After that, the data was separated based on salinity levels and a two-way ANOVA was performed to see whether there was an effect of fungi and time at 150 mM and 650 mM of NaCl. In the case of significant differences, multiple pairwise comparisons were applied at each measurement event to test possible differences between each fungi.

For aboveground and belowground biomass, we used a two-way ANOVA with salinity (with two levels: 150 or 650 mM NaCl) and fungal isolate (with five levels: a control, *Alternaria conjuncta*, *Phoma* sp., *Stemphylium* sp. and a fungal mixture) as factors. After that, a separate one-way ANOVA was conducted, followed by a post hoc Tukey test, on each salinity level, to test whether the different fungal treatments had an effect on above and belowground productivity. For N and P concentrations in shoots and roots, the same approach was used. Separate two-way ANOVAs were performed for N and P in shoots and N and P in roots. After that, we conducted a separate one-way ANOVA, followed by a post hoc Tukey test, on each tissue and salinity level, to test whether the different fungal treatments had an effect on N and P concentrations.

5 Results

5.1 Fungal growth at different salt concentrations

The growth of the fungal isolates on PDA medium was affected both by salinity ($p < 0.01$, two-way ANOVA), isolate identity ($p < 0.01$) and their interaction ($p < 0.01$). All three isolates exhibited a high ability to grow in PDA medium at all tested NaCl concentrations (Table 1; Fig. S1). However,

growth of *Alternaria conjuncta* and *Phoma* sp. were inversely related to NaCl concentration. The growth of *Stemphylium* sp. seemed not to be affected by the presence of NaCl in the growth medium. *Alternaria conjuncta* showed the fastest growth with higher growth than other isolates in all NaCl treatments, especially at 600 and 800 mM NaCl.

5.2 Plant biomass

Growth was significantly affected by the inoculation with endophytes. The final aboveground plant biomass showed differences related to fungal isolate ($p < 0.01$) and salinity ($p < 0.01$), while the interaction was not significant ($p = 0.17$). Plants inoculated with *Stemphylium* sp. at 150 mM NaCl showed higher aboveground biomass than controls ($p = 0.017$) (Fig. 1; Fig. 3). No fungal treatment-related differences were detected in plants growing at 650 mM NaCl ($p = 0.81$). Overall, plants growing at 150 mM NaCl grew faster than those at 650 mM NaCl ($p < 0.01$).

For belowground biomass, the results of the two-way ANOVA indicated significant differences within the different fungal treatments ($p < 0.01$), salinity ($p < 0.01$) and their interaction ($p < 0.01$). The significant interaction term indicates that plants responses to fungal treatments were dependent on salinity level. As observed for aboveground biomass, for belowground biomass, plants growing at 150 mM NaCl that were inoculated with *Stemphylium* had higher root biomass when compared to non-inoculated ones growing at both 150 ($p = 0.049$) and 650 mM of NaCl ($p < 0.01$) (Fig. 1). When both root and shoot biomass were combined in total biomass, differences within fungal ($p < 0.01$) and salinity ($p < 0.01$) treatments were observed, although the interaction between the two factors was not significant ($p = 0.1$). In this case, plants inoculated with *Stemphylium* sp. differed significantly from controls ($p = 0.01$) (Fig. 2) regardless of salinity. Plants growing under a salt concentration of 150 mM NaCl had a higher total biomass compared to those growing under 650 mM NaCl ($p < 0.01$) Fig. 3.

Table 1 Colony diameter (mm) of fungal isolates measured at 10 days of growth in PDA medium amended with different NaCl concentrations. Values are means of five replications and standard deviation. Means

followed by the same upper case letters in the same column or lower case letters in the same row do not differ significantly from each other (Tukey's test at $p < 0.05$)

NaCl conc. (mM)	Colony diameter (mm)		
	<i>A. conjuncta</i> (DRG96)	<i>Phoma</i> sp. (DRG94)	<i>Stemphylium</i> sp. (DRG97)
0	75.8±0.8 Aa	65.0±1.9 Ab	47.2±3.2 Ac
200	57.0±0.7 Ba	63.6±2.3 ABa	46.6±2.7 Ab
400	52.8±1.9 Bab	57.2±8.3 Ba	45.8±3.5 Ab
600	53.0±1.4 Ba	42.4±1.8 Cb	45.0±3.9 Aab
800	54.6±0.5 Ba	38.0±3.5 Cb	43.6±1.4 Ab

Fig. 1 Effects of inoculation of different endophytes on above and belowground biomass in *Salicornia* sp. growing under different NaCl concentrations. Differences in treatments were checked with a Tukey's test at $p < 0.05$. Different letters indicate differences within treatments.

5.3 Nitrogen and phosphorus concentrations in shoots and roots

Nitrogen concentration in shoots decreased with salinity ($p < 0.01$). Fungal isolates showed different effects on shoot N ($p = 0.050$). At 150 mM NaCl, shoot N concentration in plants inoculated with *Stemphylium* sp. was higher than in plants inoculated with *Phoma* sp. ($p = 0.050$), but no differences occurred with controls or those inoculated with other strains (Fig. 4). At 650 mM NaCl, no differences were observed on N concentration in shoots ($p = 0.36$). Phosphorus

concentration in shoots was affected by salinity ($p < 0.01$) and fungal isolates ($p < 0.01$), whereas their interaction was not significant ($p = 0.64$). Plants growing at 150 mM NaCl had a higher P concentration ($p < 0.01$) compared to those growing at 650 mM NaCl, regardless of the inoculated fungus. When the different fungal treatments were analysed, at both 150 and 650 mM of NaCl, the lowest P concentration was detected in plants inoculated with the fungal mixture, which significantly differed from those of the controls at 150 mM NaCl ($p < 0.01$) and 650 mM NaCl ($p < 0.05$) (Fig. 4).

Nitrogen concentration in roots was not affected by salinity ($p = 0.06$) but by fungal isolates ($p < 0.01$) and their interaction ($p = 0.046$). Plants growing at 150 mM NaCl inoculated with *Stemphylium* sp. had higher N concentration compared to controls ($p = 0.04$). At 650 mM NaCl, plants inoculated with a fungal mixture showed higher N concentration compared to control ($p < 0.01$) (Fig. 5). For P concentration in roots, no differences among fungal treatments were observed at 150 ($p = 0.08$) and 650 mM NaCl ($p = 0.11$) (Fig. 5).

Fig. 2 Effects of inoculation of different endophytes on total biomass in *Salicornia* sp. growing under different NaCl concentrations. Differences in treatments were checked with a Tukey's test at $p < 0.05$. Different letters indicate differences within treatments. Boxes cover the first and third quartiles and horizontal lines denote the median. Whiskers represent the ranges for the bottom 25% and the top 25% of the data values. Black dots are outliers

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5.4 Chlorophyll fluorescence

Chlorophyll fluorescence was influenced by the interaction among salinity, fungal isolate and time after inoculation ($p < 0.01$). The interactive effects of fungal isolate and time influenced fluorescence yield at both 150 ($p < 0.01$) and 650 mM NaCl ($p < 0.01$). At 150 mM of NaCl, significant fungal isolate-related differences were detected 4 weeks after the experiment started: plants inoculated with *Phoma* sp. had higher Fv/fm compared to those inoculated with the fungal mixture ($p = 0.04$). In addition, at the last week before harvesting, plants inoculated with *Phoma* sp. had higher Fv/fm

Fig. 3 **a** *Salicornia* sp. inoculated with *Stemphylium* sp. (St150) and non-inoculated ones (Co150) growing at 150 mM NaCl. **b** Non-inoculated *Salicornia* sp. growing at different salt concentrations: Co650: 650 mM NaCl and Co150: 150 mM NaCl

compared to controls ($p = 0.002$) (Table S2). At 650 mM of NaCl, non-inoculated plants had higher Fv/fm in the last measurement event compared to individuals inoculated with *Phoma* sp. ($p = 0.04$) and *Stemphylium* sp. ($p = 0.01$) (Table S2).

5.5 Detection of fungi in the roots and re-isolation

We observed the presence of the three isolates in roots of *Salicornia*, although *Phoma* sp. colonization was not as frequent as that of *Alternaria conjuncta* and *Stemphylium* sp. (Figure S2). When a subset of *Salicornia* roots from each treatment were plated in PDA medium for fungal isolation, developing colonies resembled morphologically the three isolates previously inoculated were detected. Nevertheless, for the treatments consisting of a mixture of the three isolates, only *Stemphylium* sp. and *Alternaria conjuncta* were morphologically detected. No colonies were observed in the mock inoculated treatments.

6 Discussion

Root endophytes occur ubiquitously among plant species (Rodriguez et al. 2009). However, their role in this association remains poorly understood and controversial in most cases. Their effect on plant growth can vary from positive (Santos et al. 2017; Furtado et al. 2019b; Mateu et al. 2020) to neutral (Mayerhofer et al. 2013) and sometimes negative (Stoyke and Currah 1993; Tellenbach et al. 2011; Mayerhofer et al. 2013). Our study demonstrates that under salt stress, the presence of endophytes does not significantly improve biomass production in *Salicornia*. However, under optimum salinity condition, an increase in biomass was observed. Furthermore, the positive effects of endophytes on plant biomass and N concentration varied with fungal isolate and interaction among different fungi.

Salicornia species are among the most salt-tolerant plants, surviving up to 1 M of NaCl in the soil (Ushakova et al. 2005). Salt exclusion, accumulation and excretion are some of the strategies adopted by *Salicornia* to survive and thrive under extremely saline environments (Meng et al. 2018). In the same way, endophytic fungi evolved different adaptations to high salt concentration (Ruppel et al. 2013). Association of plants with fungal endophytes has been shown to reduce the impact of salt stress and other abiotic stresses by inducing several plant responses, such as inducing systemic resistance, production of beneficial metabolites and phytohormones (Gupta et al. 2020). However, in the case of halophytes, the influence of a plant's mycobiome on salt tolerance is poorly investigated. One study that assessed the effect of fungal inoculation on the growth of a facultative halophyte (*Phragmites australis*; Mateu et al. 2020) demonstrated that inoculated plants had higher aboveground biomass compared to controls, although neutral effects of fungal inoculation on nitrogen concentration and photosynthesis were observed.

Fungal endophytes can promote plant growth under salt stress with different metabolic and genetic strategies. One of the main physiological changes that occurs in inoculated plants is the modification of root architecture by increasing its biomass and nutrient absorption (Gupta et al. 2020). This has been shown to occur in different plant species, for example *Zea mays* L. (Rho et al. 2018), *Oryza sativa* L. (Saddique et al. 2018) and *Vochysia divergens* Pohl (Farias et al. 2019). In our study, plants inoculated with *Stemphylium* sp. had greater above- and belowground biomass compared to non-inoculated plants at both salt concentrations. However, significant differences were observed only at 150 mM NaCl. The same isolate improved N concentration in roots only at 150 mM NaCl. Although *Stemphylium* sp. grew in all in vitro tested NaCl concentrations, colony growth slightly decreased with an increase in salinity. It appears that at 650 mM NaCl, although still present in *Salicornia* roots as confirmed by our microscopical observations, the ability of

Fig. 4 Effects of inoculation of different fungal endophytes on N and P concentrations in shoots of *Salicornia* sp. growing under different NaCl concentrations. Differences in treatments were checked with a Tukey's test at $p < 0.05$. Different letters indicate differences between treatments. Boxes cover the first and third quartiles and horizontal lines denote the median. Whiskers represent the ranges for the bottom 25% and the top 25% of the data values. Black dots are outliers

Stemphylium sp. to enhance plant growth decreased. This has been observed in other studies in which inoculated plants growing under salt stress were colonized by fungi, although positive effects depended on fungal isolate and were observed only in low salt stress conditions (Pereira et al. 2019; Mateu et al. 2020). For example, Mateu et al. (2020) observed positive effects of fungal inoculation in growth of the facultative halophyte *Phragmites australis* only at 200 mM NaCl but not at 400 mM NaCl. However, it is still unclear why under high salt stress the ability of endophytes to promote growth decreases, even though they are still able to colonize plant tissues. It is possible that under salt stress condition, either the mutualistic interaction shifts towards parasitic interaction or the fungi become metabolically inactive. In this regard, further

work is necessary to investigate how salt stress affects fungal physiology and consequently the outcome of the plant-fungus symbiosis.

Phosphorus (P) and nitrogen (N) are two of the most important nutrient elements for plant growth. However, their absorption is compromised by salinity due to N immobilisation and P precipitation. Endophytes have been shown to play a role in ameliorating these effects. For example, Yadav et al. (2010) showed that the endophytic fungus *Piriformospora indica* produces a phosphate transporter (PiPT) actively involved in phosphate transport to the plant. The same fungus has been associated with improved N assimilation of plants by stimulating the key enzyme for nitrate assimilation (Sherameti et al. 2005). In our study, N and P

Fig. 5 Effects of inoculation of different fungal endophytes on N and P concentrations in roots of *Salicornia* sp. growing under different NaCl concentrations. Differences in treatments were checked with a Tukey's test at $p < 0.05$. Different letters indicate differences between treatments. Boxes cover the first and third quartiles and horizontal lines denote the median. Whiskers represent the ranges for the bottom 25% and the top 25% of the data values. Black dots are outliers

concentrations in shoots and roots were strongly affected by salinity, whereas fungal inoculation had a positive effect only on N concentration in roots, at both salinities. Although small or no effects of fungal inoculation on nutrient concentration have been reported before (Ding et al. 2016), most studies indicated that DSE fungi act as plant growth promoters by increasing N and P concentrations in plant tissues (Della Monica et al. 2015; He et al. 2020).

Overall, plants growing under 150 mM NaCl had higher N and P concentrations in shoots and roots when compared to those growing at 650 mM NaCl, which is consistent with the decreasing nutrient absorption with increasing salinity (Ruiz et al. 1997; Hu and Schmidhalter 2005; Amiri et al. 2010). Surprisingly, fungal mixture inoculation negatively affected P

concentration in shoots in comparison with plants inoculated with individual isolates or not-inoculated at both salinities. The same pattern was observed by Aguilar-Trigueros and Rillig (2016) who demonstrated that individuals of *Arrhenatherum elatius* (Poaceae) inoculated with a fungal mixture (strains belonging to *Fusarium*, *Gibberella*, *Microdochium*) produced 17% less biomass compared to non-inoculated ones. A weak parasitism in the sense of increased competition for a rare resource or an induction of host resistance through the allocation of carbon to the production of defence compounds, instead of investing in vegetative growth, has been suggested as reasons for such a decrease in biomass in treatments inoculated with a fungal mixture and

might as well explain our findings (Aimé et al. 2013; Aguilar-Trigueros and Rillig 2016).

In addition to biomass increase, and N and P concentrations, photosynthesis has been also shown to be improved in plants colonized by endophytes, particularly those growing under salt stress (Ghorbani et al. 2018; Molina-Montenegro et al. 2020). The actual mechanisms vary. For example, endophytes may facilitate the absorption of Mg^{2+} , an essential nutrient involved in many photosynthetic processes (Jogawat et al. 2013; Yin et al. 2014). In addition, Kumar et al. (2012) showed that *Piriformospora indica*, a dark septate endophyte, improved photosynthesis of Brassicaceae species by modulating the defense system, especially the ascorbate-glutathione (ASH-GSH) cycle, maintaining a high antioxidative environment during salt stress. In general, our results did not show a significant effect of fungal inoculation on photosynthetic performance of *Salicornia*, although weak positive and negative effects were observed at particular measurement times. This is consistent with results reported by Wezowicz et al. (2017) on *Verbascum lychnitis* plants inoculated with a strain belonging to *Phoma*. Interestingly, when the same plant species was inoculated with other fungi (i.e., *Xylaria* sp.), positive effects were observed, which emphasizes the importance of fungal identity on plant performance. In addition to the effect of fungal isolate, it is possible that, in our experiment, the intensity and duration of the salt-imposed stress was insufficient to cause severe photo-inhibitory damage at the time of sampling, as observed in other studies (Lichtenthaler et al. 2005; Ban et al. 2017; Pan et al. 2018). For example, Ban et al. (2017) showed that positive effects of fungal inoculation on photosynthesis were observed only in maize plants growing at high but not low or moderately lead-contaminated soil. An experiment with higher salinity for *Salicornia* growth and longer duration could provide more conclusive insights in that regard.

Among the three fungal isolates used in this study, *Stemphylium* sp. was found to positively alter above and belowground biomass and N concentration in roots at optimum salinity condition for plant growth. Interestingly, most of the species belonging to this genus as well as those belonging to the genera *Alternaria* and *Phoma*, are known as plant pathogens (Thomma 2003; Hanse et al. 2015; Deb et al. 2020). *Stemphylium globuriferum*, for instance, has been reported as an important pathogen of sugar beet (*Beta vulgaris* L.) (Hanse et al. 2015). However, fungal endophytes are known to shift their functional role between pathogenicity and mutualism depending on fungal genotype, host and abiotic conditions (Hardoim et al. 2015) and in the case of *Salicornia*, although endophytes are commonly present in their roots, their co-occurrence has not been associated to a functional role. Besides the potential of abiotic conditions to modulate the outcome of plant-fungus interaction, it is possible that our *Stemphylium* isolate establishes a mutualistic association with

Salicornia but a different symbiotic relationship with other salt marsh plants. The same can be the case with our *Alternaria* and *Phoma* isolates when inoculated in different salt marsh plant species. This has been observed, for instance, with pathogenic *Colletotrichum* spp., which expressed different symbiotic lifestyles ranging from mutualism to commensalism depending on the host (Redman et al. 2001). The inoculation of our *Stemphylium* isolate as well as other *Stemphylium* genotypes in different salt marsh plants under different growth conditions, would provide some important insights to understand the plasticity of the plant-fungal association.

Although we did not include in our experiment the endophytic bacteria present in *Salicornia* roots, fungi and bacteria coexist in the root microbiome and more interestingly, the bacterial community seems to influence the composition of the fungal ones (Furtado et al. 2019a). In addition, single bacterial inoculations have been shown to positively affect growth of *Salicornia* under salt stress (Bashan et al. 2000; Zhao et al. 2016; Mesa-Marín et al. 2020), whereas the effect of single DSE fungal inoculations was unknown until now. It is possible that their co-inoculation contributes to the plant's tolerance to salt stress. For example, in other plant species, arbuscular mycorrhizal fungi and bacteria seem to interact synergistically promoting plant growth by improving nutrient uptake and protection against plant pathogens (Artursson et al. 2005). Following this aspect, we provide here the first evidence that single DSE inoculations can affect growth of *Salicornia* by improving nutrient uptake and biomass production. Further work in which both microbial groups are included could provide interesting insights to understand whether they interact synergistically impacting growth of *Salicornia* and potentially contributing to its adaptation to the salt marsh conditions.

Overall, our results indicate that under greenhouse conditions *Salicornia* grows better at 150 mM NaCl than in 650 mM NaCl. This agrees with other studies in which the optimum salinity condition for growth ranged from 100 to 200 mM NaCl (Algharib et al. 2016) while effects of salt stress on plant growth were observed in a range of 300 to 600 mM NaCl (Aghaleh et al. 2011; Torabi and Niknam 2011). An important aspect to consider in future studies is the variation in ploidy among *Salicornia* species. Polyploidy results from an evolutionary process in which two or more genomes are brought together into the same nucleus, causing phenotypic changes, which may reflect on organism's interactions (Segraves and Anneberg 2016). This is a common phenomenon that can lead to decisive morphological and physiological differences (e.g., salt tolerance) in *Salicornia*, as well as in species ecological interactions (Teege et al. 2011). In our study, the ploidy of a subset of individuals was analysed revealing that approximately 70% of the individuals were tetraploid (*S. procumbens*) and 30% diploid (*S. europaea*). In

addition to ploidy, seed dimorphism in *Salicornia* is another aspect with the potential to alter the outcome of the experiment. *Salicornia* species produce two different types of seeds that differ in size: central seeds in the inflorescence are larger compared to lateral seeds. Larger seeds seem to have a higher tolerance to salinity than smaller ones (Orlovsky et al. 2016). To avoid biases originating from seed differences, seeds were randomly selected from a pool containing both morphotypes.

The results of this study indicate that *Stemphylium* sp. formed a mutualistic association with *Salicornia* resulting in an increase in total biomass and N concentration in roots. However, the effects of fungal inoculation were evident only under salinity condition that was optimal for plant growth. In addition to that, inoculation of *Salicornia* with a fungal mixture had an effect on nutrient concentration in both shoots and roots, showing that the fungus-fungus interactions played a role on plant's ability to absorb nutrients. We tried to mimic the natural conditions of a salt marsh in a controlled environment, but further studies should include more complexity by including other microorganisms that co-occur with root-associated fungi in the roots of *Salicornia* or are present in the rhizosphere (e.g., bacteria). This would provide important insights into the effect of the whole endophytic microbial community versus the endophytic fungal community on plants performance.

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Code availability Not applicable.

Author contributions DRG and DCA planned and designed the experiment with suggestions provided by RP and GZ. DRG set up the experiment, collected and analysed the data and wrote the first draft. DRG, RP, GZ and DCA contributed to the final version of the manuscript.

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval Not applicable.

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