



Seasonal Dynamics of Root-Associated Fungal Microbiomes in Annual and Perennial Halophytes

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Received: 28 January 2025 / Revised: 1 September 2025 / Accepted: 2 September 2025 / Published online: 14 October 2025
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Abstract

Halophytes host diverse fungal communities that can influence plant fitness and whole ecosystem functioning. Despite their importance, general knowledge on the root-associated endophytic fungal microbiomes of halophytes is limited to few species. In our study, we investigated fungal microbiomes associated with roots of two annual (*Salicornia procumbens* and *Suaeda maritima*, both Amaranthaceae) and two perennial (*Atriplex portulacoides*, Amaranthaceae, and *Puccinellia maritima*, Poaceae) halophytes using amplicon sequencing of nuclear ribosomal DNA. We investigated the fungal communities with respect to host life history strategy, host identity and seasonality. Moreover, we investigated species-specific root anatomical traits, which could potentially influence microbiome assembly. Overall, our results revealed a strong effect of host identity on fungal microbiomes. Furthermore, variation in microbiome composition throughout seasons seems to be a species-specific process which does not depend on plant life history strategy. We observed higher richness, diversity and evenness in samples of the grass *P. maritima* compared to the three Amaranthaceae, particularly those collected in autumn. Species-specific root anatomical traits, such as larger roots cells in *P. maritima* go along with the higher diversity. The fungal orders Pleosporales and Xylariales were, in general, the most common endophytes in all species, but Glomeromycota was also detected in roots of *P. maritima* in summer. Overall, our study provides a better understanding on the dynamics of fungal microbiome assembly in roots of halophytes throughout seasons emphasizing the importance of host identity on fungal microbiome assembly.

Keywords Endophytic fungi · Metabarcoding · Plant microbiomes · Plant-fungal interaction · Salt marsh plants

Introduction

Halophytes are plants that survive and complete their life cycle in a salt concentration of ≥ 200 mM of NaCl (11.7 ppt; Flowers et al., 1986). Different species adopt different anatomical (e.g. presence of salt-excreting structures such as bladders and salt glands) and physiological (e.g. reduction of the Na⁺ influx and the compartmentalization and excretion of sodium ions) mechanisms to counter salt stress (Flowers & Colmer, 2015). A previously often overlooked aspect of plants salt tolerance concerns the root-associated bacterial and fungal microbiomes that have been shown to significantly contribute to plant reactions to salinity stress

(Christakis et al., 2021; Ruppel et al., 2013). Fungal and bacterial mutualists were shown to enhance salt tolerance in halophytes by, for example, improving plant nutrient uptake (Zhang et al., 2014), and in the case of bacteria, the accumulation of Na⁺ in the plant shoots to stimulate growth and photosynthesis (Navarro-Torre et al., 2017; Redondo-Gómez et al., 2009). Thus, many central processes in halophyte communities depend on the interaction of plants with their microbiome. Consequently, a rising number of studies investigate microbiome composition of plant species and the species-specificity of these interactions, which has only been possible to analyze with the advent of metabarcoding strategies using DNA and, less frequently, RNA sequencing. However, general knowledge on the factors influencing the composition and especially the dynamics of the microbiome associated with many species of halophytes is still lacking (Lumibao et al., 2024). This is of special interest since members of the halophytic microbiome have the potential to increase salinity tolerance not only in halophytes but also in less salt-tolerant crops of economic importance (Yuan et al.,

Communicated by Just Cebrian

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2016). More fundamentally, understanding plant-associated microbiome dynamics is important because changes in its composition and structure can directly affect whole ecosystem functioning (Wagg et al., 2019).

Studies on the halophytic microbiome have mostly focused on bacteria and were limited to few plant species, including *Arthrocnemum macrostachyum* (Mora-Ruiz et al., 2016), *Suaeda salsa* (Yuan et al., 2016), *Salicornia* spp. (Ferreira et al., 2021; Furtado et al., 2019; Yamamoto et al., 2018), *Glaux/Lysimachia maritima* (Yamamoto et al., 2018) and *Limonium* spp. (Qin et al., 2018; Wang et al., 2016) and a few others (e.g., Zhou et al., 2024). With regards to fungal microbiomes, Lumibao et al. (2024) recently summarized published analyses and found the highest diversity in salt marshes compared to dunes, intertidal flats and tidal wetlands. Comprehensive metabarcoding investigations revealed the highest diversity and included those associated with *Salicornia europaea* (Furtado et al., 2019; Buhk, 2020; Gonçalves et al., 2022), *Salicornia procumbens* (Gonçalves et al., 2022), *Suaeda salsa* (Yuan et al., 2016) and few others. Different factors are known to shape microbiome structure, for example, species identity (Li et al., 2021; Rudgers et al., 2022), genotype (Leopold & Busby, 2020) and cytotype of the host (Gonçalves et al., 2022), season (Bueno de Mesquita et al., 2018; Vukicevich et al., 2019) and interactions between fungi (Furtado et al., 2019; Pan & May, 2009). Plant life history may also influence plant-microbial associations (Emmett et al., 2017). However, this has been poorly investigated for fungi. The fungal microbiome associated with halophytes seems to be dominated by Pleosporalean fungi (Furtado et al., 2019; Gonçalves et al., 2022; Maciá-Vicente et al., 2016; Yuan et al., 2016). In the only study comparing fungal interactions in annual and perennial halophytes, Maciá-Vicente et al. (2016) observed a lower diversity and slower colonization of fungal symbionts in roots of the annual *Salicornia patula* compared to the perennial *Arthrocnemum macrostachyum*, although plants of the two species were growing physically apart and under different soil conditions.

In the case of salt marshes of northern Germany, annual halophytes are restricted to the family Amaranthaceae, whereas perennial halophytes commonly present and ecologically characteristic are *Atriplex portulacoides* (Amaranthaceae) and *Puccinellia maritima* (Poaceae). These two species occur in the lower salt marsh (LSM) along with the annuals *S. procumbens* and *Suaeda maritima*. In the case of *S. procumbens*, Gonçalves et al. (2022) reported the dominance of the orders Xylariales and Pleosporales in the roots, but the microbiome of the other species was hitherto not investigated. Maciá-Vicente et al. (2016) reported slow colonization by few and poorly represented species in the annual *S. patula* but a stable, diverse community of few dominant and other rare species in the perennial *Arthrocnemum*

procumbens. This leads to the general hypothesis that annuals are colonized by few fungi and this colonization is slow over the season, whereas perennials keep their diverse community more or less throughout the year. A lack of significant seasonal variation was also reported in *S. europaea* samples collected in spring and autumn in salt marshes in Poland (Furtado et al., 2019) but diversity was not compared with other species.

In order to understand more generally the microbiome dynamics throughout the complete plant life cycle and among species and life history, we collected the annual *S. procumbens* in addition to *S. maritima* along with the perennials *A. portulacoides* and *P. maritima* in spring, summer and autumn. This allows us to test species- and family-specific (Amaranthaceae vs. Poaceae) patterns of variation in the microbiome, which often play an important role in structuring the plant-associated fungal microbiome (Botnen et al., 2020). With regards to life history, we hypothesize that perennials, due to their longer life cycle, display well-established and more diverse fungal microbiomes compared to annuals, as has previously been reported (Maciá-Vicente et al., 2016). Perennial plants have been shown to benefit more from microbial associations in comparison to annuals, likely due to the costs of establishing beneficial associations and the time-lags to receive benefits (Primieri et al., 2022), which renders fungal associations more disadvantageous for annuals due to their short life cycle. In consequence, perennials should strategically associate with more diverse fungal communities, compared to annuals, in order to potentially increase the chances of attracting a wider range of beneficial fungal partners. Based on the findings of Maciá-Vicente et al. (2016), we expected to find low turnover rates from spring to summer based on the low reliance of annuals on mutualistic fungi. However, we expected an increase in microbiome richness and diversity from summer to autumn in annuals with an increase in saprophytes.

As a result of an evolutionary adaptation to the highly dynamic salt marsh conditions, halophytes differ in several physiological, morphological and anatomical features and it is unclear which factor is responsible for differences in the microbiome. For example, species-specific root anatomical traits, which have been shown to vary considerably even among closely-related species of halophytes (e.g. *S. europaea* — 2× and *S. procumbens* — 4×; Gonçalves et al., 2022), are likely an important and frequently neglected factor directly affecting fungal colonization and establishment. Two such traits, the presence of aerenchyma and a decrease in the living cortical area, negatively impacted mycorrhizal colonization in *Zea mays* (Galindo-Castañeda et al., 2019) and in different temperate tree species (Guo et al., 2008). Given their difference in two *Salicornia* cytotypes, they are also likely at least partly responsible for differences in their microbiome assembly in the study by Gonçalves et al.

(2022). Therefore, we studied anatomical traits that may be associated with fungal colonization such as root diameter, stele diameter, thickness of the living cortical area and cell diameter. Particularly, we hypothesize that roots with a thicker living cortical area and larger cell diameter provide more space for fungal colonization, which could lead to a more diverse fungal microbiome.

Using amplicon sequencing, we investigated (i) the taxonomic and functional diversity of fungal microbiomes in the saltmarsh of Northern Germany, and in particular the differences in fungal microbiome composition and their seasonal dynamics in one community with respect to (ii) the importance of filtering by host life history strategy, (iii) host taxonomy and (iv) season. Furthermore, we analyzed host-specific root anatomical traits to address the question (v), which anatomical traits may potentially influence microbiome assembly in halophytes. To investigate these questions, we analyzed the fungal microbiome associated with two annual (*S. maritima* and *S. procumbens*) and two perennial (*A. portulacoides* and *P. maritima*) species collected in spring, summer and autumn in the lower zone of a salt marsh located on the island of Spiekeroog, Germany. In the three sampling campaigns, we collected individuals growing in close proximity and overlapping root space, in order to minimize the influence of variations in the local abiotic environment.

Methods

Study Site and Sampling

Sampling was performed during the spring (June 16th), summer (August 25th) and autumn (October 13th) of 2020 in a salt marsh located within the limits of the Wadden Sea National Park on the island of Spiekeroog, Germany (53°45'N 7°43'E). The timing of the sampling conformed largely to pre-flowering, peak-flowering, and fruiting seasons of the plants. Four different halophyte species occurring in the LSM were collected: two annuals (*Salicornia procumbens* and *Suaeda maritima*) and two perennials (*Atriplex portulacoides* and *Puccinellia maritima*). In addition to the plant samples, in each sampling event, three soil samples were collected in the lower salt marsh and their elemental chemical composition were analyzed at the Institute for Soil and Environment, Lufa Nord-West (Oldenburg, Germany). In the first sampling (spring), six sampling points (five meters apart from each other) were marked along a transect in the LSM. In each sampling point, two individuals of each plant species occurring inside a 1 m diameter circle were collected. In summer and autumn, the sampling was performed identically and new individuals were collected at the same sampling points established in spring. Overall, we,

therefore, analyzed 72 samples, six plots of four species at three sampling times. The sampling consisted of removing the plants carefully from the soil, placing them in plastics bags and keeping at 4 °C until further processing in the laboratory. In the summer sampling, 12 additional individuals belonging to each plant species (two individuals collected in each sampling point) were collected to determine their root anatomical traits.

Once in the laboratory, roots of each individual were separated from the aerial part, washed under tap water to remove any adhering soil particle and those samples selected to determine root anatomical traits were stored individually in FAA (formaldehyde: alcohol: acetic acid, 10%: 50%: 5% + 35% water) until further processing. In the case of the samples for DNA extraction, roots were cut in small pieces of about 1 cm and subsequently, root pieces were surface sterilized following the protocol described in Crous et al. (2009). We selected only secondary fine roots of approximately the same thickness for DNA extraction to guarantee comparability and which were easier to extract with less secondary compounds. Finally, for each species, roots of the two individuals collected at the same sampling point were pooled together to form a composite sample, ending up with a total of six composite root samples of each species collected in each season. After that, roots were kept at − 20 °C until DNA extraction.

Root Anatomical Investigation

In the laboratory, root samples that were preserved in FAA solution were washed with distilled water before mounting the slides. We selected only secondary fine roots of approximately the same thickness to determine root anatomical traits. For each plant species, six slides were mounted with at least three root cross-sections resulting in a total of 18 root cross-sections per species. Measurements were performed using the software Olympus CellSens Entry 2.2 (Olympus Cooperation, Tokyo, Japan) under a Reichert-Jung Polyvar (Reichert, Wien, Austria) transmitted light microscope. The following anatomical parameters were measured: maximum root diameter, maximum stele diameter, thickness of the living cortical area and maximum cell diameter. Additionally, aerenchyma was reported as present or absent. The final percentage of aerenchyma occurrence was calculated by dividing the total number of observed cross-sections by the number of cross-sections in which aerenchyma was present. For statistical analyses, a one-way ANOVA was performed using the function `aov` followed by a Tukey test to identify differences between plant species that could potentially explain differences in the microbiomes. Statistical analyses were performed in R (version 3.6.3, R Development Core Team, 2011).

DNA Extraction, PCR, Library Preparation and Sequencing

Before DNA extraction, root samples (approximately 20 mg) were lyophilized in an Alpha 1–2 LDplus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 24 h. After that, samples were milled using a Mixer Mill (Retsch, Germany) for two cycles of 1 min each with 30 oscillations per second. Genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytic Jena AG, Jena, Germany), following the manufacturer's instructions. Concentration and quality of extracted DNA were checked by absorption spectrophotometry with a Tecan infinity 200 pro (Tecan Group Ltd., Männedorf, Switzerland). Dilutions for each sample were prepared to achieve a final concentration of $2 \text{ ng } \mu\text{l}^{-1}$.

For the amplification of the ITS1 region of nrDNA, we used the primers ITS1FKyo2 (Toju et al., 2012) and ITS86R (Vancov & Keen, 2009) after initial testing of a couple of commonly used primer pairs, because it showed best amplification success across all major fungal groups. Each sample was amplified in duplicates and the reaction was prepared in a volume of $25 \mu\text{l}$ containing: $1.0 \mu\text{l}$ of DNA ($2 \text{ ng } \mu\text{l}^{-1}$), $5 \mu\text{l}$ of $5\times$ Phusion Buffer, $0.6 \mu\text{l}$ of MgCl_2 , $0.5 \mu\text{l}$ 10 mM dNTPs, $0.5 \mu\text{l}$ of each primer ($10 \text{ pmol } \mu\text{l}^{-1}$), $0.2 \mu\text{l}$ of Phusion Hot Start Flex DNA polymerase (New England Biolabs GmbH) and $16.7 \mu\text{l}$ of nuclease-free water. PCR parameters were an initial step of 98°C for 1 min and 3 cycles of 98°C for 30 s, 50°C for 30 s and 72°C for 30 s. The further 32 cycles consisted of 98°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The elongation step was at 68°C for 10 min. After amplification, products of the two reactions were pooled together and used in a second PCR reaction to attach the adapters. This reaction was performed accordingly: an initial step of 98°C for 1 min and 7 cycles of 98°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by an elongation step at 68°C for 10 min. PCR products were checked on a 1.2% agarose gel stained with ethidium bromide (Sigma-Aldrich). For the whole procedure, we included two technical replicates and four negative controls. Subsequently, amplicons were sequenced at LGC Genomics (Berlin, Germany) on an Illumina MiSeq platform (V3 chemistry, 300-bp paired-end reads) and demultiplexed.

Bioinformatics and Statistical Analyses

Sequence data was analyzed and visualized using QIIME2 v.2022.11.0 (Bolyen et al., 2019). Sequences were processed using ITSxpress plugin (Rivers et al., 2018). After that, reads were further processed using DADA2 denoise. paired plugin (Callahan et al., 2016) to identify exact Amplicon Sequence Variants (ASVs) and, alternatively, lumping sequence variants to Operational Taxonomic Units (OTU) at

99% similarity (Callahan et al., 2017). Results from analyses of ASVs and OTUs did not differ significantly, so only the latter are presented. Taxonomic assignments were performed using qiime feature-classifier classify-sklearn with a pre-trained Naïve-Bayes classifier using the database UNITE (version v.8.2) (Abarenkov et al., 2020). Before proceeding with the diversity and functional analyses, non-fungal OTU were removed from the final OTU table. The same applied to those OTUs whose relative frequency was less than 0.005% to avoid an effect of sequencing errors. For functional assignment, the FUNGuild database (Nguyen et al., 2016) was used and fungi were assigned to trophic modes. Taxa for which the information on trophic mode was not available were classified as “unidentified”.

To confirm whether sampling depth was sufficient to cover sample diversity, rarefaction curves of richness and Shannon diversity were generated in QIIME2 using the qiime.diversity.alpha-rarefaction plugin. Differences in sampling depth were accounted by rarefying samples to a sampling depth of 4500 sequences before proceeding with the diversity analyses. The basic Qiime2 statistics were imported in R (version 3.6.3, R Development Core Team, 2011) using the package Qiime2R (<https://github.com/jbisanz/qiime2R>) for further analyses. Alpha diversity was estimated based on observed richness, Pielou's evenness, Shannon diversity and Faith's phylogenetic diversity. Differences among groups, either species separated by seasons or seasons separated by species, were calculated with a Kruskal–Wallis followed by pairwise comparisons using package rstatix (Kassambara, 2019). For beta diversity, a permutational analysis of variance (Adonis) using R package vegan (Oksanen et al., 2011) was performed to check the significance of the factors host life history strategy, species, season and their two-way interaction. In order to check dissimilarities in microbial communities associated with each species in spring, summer and autumn, a Permanova test, based on 999 permutations and a Bray–Curtis distance matrix, was performed. To visualize dissimilarities in fungal microbiomes among different plant species, we used non-metric multidimensional scaling graph (NMDS) based on a Hellinger-transformed Bray–Curtis distance matrix using the R package microViz (Barnett et al., 2021). All figures were generated using ggplot2 (Wickham, 2016).

Based on the root anatomical investigation, thickness of cortical area and cell diameter in the cortex were shown to best differentiate the plant species. Therefore, we hypothesized that these traits would influence the species-specific parts of the fungal communities most. We performed a canonical correspondence analysis (CCA) in R using the package microViz (Barnett et al., 2021) to test this hypothesis based on two datasets: the abundance information of the summer occurrences of the all OTUs and a table containing the root anatomical traits measured in root samples

collected in summer. The importance of the predictor root parameters was tested using the ANOVA function with 999 permutations and Holm-Bonferroni corrections were applied to adjust the p -values.

Results

Root Anatomical Traits and Link with Fungal Microbiome Diversity

Significant differences between species were observed for maximum root diameter ($p < 0.01$), stele diameter ($p < 0.01$), thickness of living cortical area ($p < 0.01$) and

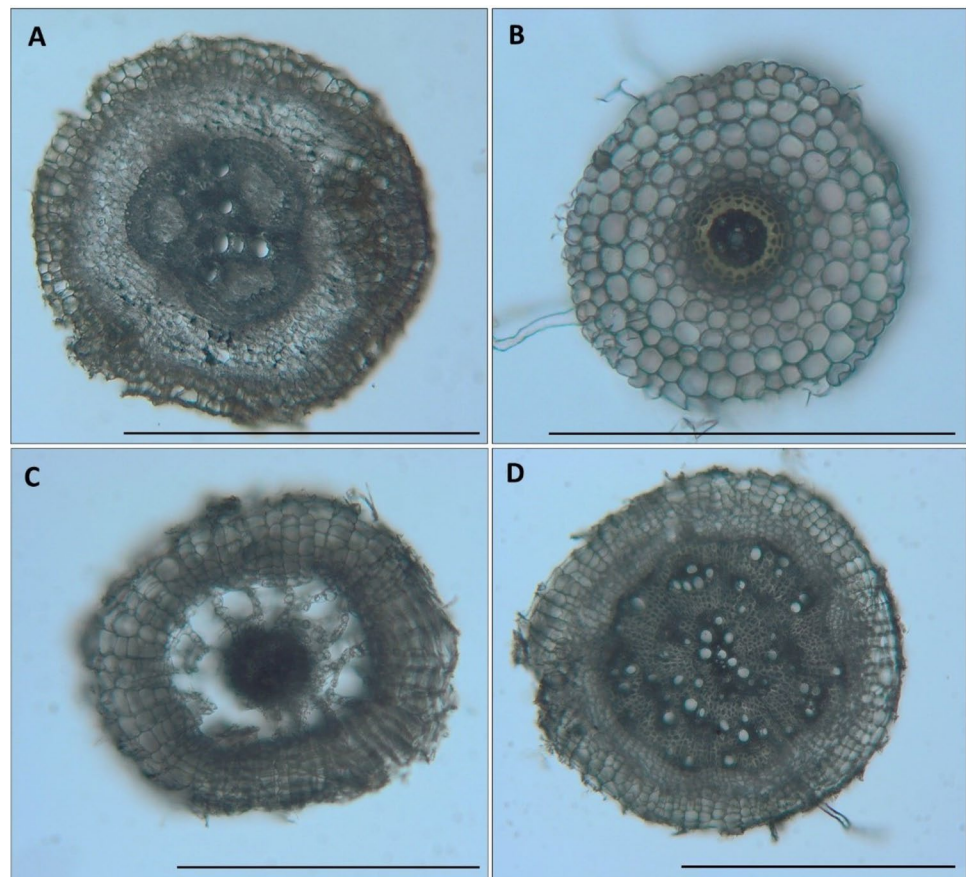
maximum cell diameter ($p < 0.01$) (Table 1, Table S1, Supplementary Material 2). The largest root and stele diameters were observed in *S. maritima*, although not significantly larger than those of *S. procumbens*. *Salicornia procumbens* had the thickest living cortical area with a consistent occurrence of aerenchyma, which was less abundant in *A. portulacoides* and *S. maritima* and absent in *P. maritima* (Fig. 1, Table S2). The largest cell diameter was observed in *P. maritima* and *S. procumbens*, which significantly differed from that of *A. portulacoides* and *S. maritima* (Fig. 1).

Table 1 Root anatomical characters measured in roots of *A. portulacoides*, *P. maritima*, *S. procumbens* and *S. maritima*

Root anatomical characters	<i>A. portulacoides</i>	<i>P. maritima</i>	<i>S. procumbens</i>	<i>S. maritima</i>
Maximum root diameter (μm)	599.3 $\pm$ 97.4 a	489.3 $\pm$ 43.3 b	719.8 $\pm$ 161.4 c	716.1 $\pm$ 123.6 c
Stele diameter (μm)	279.9 $\pm$ 30.5 a	141.9 $\pm$ 26.9 b	195.3 $\pm$ 48.1 c	451.4 $\pm$ 72.4 d
Thickness living cortical area (μm)	319.3 $\pm$ 86.5 ac	367.3 $\pm$ 55.1 a	524.5 $\pm$ 153.8 b	264.5 $\pm$ 76.4 c
Maximum cell diameter (μm)	18.3 $\pm$ 3.7 a	30.4 $\pm$ 6.7 b	26.7 $\pm$ 5.1 b	21.1 $\pm$ 2.9 c
Aerenchyma presence (%)	26.6	absent	100.0	6.6

Data represents mean values $\pm$ standard deviation. Values in the same row represented with different letters are statistically different ($p < 0.05$). Exact values can be found in Table S1

Fig. 1 Root cross sections of perennial *A. portulacoides* (A) and *P. maritima* (B) and annual *S. procumbens* (C) and *S. maritima* (D). Bars represent 500 μm



Sequencing Results and Alpha Diversity of Fungal Microbiomes

A total of 1,5360,854 high-quality sequences were obtained after denoising, merging, and chimera checking. The number of reads per sample ranged from 12,032 to 675,013. Rarefaction curves showed sufficient sequence numbers to capture sample richness and diversity (Fig. S1). Two hundred sixty-four OTUs were found altogether (Table S3). Of these, 116 associated with *S. maritima*, 122 with *S. procumbens*, 152 with *P. maritima* and 85 with *A. portulacoides*. The fewest number of OTUs were found in spring (117), more in summer (148) and the most in the autumn (167). The distribution of OTUs highlighting quantitative differences between the species in each season is shown in Fig. 2.

We observed few significant differences in OTU richness between the fungal microbiomes associated with the different plant species and life histories in different seasons (Supplementary Material 2). In general, the grass *P. maritima* had the highest OTU richness compared to the other species (Fig. 3A). Richness of microbiomes associated with the perennials *A. portulacoides* and *P. maritima* differed significantly among each other in autumn ($p=0.05$) and between *P. maritima* and *S. procumbens* in summer ($p=0.02$) (Fig. 3A). Evenness was generally similar in spring and summer but it was more even in *P. maritima* in autumn compared to those of *A. portulacoides* ($p=0.02$), *S. procumbens* ($p=0.01$), and *S. maritima* ($p=0.06$) (Fig. 3B). Shannon diversity of fungal microbiomes exhibited the same pattern as evenness with diversity of communities being similar except in the autumn, when that of *P. maritima* was more diverse than to those of *A. portulacoides* ($p=0.02$), *S. procumbens* ($p=0.01$), and *S. maritima* ($p=0.07$) (Fig. 3C). There were no significant differences in Faith's phylogenetic diversity across species or seasons (Fig. 3D). There was also no consistent difference between perennials and annuals in any of the four parameters in any season (Fig. 3, Supplementary File 2).

We also investigated, separately for each species, seasonal variation in OTU richness, diversity and evenness (Fig. S2, Supplementary Material 2). For *A. portulacoides*, there was a decrease throughout the seasons in Pielou's evenness ($p=0.02$), Shannon diversity ($p=0.04$), richness ($p=0.15$), which was less clear in Faith's phylogenetic diversity ($p=0.20$). The other perennial, *P. maritima*, showed an increase in richness from spring to the other seasons (spring–summer, $p=0.49$, spring–autumn $p=0.58$) and Shannon diversity over all three seasons ($p=0.04$) and evenness ($p=0.02$) over all three seasons (Fig. S2). In the case of the annual *S. procumbens*, no significant differences between seasons were observed but differences were larger between autumn and the other seasons than between spring and summer (Fig. S2). For *S.*

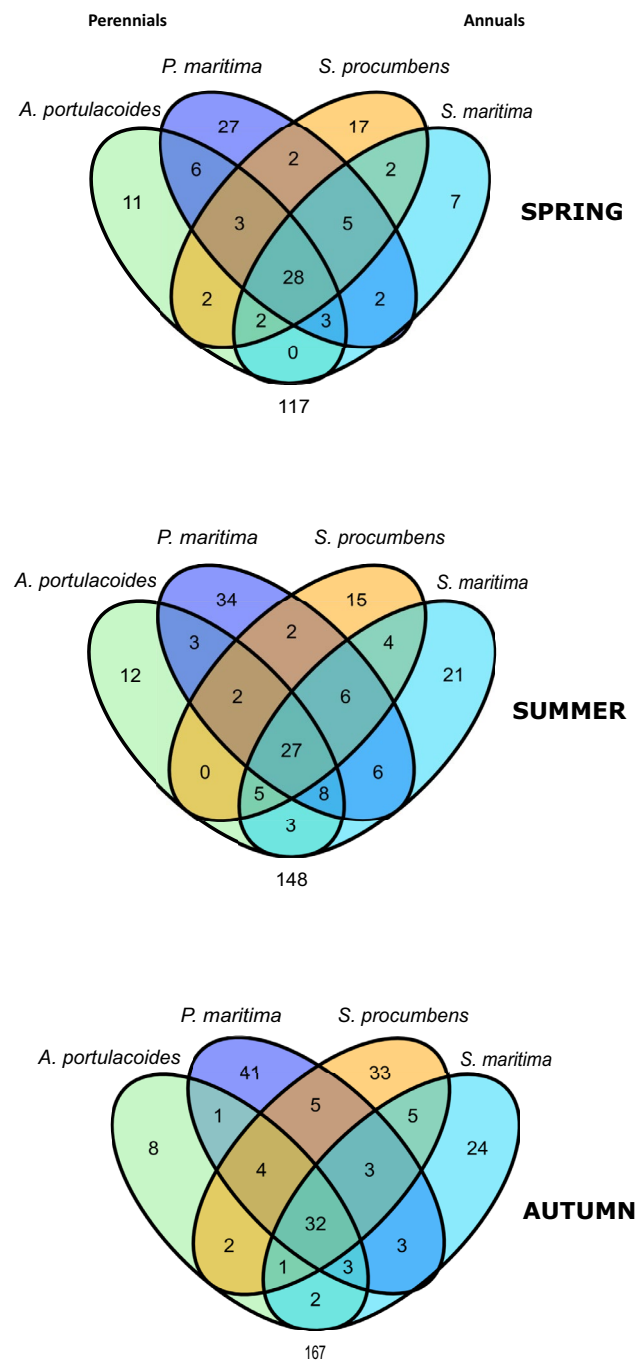


Fig. 2 Venn diagrams showing the distribution of OTUs between the different plant species in spring, summer and autumn

maritima, no differences between seasons were observed for Shannon diversity ($p=0.97$) and evenness ($p=0.94$). On the other hand, samples collected in spring had lower richness and Faith's phylogenetic diversity compared to summer ($p=0.17$, $p=0.05$, respectively) and autumn ($p=0.022$, $p=0.10$, respectively) (Fig. S2).

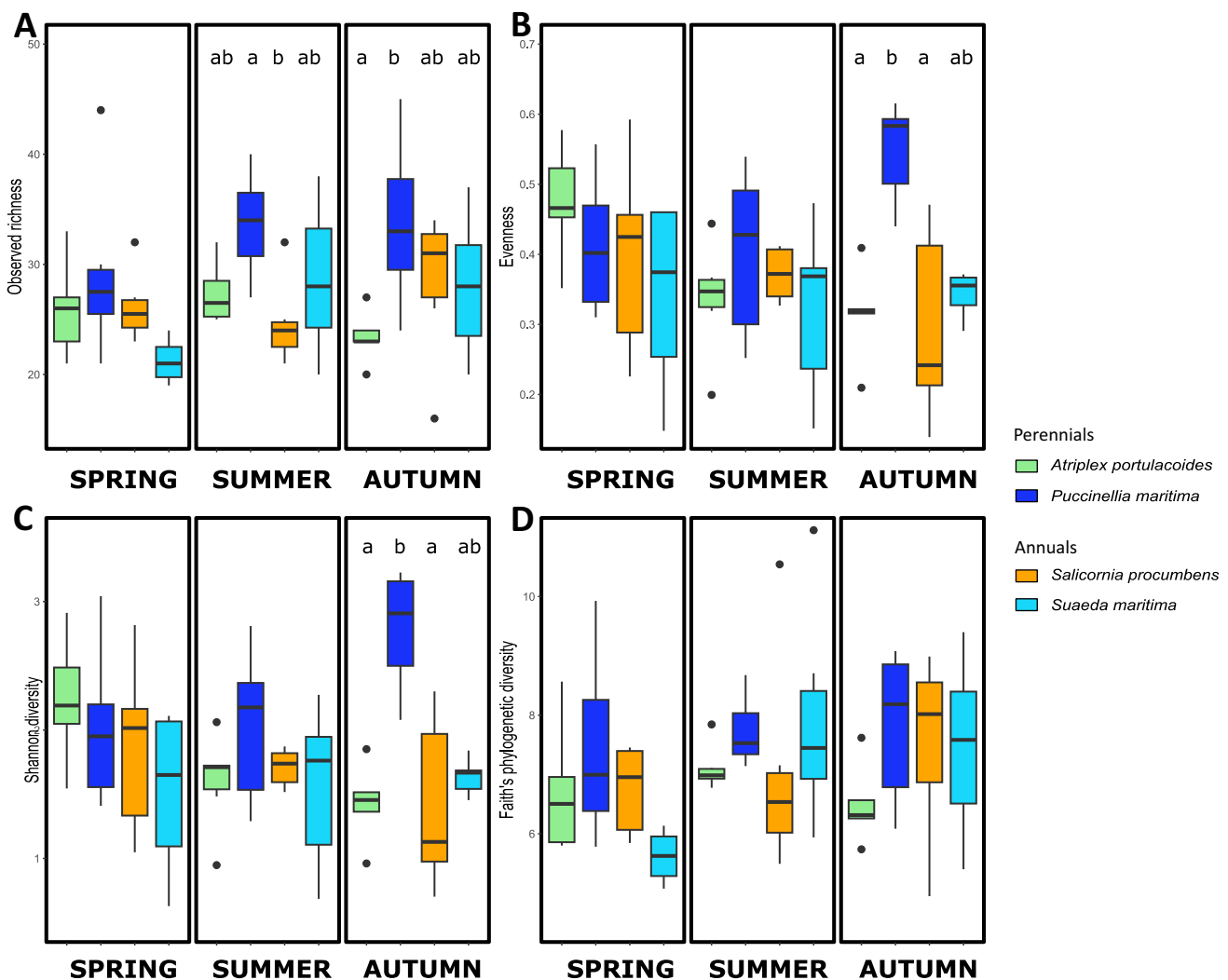


Fig. 3 Boxplots of (A) observed richness, (B) Pielou's evenness, (C) Shannon diversity and (D) Faith's phylogenetic diversity of samples of *A. portulacoides*, *P. maritima*, *S. procumbens* and *S. maritima* collected in spring, summer and autumn. Different letters represent

significant differences between plant species in each season (Kruskal–Wallis test, P -value < 0.05). The exact values can be found in Table S4

Taxonomic Composition and Functional Assignment

Our study provides the hitherto most complete overview of endophytic fungal diversity in a North Sea saltmarsh across different species, life histories, and seasons. With regards to taxonomic diversity, Ascomycota and Basidiomycota were detected in the roots of all four species in spring, summer and autumn. Glomeromycota (*Dominikia* sp.) was only detected in summer and only in *P. maritima* (Fig. 4). However, it represented only about 1% of all reads. Endophytes belonging to the orders Pleosporales and Xylariales were the most abundant in all species (Fig. 5). Seventy-seven fungal genera were detected in total. A heatmap showing the relative abundance

frequencies of the thirty most frequent OTUs is shown in Fig. 4. The three most abundant identified genera were *Parasphaeosphaeria*, *Monosporascus*, and *Alternaria* (Fig. 4, Table S2). *Alternaria chlamydospora* was the most abundant taxon for which identification to species level was possible (Fig. 4, Table S2). An unidentified Pleosporales and a species of *Parasphaeosporia* were present in all samples at more than 1%. A further seven were detected in all samples but at lower frequency. With regards to functional diversity, most of the taxa for which it was possible to assign a trophic mode were classified as pathotroph-saprotroph-symbiotroph. Fungi exclusively classified as symbiotroph (Glomerales) were only detected in roots of *P. maritima* collected in summer (Fig. S3).

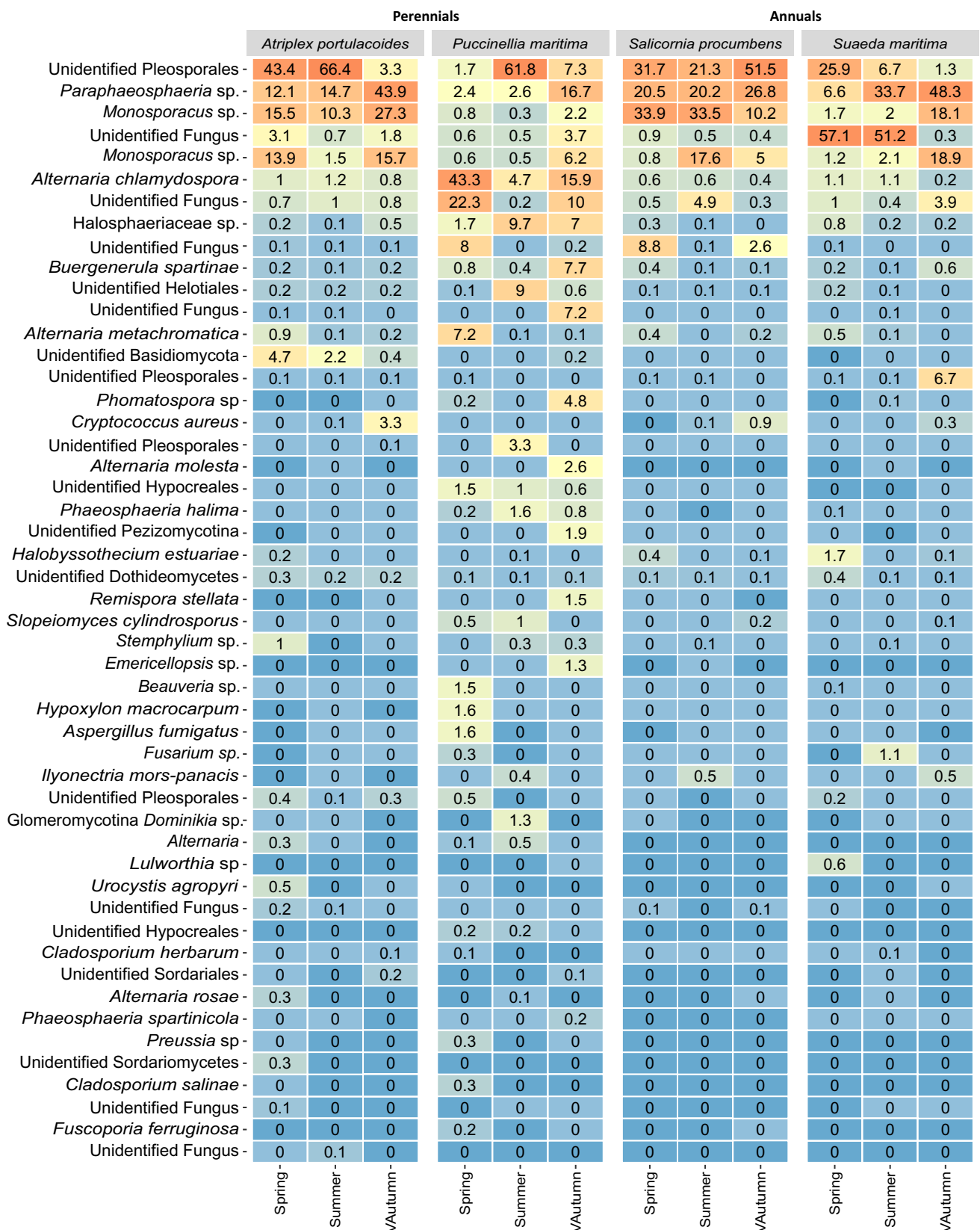


Fig. 4 Heatmap depicting the relative abundance of the 30 most abundant OTUs identified and their distribution according to the different species and seasons

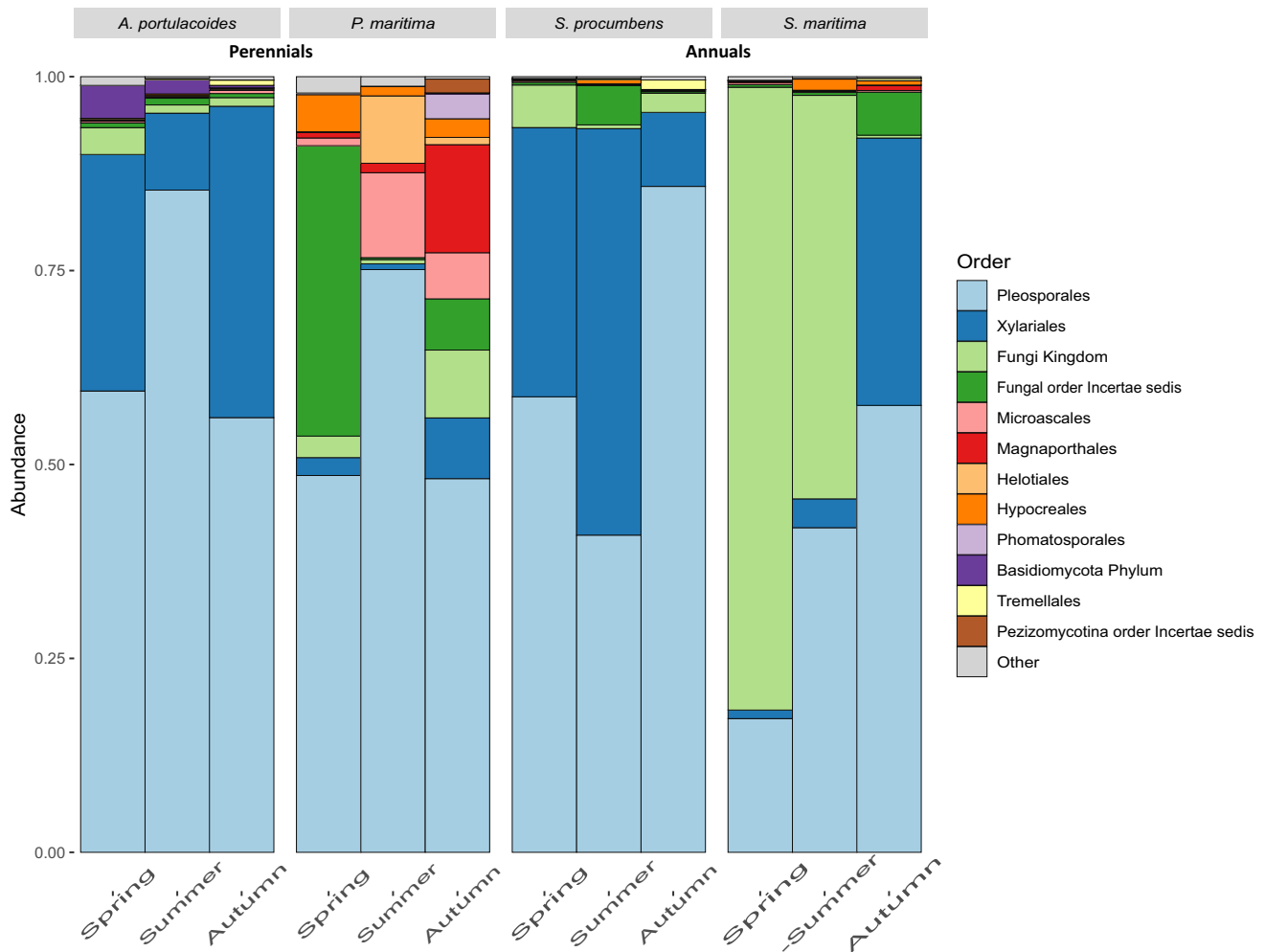


Fig. 5 Relative read abundance of fungal orders associated with *S. maritima*, *S. procumbens*, *A. portulacoides* and *P. maritima* collected in spring, summer, and autumn

Beta Diversity of Fungal Microbiomes

Differences in fungal microbiomes between seasons, host life history strategy, host species identity, and the interaction between these independent factors were statistically significant (Table 2). In spite of host life history being a significant factor, significant differences in fungal microbiomes were observed between all four species based on non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarity matrix (Permanova, $p=0.001$, Fig. 6, Supplementary Material 2).

In the case of the annual *S. procumbens*, fungal microbiomes from spring were significantly dissimilar from those of summer (Permanova, $p=0.012$) and autumn (Permanova, $p=0.001$), whereas no differences were observed between summer and autumn (Permanova, $p=0.104$). For *S. maritima*, no differences were observed between spring and summer (Permanova, $p=0.1801$), whereas spring samples significantly differed from those of autumn

Table 2 Permutational analysis of variance (ADONIS) for the factors season, life history and plant species

	Df	Sum sqs	F. model	R^2	P -value
season	2	2.297470	6.534619	0.09377357	0.001*
Life history	1	2.045831	11.637945	0.08350314	0.001*
species	2	4.707647	13.389907	0.19214776	0.001*
season:life history	2	1.411702	4.015375	0.05762066	0.001*
season:species	4	3.137682	4.462248	0.12806816	0.001*
Residuals	62	10.899839		0.44488671	
Total	73	24.500171		1	

(Permanova, $p=0.002$), and samples collected in summer significantly differed from those of autumn (Permanova, $p=0.005$). In the case of the perennial *P. maritima*, samples from spring were significantly different from those of summer (Permanova, $p=0.002$) and autumn (Permanova,

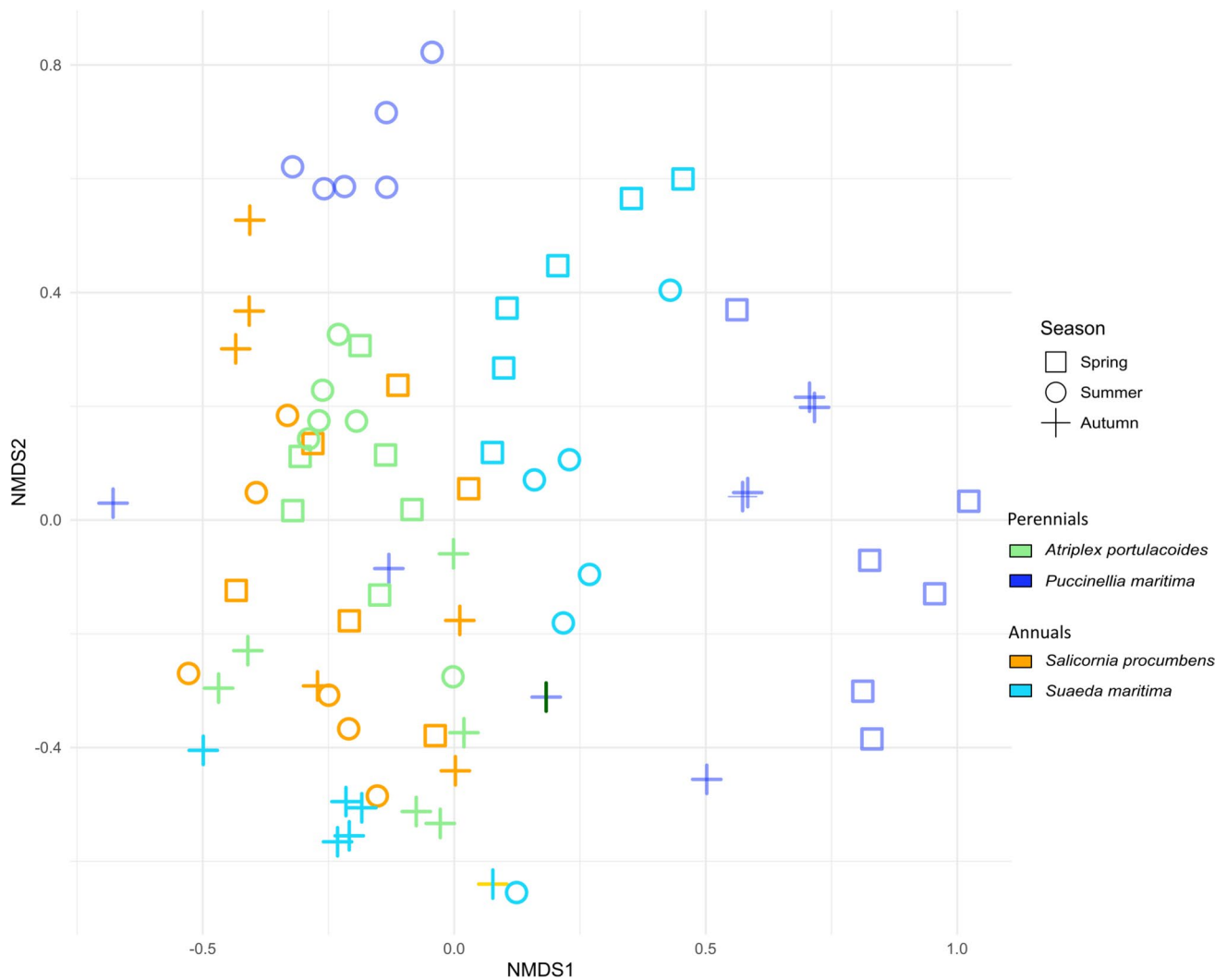


Fig. 6 NMDS plot for the samples collected from *S. maritima*, *S. procumbens*, *A. portulacoides* and *P. maritima* collected in spring, summer, and autumn

$p=0.001$) and samples from summer significantly differed from those of autumn (Permanova, $p=0.003$). For *A. portulacoides*, samples from spring did not differ from those of summer (Permanova, $p=0.351$) but those of autumn (Permanova, $p=0.011$), and samples from summer did not differ from those of autumn (Permanova, $p=0.116$). See Supplementary Material 2 for more detailed results.

The CCA (Fig. 7) demonstrates that cell diameter in the root and the thickness of living cortex area influence the presence of different fungal taxa in the roots of the salt marsh plants. However, none of the fungal taxa except for *Funneliformis geosporum* is present in the data with more than 100 reads. Thus, those fungal species with a clear niche appear to be rare species, whereas the more common species are generalists with respect to cell and cortex size. Fungal species in the upper left hand corner are predominantly those of *A. portulacoides* and *S. procumbens*,

whereas those in the lower left hand corner are those of *P. maritima*.

Discussion

The plant root-associated endophytic microbiome is a crucial determinant of plant productivity and stress tolerance in halophytes (Mateu et al., 2020; Navarro-Torre et al., 2017; Qin et al., 2018). Still, studies profiling fungal microbiomes associated with plants growing under saline environments remain scarce. Here, we investigated fungal assemblages associated with roots of annual and perennial halophytes collected in spring, summer and autumn in a salt marsh on the German North Sea coast. Our results revealed a strong effect of host identity on microbiome assembly (Figs. 4 and 6), which agrees with previous reports of microbiome variation

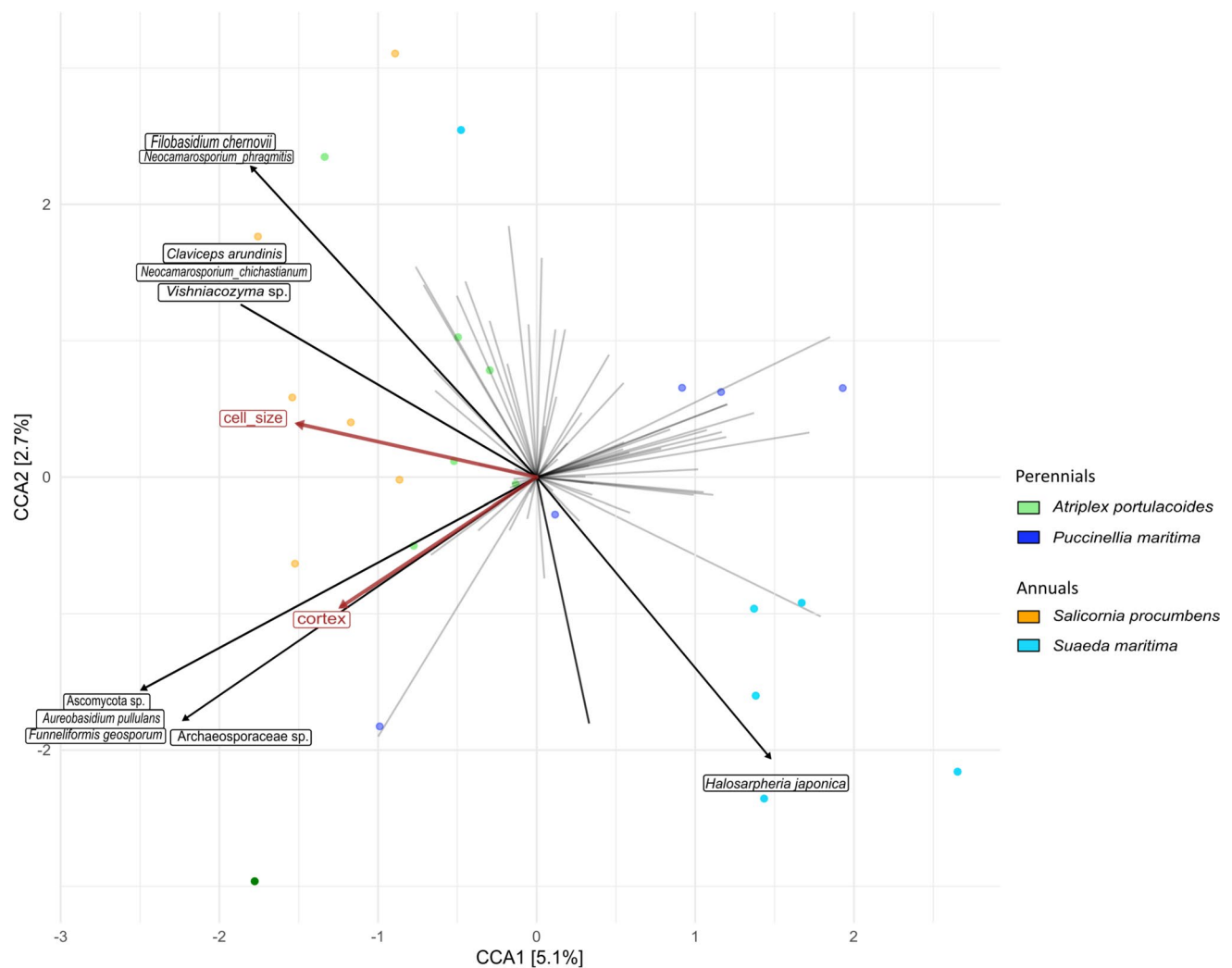


Fig. 7 Constrained Canonical Correspondence Analysis of ITS1 sequence data and root anatomical traits as explanatory variables for the divergence between OTUs detected in *A. portulacoides*, *P. mar-*

itima, *S. procumbens* and *S. maritima* collected in summer. Only the ten with the longest branch (=most explanatory power) are plotted with name

correlating with host phylogenetic distance (Botnen et al., 2020). In addition to that, we observed species-specific dynamics in microbiome composition throughout seasons but not a life history strategy-specific pattern. The difference seen in life history is weaker than the one between species (Table 2). Thus, the life history-effect seems to be driven by the difference between *P. maritima* from the remaining samples. Whereas in *P. maritima*, the fungal microbiomes of spring, summer and autumn significantly differed from each other, the same was not observed in *A. portulacoides*, although both species are perennials (Fig. 6).

Host identity has been reported as an important factor driving fungal microbiome assembly in roots of different plant species (Andreo-Jimenez et al., 2019; Li et al., 2021; Rudgers et al., 2022) but most studies included only single species, such as *Suaeda salsa* (Yuan et al., 2016) and

Salicornia europaea (Furtado et al., 2019). In the case of halophytes, there are only three studies, which compared species-specific root-associated fungal communities, yet in two closely related species (*S. europaea* and *S. procumbens*) (Buhk, 2020; Gonçalves et al., 2022; Maciá-Vicente et al., 2016). In our study, we observed that in spring, *P. maritima* associated with a richer microbiome compared to the other salt marsh plants. Our study also demonstrated a trend of increasing richness, evenness, and Shannon diversity over the year for the species (Fig. 3A–C). This trend is nothing specific to its lifeform since *A. portulacoides* had stable richness and decreasing evenness and Shannon diversity over the year (Fig. 3B, C). Thus, we consider this pattern a species- or family-specific pattern. The community of *P. maritima* has also been shown to be the most divergent community in our other analyses (Figs. 5 and 6) and may be

a Poaceae-specific community. To our knowledge, this is the first study to investigate fungal microbiomes in the salt marsh grass *P. maritima*, despite the fact that plant-associated microbiomes of grasses have been more frequently studied compared to Amaranthaceae (Lee & Hawkes, 2021; Rudgers et al., 2022). We observed a strong seasonal structuring in microbiomes associated with *P. maritima*, with an increase in richness, especially of parasites, over the summer and a subsequent replacement with autumn specialists, foremost of fungal saprophytes (Fig. S3).

In contrast to *P. maritima*, the *A. portulacoides*-associated microbiome is the least diverse and stays rather stable in diversity and composition throughout the year (Figs. 3 and 5) but becomes less even over the season as just three fungal taxa of the Pleosporales and Xylariales dominate the microbiome with more than 85% in summer and autumn (Fig. 4).

Despite the species-specific patterns in microbiome assembly evidenced by alpha diversity analyses, beta diversity analyses showed a common pattern for all four species: fungal microbiomes from spring were distinctly dissimilar from those of autumn (Figs. 5 and 6). One possible explanation for this pattern would be fungal succession, which has been reported to occur during mycorrhizal and endophytic fungal colonization of different plant species, with a shift in colonization towards saprophytes in autumn (Dang et al., 2021; Husband et al., 2002). Nevertheless, fungal succession seems not to be a general pattern with perennial *A. portulacoides* showing the weakest succession. Furtado et al. (2019) also did not observe differences in the root-associated fungal microbiomes of annual *S. europaea* collected in spring and autumn in inland salt marshes in Poland suggesting that root internal tissues are a relatively stable and protective environment, protecting the endophytic microbiomes of the harsh and fluctuating soil conditions. These observed differences between the studies suggest that, in addition to host identity, microbiome assembly could be additionally influenced by different ecological processes that are habitat-specific (Alzarhany et al., 2019) or depend highly on collection date.

Seasonal changes in fungal communities occur asynchronous in different plant species. Whereas *S. procumbens* shows the strongest shift between summer and autumn, the shift is stronger between spring and summer in *S. maritima*. This coincides with earlier development and senescence in *S. maritima* compared to *S. procumbens* (Klotz et al., 2002). The asynchronous seasonal shift in microbiome between the two species is also evident on the level of functional groups of the two microbiomes (Fig. S3). One explanation would be that earlier emergent *S. procumbens* individuals are heavily colonized by saprophytic endophytes in spring, which are then slowly replaced throughout the growing season by fewer endophyte species but with superior persistence abilities, explaining the increase in richness and decrease in evenness compared to *Suaeda* (Fig. 3A, B). We consider

it more likely that colonization of *Salicornia* by fungi is facilitated by its germination on the decaying mother plant with rich fungal community and later exclusion of many of these maternally inherited saprophytes.

Apart from the distribution of the major functional groups, we can also learn a number of important lessons from looking at specific taxonomic groups of fungi. For example, Xylariales dominate in *S. procumbens* in spring and summer (Fig. 5). This is especially noteworthy given that *S. procumbens* usually grows closer to the marine realm and Xylariales include a large number of marine taxa. It would be an interesting hypothesis to follow up on, whether *S. procumbens* is colonized more commonly by marine fungi than other plant species.

On the opposite end, typical mycorrhizal fungi are sometimes considered rare in saline environments, but have been reported colonizing salt marsh plants (Sonjak et al., 2007, 2009), including *P. maritima* (Carvalho et al., 2001; Wilde et al., 2009). In fact, Lumibao et al. (2024) suggested *Glomus* to be the most common genus in salt marshes. In the case of our study, sequences belonging to Glomeromycota represented less than 1% of the total number of sequences and were detected only in samples of *P. maritima* collected in summer. However, the occurrence of Glomeromycota may be underestimated due to primer specificity (Toju et al., 2012). The presence of Glomeromycota in the saltmarshes needs to be investigated in more detail using more specific primers. The importance of Glomeromycota, at least for the grass *P. maritima*, is further highlighted by the strong association of them (*Funelliformis geosporum*, Archaeosporaceae) with the thickness of the living cortical area (Fig. 7).

Based on our general fungi primers, Ascomycota and Basidiomycota were found to be the dominant phyla. Many of these were already reported previously from halophytes. For example, *Byssothecium* spp. were described in association with early stages of decomposition of *Spartina maritima* (Calado et al., 2015). *Alternaria chlamydospora* was reported colonizing internal root and shoot tissues of *Salicornia europaea* (Furtado et al., 2019) and *Halostachys caspica* (Zhou et al., 2024). The majority of these taxa are members of the order Pleosporales (Dothideomycetes, Ascomycota) and are grouped in the so-called dark septate endophytes (DSE), a group of fungi characterized by the formation of melanized septate hyphae. Interestingly, DSE have been reported to positively affect growth of different plant species subjected to salt stress, including those species occurring in salt marshes such as the grasses *Festuca rubra* (Pereira et al., 2019) and *Phragmites australis* (Mateu et al., 2020), which suggests that DSE and salt marsh plants likely coevolved in a mutually beneficial symbiotic association.

Our results further support the hypothesis that selective pressure caused by hosts could be partially related to host-specific root anatomical traits (e.g., cell diameter and

thickness of the living cortical area), which has been previously reported directly affecting fungal colonization in other plant species (Galindo-Castañeda et al., 2019; Guo et al., 2008). Simultaneously, fungal presence can also trigger root anatomical changes in the host. For example, Chen et al. (2021) observed an increase in the thickness of root cortex and endodermis in wild barley plants (*Hordeum brevisubulatum*) inoculated with the endophyte *Epichloë bromicola*. We observed that thickness of the living cortical area and cortex cell diameter were significant factors contributing to the observed patterns of microbiome composition in summer (Fig. 7). The largest cells were observed in *P. maritima*, which did not significantly differ from those of *S. procumbens*. *Salicornia procumbens* had the largest living cortical area with an abundant presence of aerenchyma, followed by *P. maritima*, in which aerenchyma was absent (Table 1, Fig. 1). Larger cells favor mycorrhizal colonization in maize (*Zea mays*) (Galindo-Castañeda et al., 2019), but there has been no information available whether it also benefits colonization by fungal endophytes. While not providing direct evidence for a causal relationship, we at least found an association of both. It is possible that larger cells and a relatively large living cortical area observed in *P. maritima* positively contributed to a richer and more diverse microbiome associated with this species in summer by providing more space for a wider range of different colonizers to establish. This can be exemplified by the exclusive association of some fungal taxa with *P. maritima* (Fig. 4). One example is *Phaeosphaeria halima*, a common fungus occurring in salt marsh soils (Buchan et al., 2002; d'Entremont et al., 2021). In addition to *P. halima*, an unidentified OTU belonging to Glomeromycota was only detected in roots of *P. maritima* in summer, which supports the results of Galindo-Castañeda et al. (2019) in which large cells benefited mycorrhizal colonization.

In conclusion, our results provided evidence for a strong effect of host identity and season on fungal microbiome assembly. We understand that differences in fungal microbiomes could be caused by several difficult-to-disentangle factors. We acknowledge that a sampling effort restricted to only two annual and two perennial halophytes from just one site precluded drawing a broader conclusion on the effect of host life history strategy on microbiome assembly. Importantly, further studies should also take phylogenetic relatedness of the hosts into account. Three species collected in this study (*A. portulacoides*, *S. maritima* and *S. procumbens*) belong to Amaranthaceae whereas only one (*P. maritima*) belongs to Poaceae, which limited our conclusions on the importance of phylogenetic relatedness on microbiome assembly. Another noteworthy result of this study was that fungal functional assignment is challenging not only due to limited available information, as we can see in Fig. S3, but also because functionality may change depending on

different factors. For instance, a reduced defensive capacity of a plant species may trigger a shift from a neutral to a pathogenic association. Also, functionality may differ from species to species and most studies inferred function in crop plants. Therefore, a careful interpretation of functional roles of fungal endophytes colonizing halophytes is important to not draw erroneous conclusions (Gonçalves et al., 2021).

Nevertheless, our study provides potential pathways for future research by highlighting the species-specific patterns in the microbiome and the need for a fine-scale seasonal sampling to understand microbiome turnover over time. In particular, this turnover seems to be species specific rather than occurring in parallel caused by external parameters. Our study further provides a potential mechanistic connection between microbiome variation and plant anatomy. This is particularly important since especially the symbiotic fungi show a strong association with anatomical characteristics. Consequently, we move further ahead with understanding plant-fungal-interactions in the extreme environment of a saltmarsh.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12237-025-01610-4>.

Acknowledgements We thank the administration of the Lower Saxony Wadden Sea National Park for the permission to collect the samples used in this study. We are also grateful to two anonymous reviewers for their comments and help to improve the manuscript.

Author Contribution D.C.A. and D.R.G. designed the research. D.R.G. collected the plants and conducted the DNA analysis. D.R.G. and E.M.-Q. analyzed the DNA data. A.R. conducted the anatomical analyses. D.R.G. and D.C.A. wrote the manuscript. A.R. and E.M.-Q. contributed specific parts to the manuscript. All authors revised the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. This work was funded by the German Research Foundation (DFG) in the Research Unit DynaCom (Grant FOR 2716; AL 632/17-2).

Data Availability Raw DNA sequence reads for the 72 newly sequenced samples were registered within the NCBI BioProject database (PRJNA823534). The original anatomical measurements are found in Table S2 in Supplementary Material 1.

Declarations

Competing interests The authors declare no competing interests.

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