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ORIGINAL RESEARCH

Bacterial and fungal root endophytes alter survival, growth, and resistance to grazing in a foundation plant species

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Abstract

Plants host an array of microbial symbionts, including both bacterial and fungal endophytes located within their roots. While bacterial and fungal endophytes independently alter host plant growth, response to stress and susceptibility to disease, their combined effects on host plants are poorly studied. To tease apart interactions between co-occurring endophytes on plant growth, morphology, physiology, and survival we conducted a greenhouse experiment. Different genotypes of *Spartina alterniflora*, a foundational salt marsh species, were inoculated with one bacterial endophyte, *Kosakonia oryzae*, one fungal endophyte, *Magnaporthales* sp., or co-inoculated. Within the greenhouse, an unplanned herbivory event occurred which allowed insight into the ways bacteria, fungi, and co-inoculation of both endophytic microbes alters plant defense chemicals and changes herbivory. Broadly, the individual inoculation of the bacterial endophyte increased survival, whereas the fungal endophyte increased plant growth traits. Following the herbivory event, the proportion of stems grazed was reduced when plants were inoculated with the individual endophytes and further reduced when both endophytes were present. Across genotypes, anti-herbivore defense chemicals varied by individual and co-inoculation of endophytes. Bacterial inoculation and genotype interactively affected above:below-ground biomass and *S. alterniflora* survival of ungrazed plants. Overall, our results highlight the variable outcomes of endophyte inoculation on *Spartina* growth, morphology, phenolics, and survival. This study furthers our understanding of the combined effects of symbionts and plant multitrophic interactions. Further, exploring intra and inter specific effects of plant—microbe symbiosis may be key in better predicting ecosystem level outcomes, particularly in response to global change.

Keywords Bacteria · Endophyte · Root-associated fungi · Herbivory · Salt marsh · *Spartina alterniflora*

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Introduction

Plant root endophytes, which are microbes existing intercellularly within the host plant's root tissues, are found commonly in nature. As plants are subject to complex environmental conditions, root endophytic microbes, both bacterial and fungal, can mediate fundamental plant processes including growth, nutrient uptake, and stress tolerance (Santoyo et al. 2016). The functions of these symbiotic relationships exist on a continuum of mutualistic to parasitic, dependent on context (Mandyam and Jumpponen 2014). On the positive end of the spectrum, endophytic microbes can promote plant condition through both direct and indirect mechanisms (Hardoim et al. 2008; Mandyam and Jumpponen 2015). For example, dark septate root endophytes (DSE) can directly facilitate increases in host nutrient content (Santos et al. 2021). These microbes also indirectly promote plant growth

by upregulating hormones and secondary metabolites that in turn decrease the harmful effects of pathogens and herbivores (Schulz et al. 2002; Mousa and Raizada 2013). Depending on the plant host and environmental conditions, endophytic microbes can also alter plant growth in ways that are neutral to transiently negative (Mayerhofer et al. 2013).

Extensive research has documented pairwise effects of host plants and single microbes, and more recent advances in technology have allowed studies of entire microbiomes. While these studies represent two extremes, there is a lack of focus on more nuanced effects among more than one microbial associate, such as plant–microbe-microbe interactions, which are needed to expand our mechanistic understanding of full microbiome effects. The effects of two microbes on host fitness may be additive or non-additive (positive or antagonistic), and they may occur directly between microbe and host or microbe and coexisting microbe, or indirectly through changing conditions within their shared host (White et al. 2019; Afkhami et al. 2020). For example, synergistic positive effects of arbuscular mycorrhizal fungi (AMF) and rhizobia have been noted (although they are not universal, see Larimer et al. 2010), likely due to the differential benefits they provide to the host with AMF providing phosphorus and water, and rhizobia fixing nitrogen (Liu et al. 2023). Yet, as both rhizobia and AMF receive carbon as a reward from the plant, direct competition for this resource may ultimately reduce fitness for all partners (Afkhami et al. 2014). Microbe–microbe interactions can also change the direction of the microbe-plant relationship: in a laboratory experiment exploring the effects of fungal inoculation on the flowering plant *Verbascum lychnitis,* fungal root endophytes alone had negative effects on plant survival rate and biomass, yet when co-inoculated with AMF, there was a significant increase in both measurements (Wężowicz et al. 2017). These results highlight the complex nature of tripartite interactions, which can alter the direction and magnitude of host outcomes in unexpected ways relative to each microbe alone.

Plants commonly host both bacterial and fungal endophytes, and studies that have examined cross-domain interactions highlight that they can be important and variable (Omomowo and Babalola 2019, Bastias et al., 2022). In vitro experiments exploring pairwise interactions of bacterial endophytes vs fungal root endophytes have found bacterial inoculants confer predominantly negative outcomes on fungi by reducing fungal growth when co-inoculated in culture with bacteria (Mavrodi et al. 2018; Whitaker and Bakker 2019; Christian et al. 2021). While research on bacterial endosymbionts of fungal cells found that endosymbiotic bacteria of fungi are beneficial symbionts, and that effects of ectosymbiotic bacteria on fungal performance depends on the bacterial type involved in the interaction (Bastias et al., 2020). This suggests limited knowledge and mixed results on the ways fungi and bacteria may be interacting. Furthermore, there are fewer studies on root endophytic bacterial and fungal influence on host plant outcomes are available, limiting our understanding of host-microbiome interactions.

Furthermore, studies in a variety of plant species have shown that host plant genotype influences the community composition of endophytic bacteria and fungi, but few have explored the influence of host genotype on tri-partite interactions. Plant genotype influenced the bacterial endophyte communities of crops such as potatoes (Andreote et al. 2010), olives (Müller et al. 2015), and rice (Zhang et al. 2019) and a wild member of the Brassicaceae (Wagner et al. 2016) among others. Similarly, plant genotype contributed significantly to the community composition of fungal endophytes in crops (Latz et al. 2021) and wild plant species such as cottonwoods, in which tree genetics explained 17% of the variation in continental-scale fungal endophyte community structure (Bothwell et al. 2023). While some studies explore the consequences of these community changes for plant performance, the endophyte community is often considered as a whole (e.g., Lumibao et al., 2022) rather than identifying how particular bacterial and fungal endophyte taxa interact with one another and host plant genotype. Dual inoculation studies of arbuscular mycorrhizal fungi and nitrogen-fixing bacteria in leguminous crops demonstrate that tripartite interactions can be strongly influenced by plant genotype (Liu et al. 2020), suggesting that similar responses may be observed in wild plants and their bacterial and fungal endophytes.

Bacterial and fungal endophytes may play a particularly important role for plant performance in ecosystems with stressful abiotic conditions, where positive interactions are favored (Bertness and Callaway 1994). In a meta-analysis evaluating the effects of fungal symbionts on plant responses to global change, microbial symbioses increased plant biomass in scenarios of increased drought, nitrogen addition, and temperature (Kivlin et al. 2013). While the benefits of growth-promoting microbiota have largely been studied in agricultural settings (Glick 2012; Ramakrishna et al. 2019), we predict that these effects will likely also be common in physically stressful natural environments (Kivlin et al. 2013). Salt marshes are one such ecosystem, with hypoxic, water-logged, and saline soils that create abiotically challenging environments for plants. Thus, we may expect that beneficial plant-endophyte interactions will be common in this system. Some endophytes isolated from saline environments do display mutualist function by increasing plant biomass of salt-stressed hosts (Rodriguez et al. 2008; Soares et al. 2016). However, within a greenhouse experiment, the common dark septate fungal root endophyte *Lulwoana* had consistently negative effects on aboveground and belowground traits across genotypes of the marsh grass *Spartina alterniflora* following inoculation (Hughes et al. 2020). These mixed results highlight the need for more research on

root endophyte symbioses with marsh plants, as they may be controlling critical functions in these intertidal communities.

Within this study we teased apart interactions between co-occurring bacterial and fungal root endophytes on plant growth, morphology, physiology, and survival of twelve different genotypes of the salt marsh foundation species, *Spartina alterniflora*. Specifically, we asked: Does colonization by fungal and bacterial endophytes affect *S. alterniflora* survival, morphology, biomass, and leaf chemistry? And, if so, are the combined effects of the two endophytes different than their independent effects? Previous results showed that both presence and strain of root-associated fungi affect *S. alterniflora* morphology and biomass (Hughes et al. 2020, Moore et al. 2021), so we predicted that fungal and bacterial symbionts would variable magnitudes of beneficial outcomes when inoculated in singularity and the potential for positive microbe-microbe interactions when both endophytes are present (Bastias et al., 2022). Additionally, we predicted that these effects would vary by host genotype, consistent with our previous work (Hughes et al. 2020, Hanley et al., 2021). In light of an unplanned but well documented herbivory event within the greenhouse where the experiment was conducted, we were also able to explore the complexities of multi-trophic interactions. Consequently, we asked an opportunistic question: Does root endophyte symbiosis influence the prevalence and outcomes of herbivory on the host plant? Because endophytes can alter host traits to deter herbivory and defend against pathogens (Saikkonen et al. 2013; Gange et al. 2019; Chitnis et al. 2020), we hypothesized that plants in endophyte treatments would have increased biomass, growth, and survival, as well as changes in anti-herbivore compounds, in comparison to control plants following herbivory.

Materials and methods

Study system

Spartina alterniflora is a perennial grass and a dominant plant species in low elevation salt marshes of the Gulf of Mexico and the Atlantic coast of the United States (Pennings et al. 2005). *S. alterniflora* is colonized by a diverse suite of root endophytes (Kandalepas et al. 2015; Rolando et al. 2022). For instance, *S. alterniflora* is not mycorrhizal (McHugh and Dighton 2004; Daleo et al. 2008), but it is commonly colonized by other root-associated fungi, including DSE (Kandalepas et al. 2015; Lumibao et al. 2018; Moore et al. 2021; Hughes et al. 2020). In addition, *S. alterniflora* hosts a diverse suite of endophytic bacteria in both its native and invasive range (Hong et al. 2015; Kandalepas et al. 2015). Although we have a growing understanding of the diversity of fungal and bacterial endophytes in the salt marsh, we have little understanding of their independent and combined effects on their hosts, which could have substantial impacts on marsh plant communities and the functions they provide.

We examined the function of the bacterial endophyte *Kosakonia oryzae* and the fungal endophyte *Magnaporthales* sp. (Online Resource 1) because of their importance as endophytes of grasses and their abundance in the roots of *Spartina alterniflora* in our study sites near Charleston, SC, USA. *Kosakonia* is a Gammaproteobacteria that contains several genes involved in plant growth promotion and has been observed to promote growth in plants (Berger et al. 2017; Becker et al. 2018; Shaik and Thomas 2019). Demonstrated mechanisms for growth promotion by *Kosakonia* include conferring salinity tolerance by ACC de-aminase production and biofertilization by nitrogen fixation (Liu et al. 2017; Bloch et al. 2020). However, these studies focus on crop plants, and less is known about their function in native grasses. *Kosakonia oryzae* was a common bacterial isolate in tall and short zone *S. alterniflora* at multiple salt marsh sites in South Carolina (Gehring et al., in prep). *Magnaporthales* is an order of Sordariomycetes, Ascomycota found commonly in monocots (Luo et al. 2015) including salt marsh (Kandalepas et al. 2015), that primarily displays an endophytic lifestyle within Poaceae. While more than half of this order remain taxonomically unknown, of the fungi that have been grouped, they show saprotrophic, pathogenic and endophytic lifestyles (Feng et al. 2021). In addition, a survey of endophytic fungi in wild rice identified a genus within the Magnaporthales as dark septate endophytes (DSE) that improved growth of the rice plants (Yuan et al. 2010). In the salt marshes we studied in SC, the isolate we selected for this experiment, *Magnaporthales* sp. was isolated from tall and short zone sites and was an extreme dominant (> 80% of the isolates) at one of the sites and formed DSE associations in a small inoculation trial on *S. alterniflora* (Gehring et al., in prep). Given its abundance and range of potential functions, this fungi's role as a plant root symbiont, specifically in a challenging marsh environment, is important to understand.

Despite the common perception that marsh plant species are dominated by clonal reproduction and harbor little genetic diversity, studies using a range of DNA markers show high levels of genotypic diversity in *S. alterniflora* even at small spatial scales in natural marshes (Richards et al. 2004; Edwards et al. 2005; Hughes and Lotterhos 2014; Robertson et al. 2017). In fact, levels of outcrossing and diversity in marsh plants are consistent with other outcrossing, wind pollinated grasses (Hamrick and Godt 1996; Richards et al. 2004). Genetic variation can have strong effects on *S. alterniflora* functional traits, including aboveground characteristics such as height, stem density, biomass, and clone diameter, as well as belowground features such as root/rhizome distribution and carbohydrate reserves (Seliskar

et al. 2002; Proffitt et al. 2003, 2005; Hughes 2014; Zerebecki et al. 2017). In addition, the effects of inoculation by a common DSE on *S. alterniflora* morphology and biomass allocation vary in strength and direction across plant genotypes (Hughes et al. 2020). Thus, we examined intraspecific variation in host plant responses to endophytes in this study, using plants grown from seed collected from the same sites where the endophytes were isolated.

Isolating and identifying endophytes

Although *Kosakonia oryzae* and *Magnaporthales* sp. were isolated from multiple sites, we used isolates from the roots of *Spartina* collected in a salt marsh near Fort Johnson, South Carolina. Root samples were excavated from the marsh, placed in plastic bags, and refrigerated until processing. In the lab, we rinsed the roots thoroughly with tap water to remove soil and debris, followed by rinsing in reverse osmosis (RO) processed water. After rinsing, we surface-sterilized the roots by agitating for 1 min in a 70% ethanol solution, followed by 1 min in 50% commercial bleach, and finally rinsing twice in sterile RO water. A one cm segment was removed from the ends of the root pieces and discarded. We then aseptically sectioned the roots into one cm segments and placed on Petri plates with potato dextrose agar media (PDA), four segments to each plate. One segment was streaked on PDA to check sterilization efficacy. We scored the plates daily for microbial growth and subcultured emerging bacteria and fungi, again on PDA.

To extract DNA, we removed small samples of hyphae and bacteria from the plates, avoiding inclusion of agar in the sample, and then ground samples in a Mini-G tissue homogenizer at 1500 rpm for 2 min. We extracted DNA according to Mayjonade et al., (2016) with the addition of a chloroform cleaning step after precipitation of the proteins (Mayjonade et al. 2016). We resuspended DNA in Tris buffer (pH 8.0) and diluted the genomic DNA tenfold for use in PCR amplification.

We amplified bacterial and fungal DNA by polymerase chain reaction (PCR) using the 16S primers 27F/1492R (bacteria) and the ITS primers ITS1Fxt/ITS4 mod (fungi).

We amplified the DNA with Maxima Taq (Fisher Scientific) under standard conditions with a reaction volume of 10 µL containing 1 µL of template according to White et al., and Gardes and Bruns (White et al. 1990; Gardes and Bruns 1993). We purified the PCR product using magnetic beads in an 18% PEG solution as described by (Rohland and Reich 2012). Amplified product was cycle sequenced with BigDye Terminator Mix 3.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) cleaned with magnetic beads in a 25% PEG solution and sequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, California) in the Environmental Genetics and Genomics Laboratory located at Northern Arizona University. Consensus sequences were assembled using PreGap4 and Gap4 in the Staden Package (Bonfield et al. 1995) and a BLAST search (Altschul et al. 1990) on the NCBI GenBank website was conducted to identify the organisms. These sequences have been deposited in GenBank (accession number PQ622690 for *Kosakonia* and accession number PQ622691 for *Magnaporthales sp.*). At the end of the experiment, subsamples of *Spartina* roots were collected, surface-sterilized, and plated. DNA was extracted and processed as outlined below to confirm the successful inoculation and establishment of the experimental bacterial and fungal strains. Root samples from each of the four experimental treatments ($n = 12$–15 per treatment) were placed in tissue cassettes, cleared in 5% potassium hydroxide, rinsed in distilled water, mounted on glass slides, and viewed under a compound microscope at 200X magnification for the presence of dark septate endophytes (DSE) (Online Resource 2). The presence of melanized, septate hyphae and microsclerotia were used as indicators of DSE. DSE were quantified using the grid-line intersect method with 100 intersections per sample (McGonigle et al. 1990).

Greenhouse experiment

In summer 2018, we conducted a greenhouse inoculation experiment at the Northeastern University Marine Science Center (MSC) to test the independent and interactive effects of bacterial root endophyte (*Kosakonia oryzae*) inoculation, fungal root endophyte (*Magnaporthales* sp.) inoculation, and host plant genotype on *S. alterniflora* survival, morphology, plant chemistry, and biomass allocation. We used a suite of 12 *S. alterniflora* genotypes originally collected as seed from three sites in Charleston, S.C. in 2014 that were germinated and clonally propagated in the MSC greenhouse (see (Zerebecki et al. 2021) for details). In mid-June 2018, we isolated 24 replicate single stems of each genotype with attached root and rhizome and planted them individually in 6.4 L pots in commercial potting soil that was sterilized by autoclaving batches of soil twice, each time at 121.1℃ for 45 min. We randomly assigned pots to one of 24 plastic bins (36″ × 12″ × 6″) with 12 pots per bin (1 per genotype) across 4 water tables (6 bins per table) in the greenhouse. We grouped adjacent water tables into 2 spatial blocks. The plastic bins were then randomly assigned to one of four experimental treatments in each of these blocks (N = 3 bins per treatment per block): fungal inoculation only; bacterial inoculation only; fungal and bacterial inoculation; control. Every two weeks, bins were randomly rotated to new locations within blocks to minimize effects of greenhouse location.

In late June 2018, we inoculated each pot with six 6.35 mm plugs of either sterile potato dextrose agar (PDA; fungi absent, i.e., control as discussed above) or PDA with

the fungal culture (fungi present) approximately 2.5 cm below the soil surface in a circle surrounding the roots. We then injected 4 ml of either sterile water (bacteria absent) or bacterial inoculant (bacteria present; concentration: 1×10^8 cells/ml) to each pot at the same soil depth as the fungal plug was added. In total, every pot received six agar plugs and 4 ml liquid.

We irrigated pots with freshwater daily and flooded them with UV-sterilized, flow-through seawater from the MSC seawater system 5 days per week for ~ 8 h per day. The bins and drainage holes were elevated above the bottom of the water table and completely drained individually in a random order to prevent the mixing of water draining from the bins following irrigation. We measured salinity using soil porewater sippers installed in each pot at the beginning of the experiment (McKee and Mendelssohn 1989). These sippers were created by boring 7 small holes on 4 sides of a 2 mL pipette that was cut to be 3 inches long and topped with a stopcock. Each week, we used a refractometer (Atago Master S10M) to measure the salinity of approximately 1 mL of porewater collected by inserting a 10 mL syringe into the stopcock (after first collecting and discarding approximately 1 mL of stagnant water from the pipette tip). Pregrazing, salinity varied slightly by block (6% higher in block 1; $P = 0.008$), and over the course of the entire experiment it was slightly higher (7.1%) in the fungal treatment than the no fungal treatment ($P = 0.01$). To account for these differences, we included average salinity per pot as a covariate in our analyses (Online Resource 3).

At the time of inoculation and at monthly intervals during the experiment, we measured stem density, maximum stem height, average height, and leaf number. Initial and final measurements included height and leaf number of every stem, whereas monthly measurements included height and leaf number of the tallest stem, plus height and leaf number of three randomly selected stems. At the end of the experiment, we also collected leaf tissue samples to assess plant chemistry (organic content, percent protein, and phenolic concentrations.) Plants were then harvested and separated into aboveground (stems with attached leaves) and belowground (roots and rhizomes) portions. Biomass samples were dried at 60 °C for at least 72 h prior to being weighed.

Herbivory event

In early September, we observed grazing scars on leaves of *Spartina* in our experimental pots. We determined that the grazing was caused by caterpillars that colonized the greenhouse; individuals were removed once found and were seldom seen for the remainder of the experiment. We identified them as armyworm caterpillars (*Mythimna unipuncta*) by raising individuals through metamorphosis and confirming their identity on iNaturalist (iNaturalist. Available from https://www.inaturalist.org). Although the caterpillar is a generalist, we have not found it naturally in the marsh. However, there are other herbivores that cause similar leaf damage on spartina at our field sites (Johnson and Jessen 2008).

Grazing occurred across all treatments through the first two weeks of September, until we found and removed all of the caterpillars. To quantify grazing frequency and intensity and ensure it did not occur again, we collected data on the presence of fecal matter / frass in each pot, which was flushed out and removed on a daily basis during the inundation and draining of the pots, on Sept. 11, 12, 13, 14, 19, and 28. We also recorded the presence or absence of grazing at the pot level. At two time points (Sept 19, Oct 17) coincident with stem height measurements, we recorded the number of leaves grazed per stem in each pot, as well as the total number of grazed and ungrazed stems per pot.

Tissue chemistry

To assess the effects of bacterial and fungal inoculation on plant tissue chemistry, we measured organic, protein, and phenolic content of *Spartina* leaf tissue. These traits can affect and be affected by herbivory (Valiela and Rietsma 1984; Bärlocher and Newell 1994; Long et al. 2011), so we also compared tissue chemistry of grazed vs ungrazed stems. We measured organic content of live plants at the end of the experiment using a modified version of Long et al.'s (2011) protocol: briefly, ~ 0.5 g tissue from 5 to 15 dried *Spartina* leaves was weighed before and after being combusted at 550 °C for 3 h in a Thermo Scientific™ Thermolyne™ muffle furnace to calculate ash free dry mass (AFDM). To assess protein content, we weighed ~ 5–7 mg tissue from 2 to 5 dried *Spartina* leaves, ground samples using a Retsch mixer mill MM400, added 1 ml 1 M sodium hydroxide to each sample, and then incubated at 4 °C for 24 h to extract protein for analysis using a modified Bradford assay with a Thermo Scientific™ Coomassie Plus™ Kit and a bovine serum albumin (BSA) standard (modified from Wittyngham et al., 2019). To determine phenolic content, we weighed ~ 15–20 mg tissue from 2 to 5 dried *Spartina* leaves, ground samples using a Retsch mixer mill MM400, and extracted phenolic compounds using methanol prior to analysis with a modified protocol of the Folin-Ciocalteu method using a gallic acid standard (modified from Wittyngham et al., 2019).

Data analyses

We first analyzed the effects of our endophyte treatments on *Spartina* survival and morphology (stem number, stem height, and leaf number) at two months post-inoculation, before any grazing occurred. Statistical analyses were conducted using RStudio (version 4.3.2). We used a generalized

linear model (GLM) approach with independent and interactive effects of bacteria (present or absent), fungus (present or absent), and genotype, along with an additive block effect. We used the value of each morphological variable from the start of the experiment and average salinity over this time period as covariates.

To examine the potential for treatment effects on grazing itself, we analyzed the proportion of pots that were grazed, the number of grazing events, and the intensity of grazing for the time period after grazing occurred (months 2–4). For the proportion of pots grazed, we used a composite bivariate metric that labeled pots as grazed if either frass was observed during the experiment and/or there was clear grazing of leaves at the end of the experiment. The two measures were significantly correlated ($R^2 = 0.70$, $P < 0.001$) and their composite provided the most holistic measure of grazing during the experiment. We used the proportion of live stems grazed per pot as a measure of grazing intensity. We used a GLM approach with independent and interactive effects of bacteria (present or absent), fungus (present or absent), and genotype, along with an additive block effect and average salinity as a covariate.

We also evaluated *Spartina* density, morphology, and biomass at the end of the experiment (month 4). Although grazing was not manipulated, it occurred in 54.5% of experimental replicates (157 / 288 pots), and there was comparable replication of grazed and ungrazed pots for each endophyte treatment, although not across every genotype. Thus, to understand the effects of our endophyte treatments in the absence of grazing, as well as to maximize what we could learn from the grazing event itself, we conducted a series of complementary analyses: (1) on only pots that were not grazed; (2) on only pots that were grazed; and (3) on all pots, with grazed (present or absent) included as factor, including all possible interactions except the four-way fungi*bacteria*genotype*grazing interaction, for which there was insufficient degrees of freedom. We then ran models similar to those described above for each group separately, with independent and interactive effects of bacteria (present or absent), fungus (present or absent), and genotype, along with an additive block effect and average salinity as a covariate. For the morphological responses (stem number, stem height, leaf number), we used the value of that response at month 2 (just prior to grazing) as an additional covariate in the analyses to account for the differences that had arisen due to our treatments prior to grazing. The results were qualitatively consistent across the different analyses; we present grazed pots only and ungrazed pots only within this manuscript, with the full comparison table presented within the online resources (Online Resource 4).

To evaluate whether endophyte treatments affected *Spartina* tissue chemistry and whether tissue quality contributed to differences in herbivory across treatments, we analyzed percent organic, protein, and phenolic content for leaf tissue collected from pots with live stems at the end of the experiment. As above, we split the data into pots that were grazed and pots that were not grazed for analysis. We ran GLMs with independent and interactive effects of bacteria (present or absent), fungus (present or absent), and genotype, along with an additive effect of block and average salinity as a covariate.

In all analyses, we fit response variables to distributions based on the process that generated the distributions. Specifically, we fit *Spartina* survival and the proportion of pots and stems grazed with a binomial GLM with logit link, stem number and number of grazing events with a Poisson GLM, stem height, mean leaf number, aboveground:belowground biomass, organic content, protein content, and phenolics with a Quasipoisson GLM, and total biomass, aboveground biomass, and belowground biomass with Gaussian GLMs. We excluded dead stems from analyses of stem number, stem height, and leaf number to avoid conflating effects on survival with effects on morphology. When there were significant effects of factors with more than two levels (e.g., genotype), we performed Tukey's post hoc pairwise comparisons. For significant interactions between bacterial or fungal inoculation and genotype, we used pairwise comparisons of the endophyte treatments within genotype to test what treatments differed for each genotype. All post hoc tests were run using the emmeans package.

Results

In addition to isolating bacteria and fungi from a small subset of inoculated roots, we also confirmed the success of our fungal inoculations by measuring the percentage of the root colonized by the Magnaporthales sp, a known dark septate endophyte: DSE were observed at the end of the experiment in all of the plants that were examined in the fungal only and combined fungal and bacterial treatments (mean % root colonization (SE) = 18.54 (3.32) for plants in the fungal only group and 16.25 (1.84) for plants in the combined treatment). No colonization by DSE was observed in the bacteria only or control treatments.

After two months and prior to grazing, bacterial inoculation increased *Spartina* survival ($P = 0.008$; Fig. 1a), but decreased the number of live stems ($P = 0.04$; Fig. 1b) relative to plants without bacteria. In contrast, fungal inoculation affected only maximum stem height: stems were taller when fungi were present ($P = 0.04$; Fig. 1c). There were no interactive effects of the bacterial and fungal inoculation treatments. Each of these responses also varied independently by *Spartina* genotype (survival: $P = 0.02$, Online Resource 5a; live stem number: $P < 0.001$; Online Resource 5b; stem height: $P < 0.001$; Online Resource 5c). Number of leaves

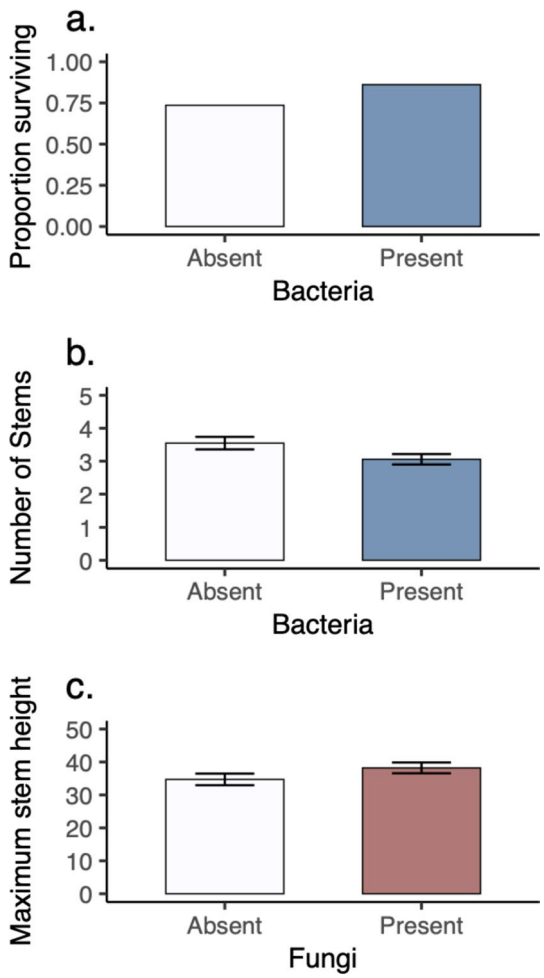

Fig. 1 Effects of endophyte inoculation treatments (blue = bacteria; red = fungi; white = none) on *Spartina alterniflora* survival and morphology pre-grazing. **a** survival (bacteria $P = 0.008$), **b** number of stems (bacteria $P = 0.04$), and **c** stem height (fungi $P = 0.04$). Error bars represent 1 SE

did not vary by any experimental treatment, independently or interactively.

Grazing by *Mythimna unipuncta* occurred at the start of month 3, particularly in block 2. Several metrics of this grazing varied by endophyte treatment. First, the proportion of pots grazed was reduced by bacterial inoculation ($P = 0.02$) and marginally by fungal inoculation ($P = 0.08$; Fig. 2a). Grazing intensity, or the proportion of live stems grazed per pot, was lowest when bacteria and fungi were both present (bacteria*fungi $P = 0.02$; Fig. 2d). There was also an independent effect of genotype on grazing intensity ($P = 0.005$).

At the end of the experiment, stem height of grazed plants, but not ungrazed plants (Online Resource 4), was greater in fungal inoculation treatments ($P = 0.03$; Fig. 3a), even when accounting for height differences that had developed across these treatments by month 2. In addition, the ratio of aboveground to belowground biomass of grazed plants was marginally higher with fungal inoculation ($P = 0.07$; Fig. 3b). Bacterial inoculation only affected ungrazed plants, and these effects varied by genotype for survival and biomass allocation (bacteria*genotype: aboveground:belowground $P = 0.02$, Fig. 3c; survival $P < 0.01$, Fig. 3d). Genotypes D and H had higher above to belowground biomass allocation when inoculated with bacteria relative to the control (Fig. 3c). None of the contrasts between bacterial inoculation and control for individual genotypes were significant for survival, despite a significant interaction in the overall model (Fig. 3d). In addition, the number of live stems in pots that were not grazed was higher in bacterial inoculation treatments ($P = 0.02$). Of plants that were grazed, genotypes varied in morphology (live stems $P < 0.001$, Online Resource 6b; stem height $P < 0.001$, Online Resource 6c; number of leaves $P < 0.001$, Online Resource 3d) and biomass (aboveground $P = 0.04$; Online Resource 7a, aboveground:belowground $P < 0.001$; Online Resource 7b) at the end of the experiment.

Multiple metrics of tissue chemistry varied by endophyte inoculation treatment. For grazed plants, percent protein was lower in fungal inoculation treatments ($P = 0.005$; Fig. 4a) and also varied across genotypes ($P = 0.004$; Online Resource 7c). Phenolic content of grazed plants differed interactively by fungal inoculation, bacterial inoculation, and genotype ($P = 0.02$; Fig. 4b): for genotypes B and D bacterial inoculation increased phenolic content relative to the control, and for genotype L, co-inoculation reduced phenolic content relative to inoculation with fungi alone (Fig. 4b). Leaf organic content varied across genotypes for grazed plants ($P = 0.0002$; Online Resource 7d), with no significant differences among fungal or bacterial inoculation treatments.

Discussion

Bacterial and fungal endophyte inoculation affected *Spartina* growth, morphology, and survival. In general, the bacterial endophyte increased survival, whereas the fungal endophyte increased plant growth. In contrast to our initial hypothesis, we found little evidence of interactions between endophytes within plants before the grazing event. In addition, when examining differences among *S. alterniflora* genotypes, we found differences in plant morphology to be primarily independent of the endophyte treatments. Although unplanned, the grazing event by *Mythimna unipuncta* revealed notable insights. The prevalence of grazing was reduced in the presence of each endophyte, and particularly with the bacteria. We saw increases in plant defense phenolics across all endophyte treatments, although the magnitude varied by genotype. In addition, the interaction of both endophytes decreased grazing intensity (proportion of stems grazed per pot), providing further insight into the combined effects of

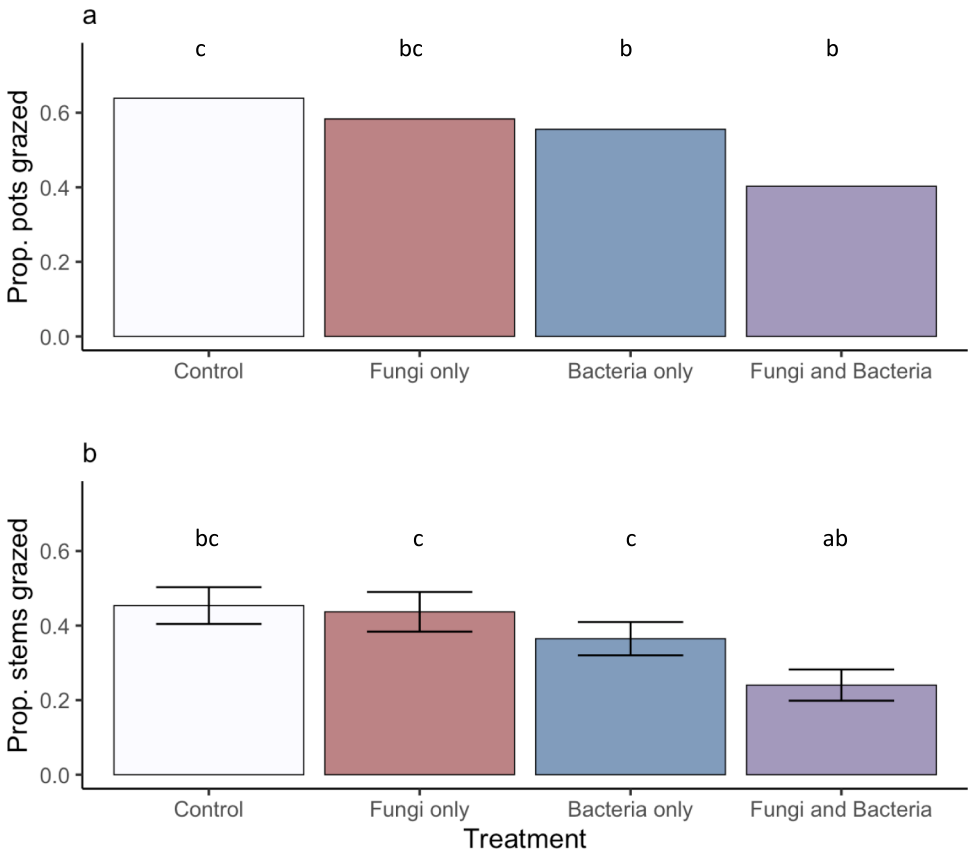

Fig. 2 Effects of endophyte inoculation treatments (blue = bacteria; red = fungi; white = none; purple = co-inoculaiton) on herbivory of *S. alterniflora*. Proportion of S. alterniflora pots grazed was reduced independently by bacteria ($P = 0.02$) and marginally by fungi ($P = 0.07$). **b** Proportion of live stems grazed was most reduced in the co-inoculation treatment (bacteria*fungi $P = 0.02$). Error bars represent 1 SE. Letters indicate significant differences ($P < 0.05$) based on post hoc pairwise comparisons with Tukey's HSD

symbionts on plant success. Despite differential grazing across endophyte treatments, grazed and ungrazed plants showed similar responses to bacterial and fungal presence at the end of the experiment. Overall, we saw stronger and more numerous effects of the bacterial isolate on plant traits than of the fungal endophyte.

Our pre-grazing results showed differential outcomes between endophyte types, with no significant interactions when plants were inoculated with both endophytes. For example, we found positive effects of the bacterial endophyte, *Kosakonia oryzae*, on proportion of surviving plants. *K. oryzae* is known to be a plant growth-promoting and nitrogen-fixing bacterial endophyte (Peng et al. 2009; Xianfa et al. 2015). Temperate marshes are often most limited by nitrogen (Crain 2007). Thus, these endophytic bacteria may facilitate nitrogen uptake that can be used for the host plant's development and survival (Cocking 2003; Afzal et al. 2019), and thereby contribute to the resulting positive outcomes of this plant-endophyte symbiosis. Although we did not see an increase in percent plant protein within bacterial conditions, we see value in pursuing the potential of *K. oryzae* as a nitrogen-fixing, plant-growth promoting bacteria in the future. In contrast to other studies of plant growth-promoting bacteria (Hayat et al. 2010), we did not find plants inoculated with the bacterial endophyte to have increased growth, and instead we saw a decrease in the number of *S. alterniflora* tillers. The growth-survival trade-off is a core ecological concept for organisms in poor environmental conditions (Stearns 1989; Reich 2014). As our system is characterized by various stressful abiotic conditions, these results could suggest a trade-off between host survival versus increased growth, even when ameliorated with a plant growth-promoting bacteria. Although, it is worth noting that our greenhouse environment was not as stressful as native saltmarsh, and therefore, we may subsequently be underestimating beneficial effects if the outcome is stress dependent.

The DSE belonging to the *Magnaporthales* order increased stem height of the host, but it had no other effects on *S. alterniflora* growth and morphology pre-grazing. While the function of dark septate endophytes is heavily variable and multifunctional, there is evidence in multiple cases that these fungi may be acting as "psuedomycorrhizae", by alleviating stressors and increasing colonization in stressed plant hosts (Jumpponen 2001; Li and Guan 2007; Upson et al. 2009; Santos et al. 2017; Liu et al. 2021). For instance, a meta-analysis found positive relationships between fungal endophytes and plant growth responses including total biomass, root biomass, shoot biomass, and root:shoot biomass (Newsham 2011). However, additional reviews highlight the context dependent nature of this symbiosis, where fungal strain, plant type, host plant species, and ecosystem conditions govern

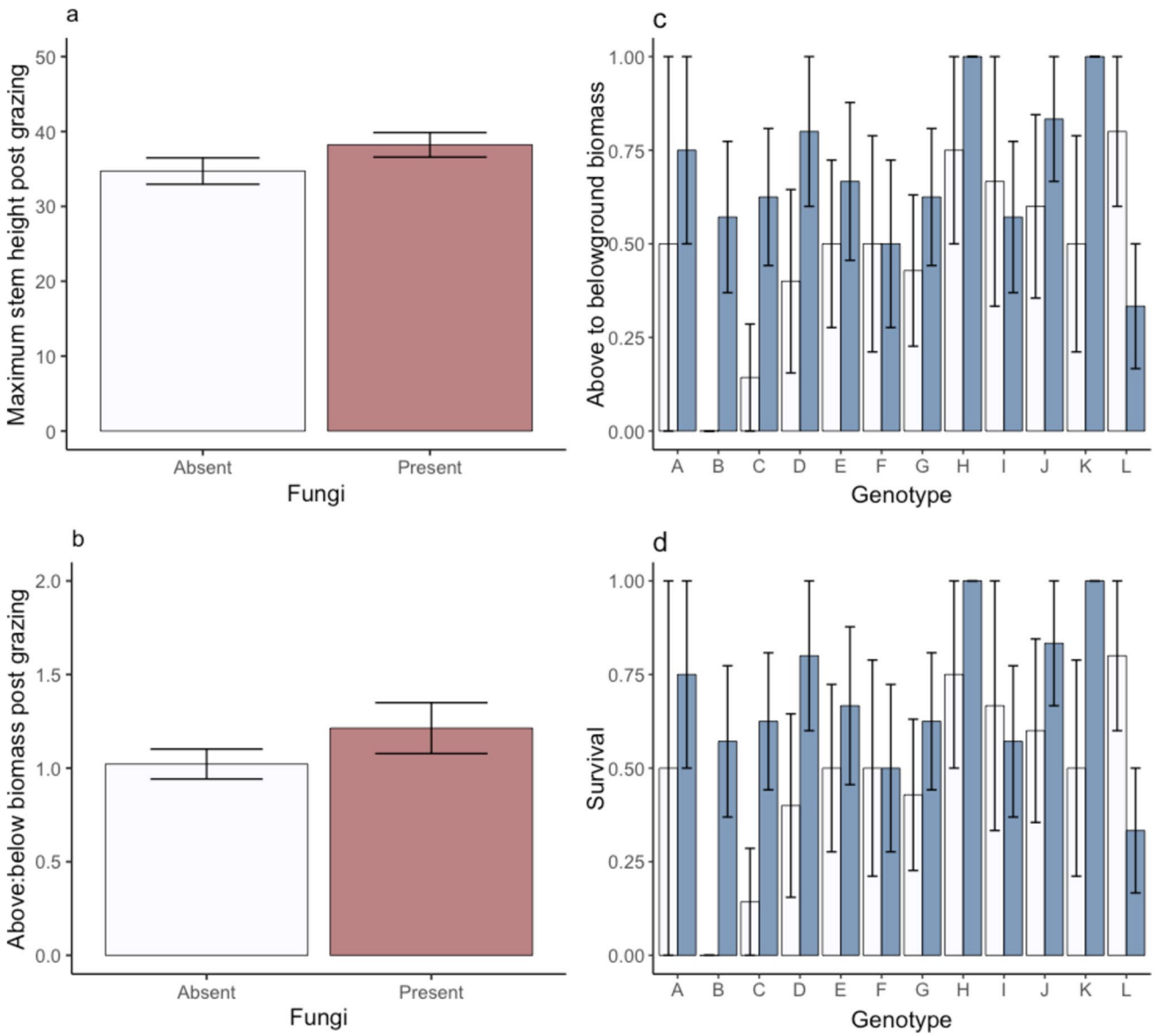

Fig. 3 *S. alterniflora* morphology and biomass at the end of the experiment for (**a**, **b**) plants that were grazed and (**c**, **d**) plants that were not grazed. Fungal inoculation increased (a) maximum stem height ($P = 0.03$) and marginally increased (**b**) above:below-ground biomass ($P = 0.07$) of grazed plants. Bacterial inoculation and genotype interactively affected (**c**) above:below-ground biomass ($P = 0.02$) and (**d**) S. alterniflora survival ($P < 0.01$) of ungrazed plants. Blue = bacteria inoculation; red = fungi inoculation; white = no inoculation. Error bars represent 1 SE. Asterisks in (**c**) indicate significant differences ($P < 0.05$) based on post hoc pairwise contrasts of bacterial treatment within genotype. In (**d**), there were no significant post hoc differences despite a significant interaction between bacteria and genotype in the overall model

the relationship on a mutualism-parasitism continuum (Mandyam and Jumpponen 2014; Akhtar et al. 2022). In particular, of the around 200 species that have been identified as phylogenetically distinct within this order, approximately 50% are considered parasitic, with most of the remaining endophytes being not well described (Feng et al. 2021; Luo and Zhang 2022). While the plant-fungal outcomes varied across our trait measurements, our results indicate the potential growth-promoting mechanism of *Magnaporthales*, as well as neutral to opportunistic behavior, representing a continuum of functional outcomes.

An unplanned but well-documented grazing event identified additional effects of endophyte inoculation in this system. Inoculation with bacteria decreased the proportion of plants grazed by 32%. Further, there was a significant interaction between the bacterial and fungal endophytes: plants inoculated with both had a lower proportion of stems grazed than predicted based on the effects of either endophyte alone.

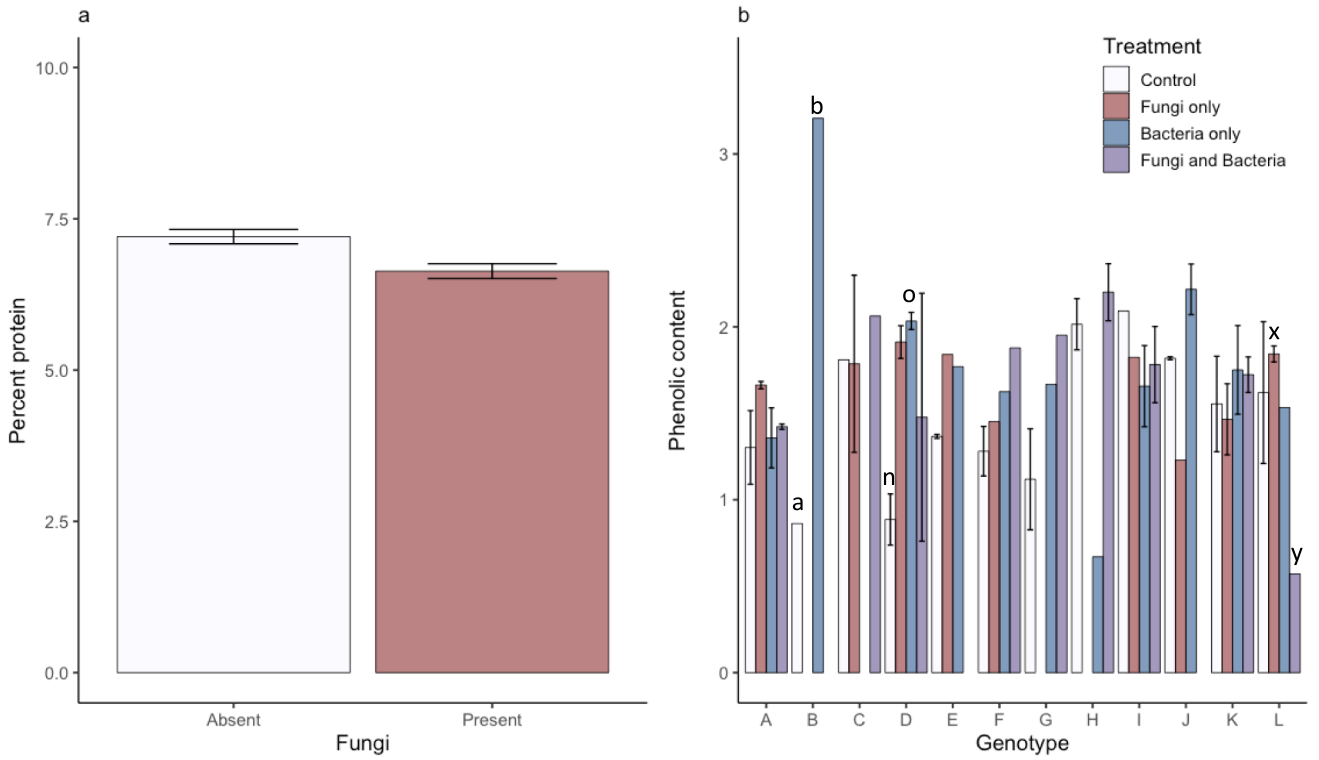

Fig. 4 Variation in *S. alterniflora* leaf tissue chemistry at the end of the experiment. **a** Fungal inoculation decreased percent protein in leaf tissue of grazed plants ($P = 0.005$). **b** Bacterial and fungal endophyte inoculation interacted with S. alterniflora genotypic identity to affect leaf phenolic content of grazed plants (bacteria*fungi*genotype $P = 0.02$). Error bars represent 1 SE. Letters in (b) indicate significant differences ($P < 0.05$) based on post hoc pairwise contrasts of bacterial and fungal treatments within genotype

These effects of endophyte treatments on herbivory were likely mediate by observed changes in plant chemistry, as in other systems (Contreras-Cornejo et al., 2018). For example, fungal inoculation increased phenolic content both individually and in co-occurrence with the bacterial endophyte. We also saw a general increase in phenolics when the bacterial endophyte was present, although the magnitude of this effect varied by genotype. Many endophytes can elicit plant defense responses through secondary metabolites such as phenolics, which in turn provide indirect plant benefits when subject to various stressors (Bamisile et al., 2018; Rodriguez et al., 2009; Santoyo et al. 2016). Specifically, the production of defense chemicals has been shown to deter both vertebrate and insect herbivores (Clay & Schardl, 2002). In addition, plants infected with fungal endophytes can cause increased rates of mortality and reduced relative growth rate of their aboveground pests (Raps & Vidal, 1998; Resquín-Romero et al., 2016; Sánchez-Rodríguez et al., 2018). These studies show a clear influence of belowground symbionts on crucial aboveground processes that influence the success of these foundational marsh grass species.

Following the grazing event, inoculation with the fungal endophyte increased plant stem height and aboveground:belowground biomass. These effects followed similar patterns to the pre-grazed plants. Plants inoculated with the fungal endophyte also had an 8% decrease in protein content in comparison to control plants at the end of the experiment. Such an effect of fungal endophytes on plant protein content has been commonly observed in past studies, although the direction and magnitude of this effect is variable by plant and fungal species (Lledó et al. 2015, 2016; Santamaria et al. 2017; García-Latorre et al. 2021). Because the herbivory event in our experiment was unplanned and we only have protein content data from the end of the experiment, we are unable to disentangle whether the decrease in plant protein influenced or was a response to grazing.

Genotype was a significant predictor of plant performance and traits throughout the experiment, as demonstrated in past studies (Hughes 2014; Zerebecki et al. 2017, Hanley et al., 2021). Interestingly, this genotypic variation was generally independent of endophyte treatment (Online resource 6, online resource 7), in contrast to the substantial interactions between fungal endophyte presence and host genotype observed previously (Hughes et al. 2020). There was some evidence of differential responses across host genotypes: the effects of bacterial inoculation on survival and biomass allocation varied by genotype post-grazing, but only for ungrazed plants. For most genotypes, bacteria

increased *S. alterniflora* survival and decreased or did not affect aboveground- to belowground biomass. These two responses may be linked: a shift to greater belowground biomass may increase survival through increased nutrient uptake by roots. In addition, increases in belowground biomass may increase plant competitive ability in a natural setting (Emery et al. 2001). Although bacterial inoculation generally increased *S. alterniflora* survival, the direction of the effect was reversed for some genotypes, suggesting caution is needed in extrapolating these benefits widely in a restoration context (McHugh and Dighton 2004). Further, our results highlight the importance of continuing to incorporate genotypic responses when evaluating microbial interactions to gain better understanding of the potential for plant-fungal interactions to aid in local adaptation across an ecosystem.

Conclusions

Salt marshes are key coastal ecosystems providing valuable ecosystem functions. As global change continues to threaten these ecosystems through sea level rise, habitat loss, and eutrophication, it is crucial to better understand the complexities of microbial species interactions that may be contributing to the success of foundational plants such as *S. alterniflora*. In addition, while we recognize the experimental challenges, it is critical to understand the role of intra- and inter-specific variation in these interactions to increase our predictive ability. Our findings suggest that fungal and bacterial endophytes may affect different plant responses, with relatively few interactive effects. However, plant-bacterial-fungal interactions were more prominent during and following an unplanned herbivory event, with shifts in plant tissue chemistry coincident with cascading effects on herbivory, suggesting that our controlled greenhouse experiment may have underestimated the complexity of this system. Continuing to examine tripartite and multitrophic interactions under a range of environmental conditions will be essential for our fundamental understanding of these systems, as well as our ability to conserve and restore them in the future.

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Author contribution statement ARH, CG, TCH, and CR conceived the ideas and designed methodology; ARH, TCH, AN, RD, and CG collected the data; ARH analyzed the data; KRG and ARH led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability Data supporting the results will be available at Northeastern University's Digital Repository Service pending manuscript approval.

Code availability The code used during the current study is available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

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