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Fungal endophytes from *Thalassia testudinum* show bioactivity against the seagrass pathogen, *Labyrinthula* spp.

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Thalassia testudinum has undergone die-offs in the past century due to seagrass wasting disease caused by *Labyrinthula* sp. Little is known about how seagrasses resist *Labyrinthula* infections, but metabolites that inhibit *Labyrinthula* were previously extracted from seagrass leaves. Furthermore, leaf fungal endophytes from seagrasses possess antipathogenic potential, but their activity against *Labyrinthula* is unknown. Here, we aimed to identify whether fungal endophytes of *T. testudinum* can aid in disease defense against *Labyrinthula*. Through Illumina amplicon sequencing of the leaves' mycobiome, we identified fungi that are known to produce antimicrobials. We also isolated and extracted organic compounds from endophytes to test their anti-*Labyrinthula* potential using disk diffusion assays. There were 22 isolates that inhibited *Labyrinthula*, from which two isolates, *Trichoderma* sp. P1a and *Diaporthe* sp. M14, displayed strong inhibition. LC-HRMS/MS analysis determined the likely bioactive compounds of *Trichoderma* as peptaibols and of *Diaporthe* as cytosporone B. Cytosporone B was confirmed bioactive against *Labyrinthula* via disk diffusion assays. While these organisms are low in abundance in the mycobiome, this study demonstrates that seagrass endophytes have the potential to play an important role in defense against *Labyrinthula*.

KEYWORDS

seagrass, *Thalassia*, metabolites, fungi, *Labyrinthula*, endophytes

Introduction

Within the phylum Labyrinthulomycota, the genus *Labyrinthula* contains colorless or yellowish protists that form slime nets containing an ectoplasmic network of hairlike structures in which they glide (reviewed in [Bennett et al., 2017](#)). *Labyrinthula* spp. are generally saprotrophs and have been described as either commensals, mutualists, or parasites (reviewed in [Bennett et al., 2017](#)) and include the pathogenic species that causes

seagrass wasting disease, *Labyrinthula zosterae* (Martin et al., 2016). *Labyrinthula* spp. degrade the cell walls of the host using extracellular enzymes (Muehlstein, 1992), and seagrass wasting disease is characterized by splotching and dark streaking on the leaves (Renn, 1935; Duffin et al., 2020). Several consequences arise from *Labyrinthula* infections in seagrasses, including carbon deficiency, increased respiration, and smaller biomass, making the seagrass susceptible to sulfide, hypoxia, and possible secondary infections (Renn, 1936; Durako and Kuss, 1994; Trevathan-Tackett et al., 2013; Lohan et al., 2020). Die-offs of *Thalassia testudinum* in Florida Bay have been attributed to wasting disease in the past (Robblee et al., 1991; Durako, 1994), and it is important to understand how the *T. testudinum* holobiont responds to *Labyrinthula* infections.

The holobiont of a plant is composed of the plant host and its microbiome, including the phyllosphere and rhizosphere, and these factors act as a biological unit for the survival of the whole (Jones et al., 2019). The microbiomes of plants are known to provide several benefits to the plant host, including improved environmental stress tolerance, plant growth, and pathogen resistance (reviewed in Classen et al., 2015; Berg and Koskella, 2018; Jones et al., 2019). In terrestrial plants like *Arabidopsis*, pathogenic infections can trigger an immune response like the exudation of jasmonic acid, which can affect the abundance and/or composition of the microbial community (Carvalhais et al., 2015) and also support microbes that may have beneficial effects on the plant, including increased biofilm formation that can protect against pathogens (Berendsen et al., 2018). Furthermore, terrestrial plants can “precondition” their soil into a “protective” soil after pathogenic infections, so that new, uninfected plants of their species can obtain heightened resistance against pathogens, likely provided by soil microbial communities (Berendsen et al., 2018). Studies in which the microbiome can provide disease defense to the plant host have been reported for some aquatic plants. For example, bacterial isolates from *Vallisneria americana*, which is closely related to *T. testudinum*, were shown to inhibit the growth of the pathogenic oomycete, *Pythium aphanidermatum* (Kurtz et al., 2003). Although the seagrass microbiome has not been clearly shown to play a role in disease defense, studies have suggested that the microbiome can provide many benefits to the host. For instance, gene expression studies on the seagrass microbiomes show that they have the potential to detoxify harmful compounds such as sulfide, methanol, and ethanol, as well as fix nitrogen, and produce plant-growth hormones and agarase, which might inhibit algal competition (Crump et al., 2018). Additionally, seagrass endophytic isolates of *Aspergillus* spp (Notarte et al., 2018), *Trichoderma* spp., *Penicillium* spp., *Fusarium* spp., and *Stephanonetria* spp (Supaphon et al., 2013). have shown antipathogenic potential against human pathogens, but it is unknown whether this activity applies to seagrass pathogens.

Endophytic fungi are usually transmitted to plants via either vertical transmission to the seeds from the female plant or horizontal transmission to aboveground portions of the plant via biotic or abiotic dispersal, spores, or hyphae (Yan et al., 2019). For many years, it has been known that fungal endophytes provide many benefits to the plant host, including increased resistance to abiotic stressors, like high salinity, drought, and heavy metal exposure, and biotic factors such as herbivores and pathogens (reviewed in Yan et al., 2019 and Qin et al.,

2023). Fungal endophytes can release secondary metabolites that can alter the host plant’s gene expression and biochemical pathways as a way to increase pathogen resistance (Qin et al., 2023). For example, the fungal endophyte *Acrophialophora jodhpurensis* was able to increase the activity of several immune responses on tomato plants against *Alternaria alternata* infections, including phenolic and ROS production, among other responses (Daroodi et al., 2021). Endophytic fungi also produce several antimicrobial compounds including antibiotics such as penicillin, trichodermin, and cell wall-degrading enzymes like cellulases, lipases, and proteases, all of which can offer defense against plant pathogens (Ghorbanpour et al., 2018; Fadji and Babalola, 2020).

Few studies have examined the effects of endophytic marine fungi against diseases in marine plants; however, a few studies show that metabolite extracts from marine plants can potentially deter marine pathogens. A study on the seaweed *Agarophyton vermiculophyllum* demonstrated that seaweed metabolites can deter opportunistic pathogens *in vitro* while attracting bacteria that displayed defensive properties against disease-causing bacteria (Saha and Weinberger, 2019). Additionally, seagrasses are known to produce many secondary metabolites that have antimicrobial activity against medically and agriculturally important pathogens (reviewed in Zidorn, 2016), and antifungal activity (Ross et al., 2008). Anti-labyrinthulid activity has also been observed from phenolic acids and an unknown small molecule that was isolated from *T. testudinum* (Steele et al., 2005; Trevathan-Tackett et al., 2015). Furthermore, a study by Castro-González et al. (2022) showed that there are differences in the metabolic profiles of healthy vs. *Labyrinthula*-infected seagrasses, meaning that the pathogen infection changes the metabolic profile of the plant. Although these studies suggest that the secondary metabolites arise from the seagrass plant, the endophytic fungal secondary metabolites must be considered, due to their presence within the leaves and potential antipathogenic activity (Supaphon et al., 2013; Notarte et al., 2018). To date, no studies have determined the effects of the metabolic extracts from *T. testudinum* fungal endophytes on the growth of *Labyrinthula* spp. The aim of this paper is to determine whether fungal endophytes of *T. testudinum* have the potential to produce anti-labyrinthulid compounds. We first examined the potential of the fungal community to produce antipathogenic compounds based on the taxonomy of the endophytic fungi (within the leaves) and epiphytic fungi (on the leaf surface). We then cultured fungal endophytes from apparently healthy leaves, and their extracted organic compounds were tested for their activity against a strain of *Labyrinthula*. Finally, we identified the bioactive compounds in the two most bioactive extracts.

Methods

Amplicon sequencing of fungal communities in field collected *Thalassia testudinum* leaves

There were 50 healthy-appearing leaves of *T. testudinum* that were collected from Hobie Island Beach Park from Virginia Key, Miami, FL, USA, in November 2022. The leaves were placed in a

ziplock bag with seawater while transferring to the lab. The leaves were rinsed with seawater to remove loosely attached sediment and scraped with a sterile blade on both sides of the leaf to collect the epiphyte samples, which were transferred to a 1.5-mL Eppendorf tube and stored in -80°C until DNA extractions. The leaves, which were considered surface sterilized, were then stored at -80°C overnight, and afterward transferred to a lyophilizer for 3 days. The freeze-dried leaves were crushed to almost powder and transferred to a 1.5-mL Eppendorf tube for DNA extractions. The crushed leaves were considered the endosphere samples. DNA was extracted using the Qiagen DNeasy PowerLyzer Soil Kit (Qiagen, Hilden, Germany) following manufacturer-recommended protocols, including a 10-min incubation step at 70°C after step 3 to increase DNA yield. The DNA was sent to Novogene (Beijing, China) for amplicon sequencing of the fungal ITS1-1F region (primers: ITS1-1F-F CTGGTCATTTAGAGGAAGTAA and ITS-1F-R GCTGCGTCTTCATCGATGC).

The preprocessed sequences were run through dada2 in R (v 4.2.2; [R Core Team, 2013]), using default settings. The amplicon sequence variants are available in the [supplementary material](#) as a fasta file. Taxonomy was assigned using the assignTaxonomy function in dada2 (v 4.2.2; [R Core Team, 2013]), with the UNITE database (version 9.0, [Abarenkov et al., 2022](#)) as reference. The R packages phyloseq ([McMurdie and Holmes, 2013](#)), vegan ([Oksanen et al., 2018](#)), ggplot2 ([Wickham, 2009](#)), dplyr ([Wickham et al., 2023](#)), ape ([Paradis and Schliep, 2019](#)), and microbiome ([Lahti et al., 2017](#)) were used for visualizations and statistical analysis including alpha (Shannon and Chao1) and beta diversity (UniFrac PCoA) plots to compare the endophytic and epiphytic fungal communities. Permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations using the Adonis command in the base package in R (v 4.2.2; [R Core Team, 2013]) was used to determine significant differences in the alpha and beta diversity between the epiphyte and endosphere samples. Phyla labeled “incertae_sedis” were removed from the dataset, as the purpose of this approach was to identify known producers of bioactive compounds. The top 20 most abundant genera were plotted using the plot_bar function in phyloseq after normalizing and filtering out “NA” genera.

Cultivation and isolation of endophytic fungi

Samples of *Thalassia testudinum* leaves were collected off the shores of Hobie Island Beach Park in Virginia Key in Miami, FL, USA, in late June 2021. Only green leaves that contained no apparent lesions were selected for isolation of potentially beneficial fungal endophytes. The leaves were collected haphazardly during low tide, with gloved hands, and were placed in ziplock bags containing seawater. The ziplock bags were then placed in a cooler with ice during transportation to the lab to be processed within 4 h.

The protocols of [Mata and Cebrián \(2013\)](#); [Bibi et al. \(2018\)](#), and [Ettinger and Eisen \(2020\)](#) were modified to isolate endophytic fungi. Briefly, 48 leaves were swabbed with sterile cotton swabs to

remove ectosymbionts and to surface sterilize the leaves. Half of the leaves ($n = 24$) were then further sterilized by submersion in 0.5% NaOCl for 5 min and then rinsed with sterile salt water (Instant Ocean, VA, USA) for 5 min. All leaves were then cut into 1–2-cm pieces and plated on potato dextrose agar (PDA) and malt extract agar (MEA) media plates, one leaf per plate. This resulted in 24 plates of only scraped leaves (12 in PDA and 12 in MEA) and 24 plates of scraped and bleached leaves (12 in PDA and 12 in MEA). PDA plates were prepared with 39 g/L of PDA in DI water, and, after autoclaving, the addition of 25 mL/L of penicillin–streptomycin (10,000 units penicillin and 10 mg streptomycin per mL; Millipore Sigma). MEA plates were made with 30 g/L of malt extract and 15 g/L of agar in DI water, followed by adding 25 mL/L of penicillin–streptomycin (10,000 units penicillin and 10 mg streptomycin per mL; Millipore Sigma) after autoclaving. Once the leaves were plated, the plates were parafilm and incubated at room temperature ($\sim 23^{\circ}\text{C}$), in the dark for 3–4 weeks, checking for new growth weekly. Plugs of emerging fungi were plated in the center of their respective fresh media plates (either PDA or MEA) and allowed to grow until the plate was covered. Plugs were taken from each plate until colonies were isolated.

DNA extractions, PCRs, and sequencing of fungal isolates and *Labyrinthula* spp.

DNA was extracted from each fungal isolate using the Qiagen PowerLyzer Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer-recommended protocols with slight modification. Briefly, a sterile needle was used to collect mycelia, tissue, and/or spores from each fungus and to place them in the bead beating tubes (provided by the kit) containing the first buffer and solution SL. A 10-min incubation step at 70°C was added to increase DNA yield. Bead-beating was performed at 4.0 m/s for 20 s, twice, with a FastPrep-24 bead beater (MP Biomedicals). The ITS-28S region was amplified with primers ITS5 (5'-GGAAGTAAAAGTCGT AACAAGG; [White et al., 1990](#)) and LR3 (5'-GGTCCG TGTTTCAAGAC; [Vilgalys and Hester, 1990](#)), as used in [Ettinger and Eisen \(2020\)](#). The PCR program was as follows: 95°C for 5 min, 35 cycles of 94°C for 30 s, 52°C for 15 s, 72°C for 1 min, and a final extension at 72°C for 8 min ([Ettinger and Eisen, 2020](#)).

All PCR products were sent for cleanup and Sanger sequencing with the same primer set, ITS5 and LR3 (MCLAB, San Francisco, CA, USA). Forward and reverse sequences were merged for all samples using the MegAlign program from Lasergene (v.17.4, DNASTAR). Sequences are available in GenBank (accession numbers OR355682–OR355766). NCBI BLAST was used for initial identification of all cultures. The ITS sequences obtained from the Sanger sequencing run of the most bioactive fungi were trimmed to match the ITS1-1F amplicons using Geneious v.R8.1.9 (Biomatters, Auckland, New Zealand). Amplicon sequences were used as a custom BLAST database in Geneious v.R8.1.9 and were blasted against the trimmed ITS sequences to find 100% matches, confirming the presence of the strains in our amplicon dataset. Furthermore, ASVs that matched the taxonomical assignment of the isolates that showed the most bioactivity against *Labyrinthula*

were subset from our datasets using the `subset_taxa` function in `phyloseq` to determine their relative abundance (McMurdie and Holmes, 2013). Where appropriate, a standard two-sample T-test was performed on log-transformed data to compare the abundance of the most bioactive fungi between epiphytic and endophytic samples using the base package in R (v 4.2.2; [R Core Team, 2013]).

Metabolite extractions of fungal isolates

Fungi were grouped based on sequence data and colony morphology. A representative from each group was selected for metabolite extractions. If a culture belonged to the same taxon based on sequence data but differed in colony morphology, it was also selected for extractions. The protocols from Supaphon et al. (2013) and Notarte et al. (2018) were followed with modifications to extract metabolites from the fungal endophytes. To prepare the cultures for metabolite extractions, 17 plugs (5 mm wide) of each representative strain were placed in 125-mL flasks containing 50 mL of malt extract media (30 g/L in DI water). Malt extract media were used for growing both MEA- and PDA-derived fungal isolates because of its better yield (Supplementary Table 1). Cultures were placed in incubated mini shakers (VWR International, LLC.) set to 28°C and 130 rpm. Cultures were allowed to grow for a week, and if no growth was observed, the cultures were moved to static conditions at 28°C. If the cultures remained in the incubated shakers, they were left for an additional week, and if they were in static conditions, they were left for an additional 2 weeks.

The cultures were filtered into a 50-mL filtration unit using a GF/F Whatman filter (47-mm diameter; [®]Cytiva). All glassware was rinsed with HPLC Plus grade ethyl acetate (Sigma-Aldrich, Darmstadt, Germany) between samples. For the liquid-liquid extractions, a total of 150 mL of HPLC Plus grade ethyl acetate was used to extract metabolites from the culture filtrate, three times. For the first extraction, 50 mL of ethyl acetate was mixed with the filtrate (~50 mL) and shaken vigorously for 5 min. The second and third extractions were also done with 50 mL of ethyl acetate and shaken vigorously for 2 min each. For the biomass extractions, the cultures were scraped off the GF/F Whatman filter and stored at -80°C overnight. The biomass was then lyophilized for 3 days and stored at -80°C until further processing. The biomass was then crushed with a mortar and pestle, placed in a glass beaker with 50 mL of ethyl acetate, and homogenized with a tissue homogenizer (TH Tissue Homogenizer, Omni International, USA). After 30 min in ethyl acetate, intermittently shaking the beaker, the crushed biomass was filtered through a ceramic funnel lined with a Whatman filter paper (ashless, 150 mm) shaped to fit the funnel, into a glass flask attached to a vacuum pump. The extracts derived from the supernatant and biomass were dried in vacuum using a Rotavapor (RE111, Büchi, Switzerland) at 46°C, until the ethyl acetate mostly evaporated. When the volume was near 5 mL, the extracts were transferred to scintillation vials and further evaporated on the Rotavapor. Samples were left to air-dry overnight and then stored at 4°C until assayed (between 1 and 2 weeks).

Labyrinthula isolation and identification

The protocols of Martin et al. (2009) and Sullivan et al. (2017) were modified to culture *Labyrinthula* spp. from field-collected leaves. Briefly, green leaves that contained lesions were collected during low tide and placed in ziplock bags containing seawater. Upon arrival to the lab, the leaves were rinsed with seawater and swabbed with sterile cotton swabs to remove ectosymbionts. The leaf lesions were then cut into 1–2-cm pieces and placed in serum seawater agar plates (SSA, Muehlstein et al., 1988), which were prepared with modifications as follows. Twelve grams of agar was added to Instant Ocean (Spectrum Brands, Blacksburg, VA, USA; prepared according to manufacturer-recommended protocols) per liter of SSA media. After autoclaving, 10 mL of horse serum (Gibco), 25 mL of penicillin-streptomycin (10,000 units penicillin/mL, 10 mg streptomycin/mL; Millipore Sigma), and 2 mL of a germanium dioxide solution (1.5 mg/mL in DI water; filter-sterilized) were added per liter of SSA media. After isolating the strains, they were maintained in Enhanced SSA (ESSA) media (Martin et al., 2016). ESSA was prepared with modification as follows: 12 g of agar, 1 g of glucose, 0.1 g of peptone, and 0.1 g of yeast extract per liter of ESSA were autoclaved in Instant Ocean. After autoclaving and cooling, 10 mL of horse serum (Gibco) was added per liter of ESSA. After *Labyrinthula* colonies were isolated, they were grown in 10 mL of liquid SSA media in sterile petri dishes for 14 days in preparation for DNA extractions using the Qiagen PowerLyzer Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer-recommended protocols. PCRs of the 18S rRNA gene were then performed using primer sets A, R3 and F2, B (Martin et al., 2016). PCRs were sent for Sanger sequencing using the same primers (MCLAB, San Francisco, CA, USA). Sequences were assembled using the MegAlign program from Lasergene (v.17.4, DNASTAR).

The *Labyrinthula* strain that grew the best was L 7.2 (GenBank accession OR356117). To determine the pathogenic probability of this strain based on Martin et al. (2016), who defined different phylogenetic clades for the pathogenic and non-pathogenic strains of *Labyrinthula* spp., 26 near full-length 18S rRNA gene sequences representing three distinct *Labyrinthula* clades (pathogenic, non-pathogenic, and terrestrial) were retrieved from the GenBank nr database. These sequences (including two outgroup sequences) were aligned using MAFFT v7.271 (Katoh and Standley, 2013) with default parameters, and gaps were masked using trimAl v1.4 (Capella-Gutiérrez et al., 2009). Phylogenetic inference was made by Maximum Likelihood methods implemented in RAxML v8.0.0 (Stamatakis, 2014) under gamma-corrected GTR model of evolution with 1,000 bootstrap replicates based on 935 homologous positions, and the final tree was produced with FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>).

Antagonism assays

Several ESSA plates were inoculated with a plug (5-mm diameter) of *Labyrinthula* strain L7.2. When the *Labyrinthula* colonies grew at least 5 mm beyond the plugs, they were considered ready for assay.

Only colonies that were actively growing were chosen. The metabolite extracts were resuspended in 400 μ L methanol, from which 20 μ L was pipetted onto sterile Whatman filter disks, cut with a hole puncher (6-mm diameter), in triplicates. 20 μ L of methanol was used for control. The disks were airdried overnight within a closed biosafety cabinet. The disks were then placed on the edge of the *Labyrinthula* colony. For each replicate, both the liquid extract and the biomass extract disks from the same culture were placed at the edge of the *Labyrinthula* colony. The plates were checked every other day for 15 days for zones of inhibition or any visible changes to the colony, which were assessed each time. Due to the irregular and asymmetrical nature of *Labyrinthula* colony growth on agar media (Martin et al., 2009), measurements of colony growth can be difficult to compare between colonies; therefore, we rated bioactivity levels as strong (the colony was pushed away from the disk), moderate (the colony ceased growth), or none (the colony grew over the filter disk). The fungal extracts with the strongest bioactivity levels were selected for further analysis.

LC-HRMS/MS analysis of extracts

Dried fungal extracts were resuspended in 100% MeCN and diluted (1:100) in LC-HRMS starting conditions, and experiments were conducted on an Agilent 1290 Infinity II series UPLC coupled to an Agilent 6546 QTOF mass spectrometer with an electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, USA). Chromatography was performed using a Kinetex[®] C18 column (50 \times 2.1 mm, 2.6, Phenomenex, Torrance, CA, USA), oven temperature was set to 40°C, and the sample injection volume was 1 μ L. A binary gradient consisting of MeCN (eluent A) and water (eluent B) (both + 0.1% formic acid (FA)) at a constant flow rate of 400 μ L/min was used. The gradient was applied as follows: 0 min–0.25 min, 10% A; 10.00 min, 95% A; 13.00 min, 95% A; 13.1 min, 10% A; 15.00 min 10% A. The MassHunter software (Agilent Technologies, Santa Clara, CA, USA) was used for data acquisition and subsequent qualitative and quantitative analysis. All parameters regarding the QTOF are listed in Supplementary Table 2.

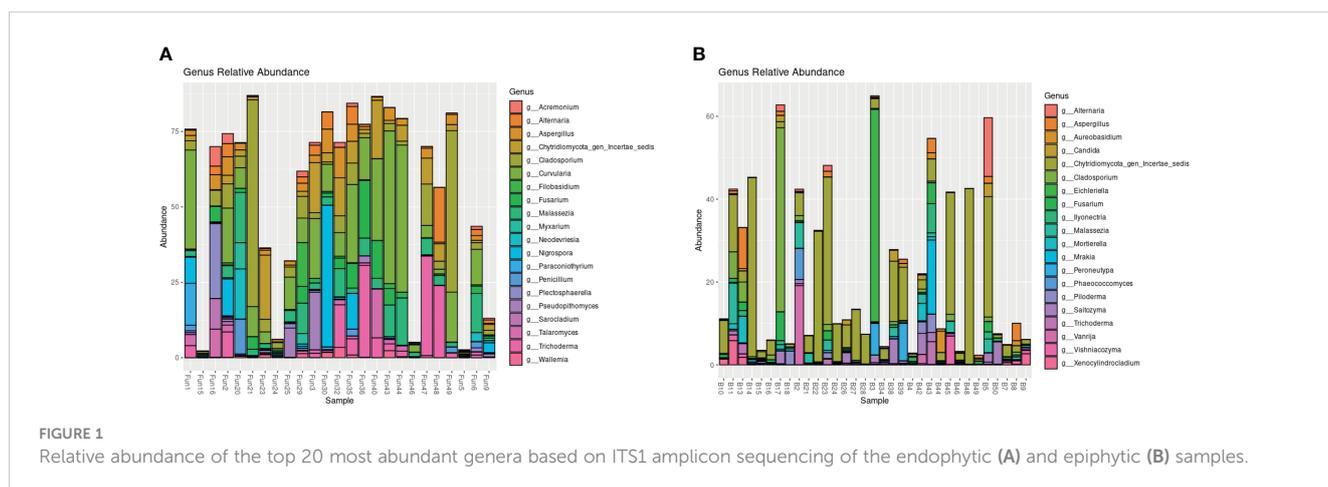
Cytosporone B assay

Cytosporone B was identified in extracts from colony M14 (*Diaporthales* sp.) and was purchased as a chemical standard to confirm its bioactivity against *Labyrinthula*. Around 3 mg of cytosporone B (5 mg, Santa Cruz Biotechnology, Inc., USA) was resuspended with 60 μ L of methanol, from which 20 μ L was aliquoted onto three Whatman filter disks (6 mm diameter), totaling around 1 mg each. The filter disks were left to air-dry overnight before placing them at the edge of *Labyrinthula* colonies. A filter disk with 20 μ L of methanol was used for control. The assays were monitored every other day for 15 days for any changes in the colonies.

Results

Fungal diversity and abundant genera inhabiting the leaves

The endophytic samples had a higher average alpha diversity compared with the epiphytic samples in both Shannon and Chao1 diversity indices (PERMANOVA, $p = 0.001$, $p = 0.007$, respectively; Supplementary Figure 1). The communities also differed in beta diversity, with clear, separate clusters shown by the UniFrac distance matrices (PERMANOVA, $p < 0.0001$; Supplementary Figure 2). In general, the top 20 most abundant fungi of the epiphytic samples differed from the endophytic samples (Figure 1). Several of the fungal genera that were isolated and cultured from the endosphere were present in the amplicons: *Aspergillus*, *Cladosporium*, and *Trichoderma* in both the endophytic and epiphytic samples, and *Penicillium* and *Sarocladium* in the endophytic samples (Figure 1, Supplementary Table 3). Many of the most abundant genera of the endosphere, compared with only a few of the abundant genera in the epiphytes, included taxa that have previously been described to produce antipathogenic compounds (Supplementary Table 3).



Fungal isolates

A total of 104 fungal isolates were obtained (43 from PDA and 61 from MEA) from *T. testudinum* leaves. A total of 56 isolates from MEA and 30 from PDA could be successfully identified using sequencing of the ITS-28S rRNA region. The remaining cultures either had poor DNA quality after extractions or poor sequence quality after Sanger sequencing. Many sequences were identical or very similar; therefore, isolates were clustered into taxa. On MEA, there were cultures from 15 taxa: *Aspergillus*, *Cladosporium*, Diaporthales, Dothideomycetes, *Eutypella*, *Microsphaeropsis*, *Myrothecium*, *Ohleria*, *Paraphaeosphaeria*, *Parastagonospora*, *Penicillium*, *Pestalotiopsis*, *Phoma*, Pleosporales, and *Trichoderma*. On PDA, strains from nine taxa were isolated: *Aspergillus*, *Cladosporium*, Diaporthe, *Dothichiza*, Dothideomycetes, *Microsphaeropsis*, *Myrothecium*, *Parascedosporium*, and *Trichoderma*. In addition to NCBI's GenBank, the UNITE database was also used to determine the taxonomy of the sequences (Supplementary Table 1). Although most of the cultures were assigned to at least the same genus on both databases, 29 of the 86 cultures had different taxonomic assignments from each database. Although the assigned taxonomy differed in each database, the taxa belonged to at least the same family, order, or class for 26 of the 29 cultures (Supplementary Table 1). Because NCBI's GenBank yielded better %ID matches to many of the isolates compared with the UNITE database, we assigned taxonomy based on the BLAST results.

Antagonism assay

Labyrinthula strain L 7.2 was determined to be potentially pathogenic based on the similarity of its 18S rRNA sequence to the strains that were identified as pathogenic in Martin et al. (2016) (Figure 2). This strain was selected for the antagonism assays due to its faster growth rate and its potential pathogenicity.

Out of 50 fungal strains tested, 22 strains showed activity against *Labyrinthula*. Two strains showed strong activity, meaning the *Labyrinthula* colonies were cleared away from the disks (Supplementary Figure 3; Table 1), whereas 20 strains showed moderate activity, meaning the *Labyrinthula* colonies neither grew over the disk nor cleared away from the disk (Table 1). The strain that

showed the most activity against *Labyrinthula*, in both extracts of biomass and supernatant, was isolate M14, which belongs to the order Diaporthales per NCBI blast (97.56%; Table 1; Supplementary Table 1). Colony P1a, which belongs to the genus *Trichoderma* with 99.64% ID per NCBI blast, had strong activity only in the liquid extract (Table 1).

Fungi belonging to the genera *Trichoderma*, *Aspergillus*, *Penicillium*, *Pestalotiopsis*, order Pleosporales, and class Sordariomycetes (including Diaporthales), showed moderate activity against *Labyrinthula*. There were 28 of the strains that showed no activity (Supplementary Table 1).

LC-HRMS/MS results

The extracts from the two fungal strains that showed the most activity against *Labyrinthula* spp. (M14 and P1a) were analyzed by LC-HRMS/MS. The M14 extract showed a major component and the detection of the accurate m/z 323.1855 (Δ ppm 0.62) suggested the molecular formula of $C_{18}H_{26}O_5$. Literature revealed cytosporone B as a fit for the latter molecular formula (Chepkirui and Stadler, 2017; van Santen et al., 2019; Xu et al., 2021). Comparison of the fragmentation spectrum (MS^2) with *in-silico* prediction software (Wang et al., 2021) suggested cytosporone B as the respective metabolite. Furthermore, the presence was unequivocally confirmed with a purchased reference standard of cytosporone B (Figure 3). Cytosporones are signature compounds described for the genus *Diaporthe* (Brady et al., 2000).

The extract of P1a showed a mixture of peptaibol-like compounds (m/z 1,108–1,120, data not shown) due to characteristic neutral losses of aminoisobutyric acid (Aib, 85.059 Da). The mixture was not further characterized since both the NCBI BLAST and UNITE database analyses indicated that P1a is in the genus *Trichoderma*, which produces a vast variety of peptaibols that are known to exhibit a wide range of biological activities, including antimicrobial and cytotoxic effects (Daniel and Rodrigues Filho, 2007; Hou et al., 2022).

Cytosporone B assay

Cytosporone B was found to be a major component in M14 extracts. As cytosporone B is commercially available, an assay was

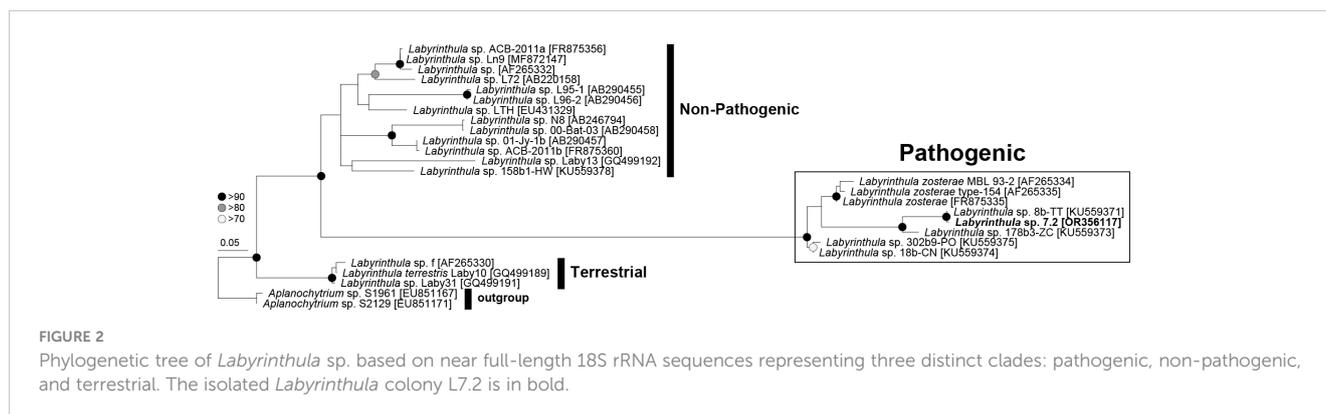


TABLE 1 List of bioactive fungal strains isolated from *T. testudinum* leaves and their predicted taxonomy.

Strain	Accession #	treated	Media	Taxa NCBI blastn	%ID	LE activity	BE activity
M1	OR355763	UB	MEA	<i>Penicillium citrinum</i> (MH990629.1)	99.81	+	+
M11	OR355749	UB	MEA	<i>Aspergillus flavus</i> (CP051065.1)	99.24	+	+
M12a	OR355750	UB	MEA	<i>Aspergillus fumigatus</i> (MN190284.1)	99.23	+	+
M13a	OR355753	UB	MEA	<i>Penicillium chrysogenum</i> (KY218687.1)	99.9	+	+
M14	OR355755	UB	MEA	<i>Diaporthales</i> (MW045842.1)	97.56	++	++
M15b	OR355756	UB	MEA	<i>Aspergillus oryzae</i> (EF634406.1)	99.73	-	+
M16	OR355714	UB	MEA	<i>Aspergillus flavus</i> (OP600483.1)	100	-	+
M22	OR355701	UB	MEA	<i>Aspergillus sydowii</i> (EF652451.1)	99.73	-	+
M28	OR355682	UB	MEA	<i>Aspergillus terreus</i> (KC119206.1)	99.91	+	+
M33	OR355692	B	MEA	<i>Penicillium steckii</i> (MT582790.1)	98.77	+	-
M34	OR355693	B	MEA	<i>Aspergillus terreus</i> (KC354516.1)	94.85	+	+
M47	OR355687	B	MEA	<i>Aspergillus terreus</i> (KC354516.1)	99.73	+	+
M48	OR355700	B	MEA	<i>Pestalotiopsis</i> (EU605881.1)	99.81	+	-
M49a	OR355706	B	MEA	<i>Pestalotiopsis</i> (MN427967.1)	99.91	+	+
M50b	OR355688	B	MEA	<i>Trichoderma afroharzianum</i> (MN644569.1)	99.82	+	+
M7	OR355745	UB	MEA	<i>Paraphaeosphaeria neglecta</i> (JX496091.1)	99.55	-	+
P15	OR355727	UB	PDA	<i>Trichoderma lixii</i> (JN943369.1)	99.91	+	+
P19	OR355731	UB	PDA	<i>Diaporthe</i> (KP306998.1)	99.91	+	+
P1a	OR355719	UB	PDA	<i>Trichoderma</i> (OM515101.1)	99.65	++	-
P4	OR355710	UB	PDA	<i>Trichoderma reesei</i> (KX664418.1)	100	+	+
P7	OR355718	UB	PDA	<i>Diaporthe</i> (KU747870.1)	97.85	+	+
P9	OR355704	UB	PDA	<i>Aspergillus versicolor</i> (MT582751.1)	99.73	+	+

UB treated leaves were scraped and not bleached before plating. B leaves were scraped and bleached before plating. MEA is malt extract agar, and PDA is potato dextrose agar. Accession numbers for the NCBI blast best hits are in parentheses. LE activity levels refer to liquid extracts, BE activity levels refer to biomass extracts. (-): no inhibition, (+): moderate inhibition, (++) strong inhibition.

performed with 1 µg of per disk in triplicates. After 15 days of running the assay, the results showed that cytosporone B displayed moderate activity, meaning that the *Labyrinthula* colonies neither grew over the disk nor receded further away from them (Supplementary Figure 4).

Abundance of bioactive fungi in field-collected samples

Exact matches for the M14 and P1a sequences were not found in the amplicon sequencing dataset of the ITS1 region. The highest match for M14 was 97.6% to ASV2969, and the highest match for P1a was 99.2% to ASV4147. The relative abundance was determined to be mostly low for both taxa that were classified as the *Trichoderma* and *Diaporthe* for P1a and M14, respectively. *Diaporthe* spp. were only present in three samples: two epiphytic samples (B11 and B15) and one endophytic sample (FUN1), with relative abundances less than 1% in

all three (Supplementary Table 4). *Trichoderma* spp. were present in 49 samples: 27 epiphytic samples, and 22 endophytic samples, with a higher average relative abundance in the endophytic samples ($2.022\% \pm 4.05$) compared with epiphytic samples ($0.16\% \pm 0.17$; Supplementary Table 5). *Trichoderma* spp. was considered rare (relative abundance <0.01%) in four epiphytic samples and one endophytic sample, moderately rare (relative abundance between 0.01% and 1%) in 23 epiphytic and 15 endophytic samples, and abundant (relative abundance >1%) in six endophytic samples. *Trichoderma* spp. were more abundant in endophytic samples than the epiphytic samples, although this difference was not highly significant ($p = 0.062$; Supplementary Table 6; Supplementary Table 7).

Discussion

In the past decade, the advancement of amplicon sequencing and other shotgun sequencing methods have made it easier to study

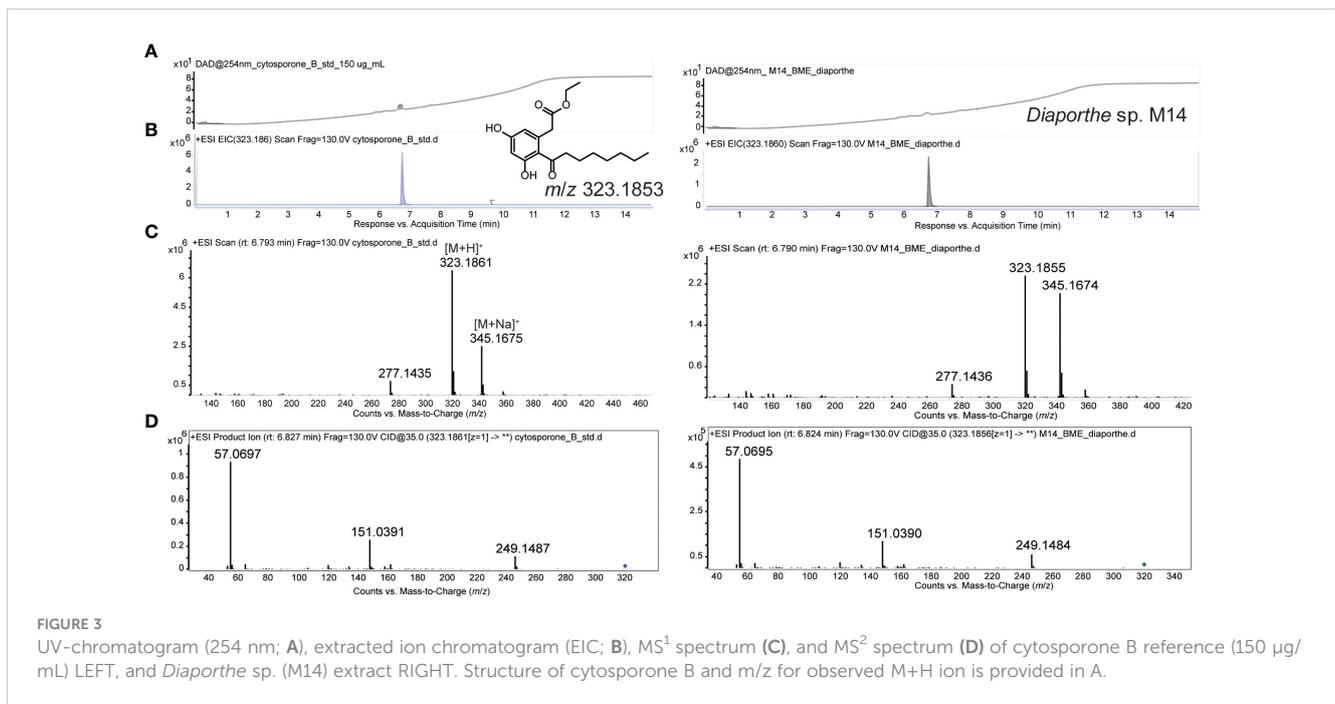


FIGURE 3

UV-chromatogram (254 nm; A), extracted ion chromatogram (EIC; B), MS¹ spectrum (C), and MS² spectrum (D) of cytosporone B reference (150 µg/mL) LEFT, and *Diaporthe* sp. (M14) extract RIGHT. Structure of cytosporone B and m/z for observed M+H ion is provided in A.

the bacterial communities of seagrasses; however, the fungal communities of seagrasses are only recently gaining attention. Recent studies on the endophytic fungi of seagrasses have shown that, like the bacterial communities, they mostly differ between sample types, at least for *Z. marina* (Ettinger and Eisen, 2019; Ettinger et al., 2021). Studies have also shown that there are differences in the fungal communities of the leaf endosphere compared with the leaf surface (Ettinger and Eisen, 2019). In this study, the epiphytic and endophytic fungi of *T. testudinum* leaves also differed in both alpha and beta diversity, with a higher diversity found in the endophytic samples. Overall, the fungal classes that have been associated with seagrasses in previous studies include Dothideomycetes, Eurotiomycetes, Sordariomycetes (Supaphon et al., 2013, 2017; Ettinger and Eisen, 2019, 2020; Ettinger et al., 2021). Several of the endophytes that occur in seagrasses have the potential to deter several pathogens through antimicrobial activities, although these were only tested on human pathogens (Supaphon et al., 2013; Notarte et al., 2018). Only a few studies have examined the effects of endophytic marine fungi against diseases in marine plants, but they found that metabolite extracts from marine plants have the potential to deter marine pathogens (Saha and Weinberger, 2019). In this study, we aimed to identify fungal taxa within the leaves that can have the potential to deter or prevent *Labyrinthula* infections.

Among the top 20 most abundant fungi based on ITS1 amplicon sequencing of *T. testudinum* leaves, we identified several fungi that can potentially provide antipathogenic benefits to the host. Five of the most abundant genera that were present as both the epiphytes and endophytes have all been previously isolated from seagrasses and shown to produce bioactive compounds against pathogens: *Aspergillus* (Hu et al., 2022, 2023), *Cladosporium* (Akmal et al., 2021; Ettinger et al., 2021), *Fusarium* (Supaphon et al., 2013; Alfattani et al., 2021), *Malassezia* (Gaitanis

et al., 2019; Ettinger et al., 2021), and *Trichoderma* (Supaphon et al., 2014; Khan et al., 2020). Interestingly, many of the most abundant epiphytic taxa have previously been associated with either marine environments or terrestrial plants, but little is known about their potential in pathogen defense and plant benefits. On the other hand, many of the most abundant endophytic fungi have previously been isolated from sessile marine animals and plants (terrestrial and marine) and were shown to produce antipathogenic compounds (Supplementary Table 3). It is likely that the epiphytic fungal communities are mostly composed of commensal fungi that adhere to the leaves from surrounding environments, and these are usually removed during sterilization for endophyte analysis. Furthermore, four of the fungal genera that displayed bioactivity in the disk diffusion assays (*Aspergillus*, *Trichoderma*, *Penicillium*, and *Sarocladium*) were part of the most abundant genera in the leaf fungal communities (Supplementary Table 3; Figure 1). This suggests that the fungi that displayed activity against *Labyrinthula* *in vitro* might partly control *Labyrinthula* infections in the field.

Several of the fungal taxa isolated from *T. testudinum* in this study have previously been isolated from *T. testudinum*, like *Penicillium*, *Cladosporium*, *Pestalotiopsis*, *Trichoderma*, and *Phoma* spp (Mata and Cebrián, 2013). In other seagrass species, such as *Zostera marina*, similar taxa have been isolated, including members of the order Pleosporales and class Sordariomycetes, and the genera *Trichoderma*, *Penicillium*, and *Cladosporium* (Ettinger and Eisen, 2020). Terrestrial fungal endophytes have been more widely studied for their potential in pharmaceutical and agricultural industries (Aly et al., 2011) and include many of the same taxa identified and isolated here, like *Aspergillus*, *Penicillium*, and *Phoma* spp., which are known to produce antifungal compounds, and *Trichoderma* which can increase immunity against pathogens (reviewed in Yan et al., 2019). In this study, these taxa, along with *Pestalotiopsis*, showed moderate activity against *Labyrinthula*, suggesting that they may also assist in disease

defense in marine systems, or at least in seagrasses. Other endophytes of marine origin that displayed activity against protistan pathogens include *Penicillium*, *Phaeosphaeria*, *Chaetomium*, and *Phoma* spp (Vallet et al., 2018), of which most were found in our dataset, although not all showed bioactivity against *Labyrinthula* (Supplementary Table 1). Although only a few studies are available on the benefits of fungal endophytes of marine plants and macroalgae, it is becoming clear that, like in terrestrial systems, the fungal communities of seagrasses can play a role in disease defense, and possibly even stress response to abiotic factors.

The two cultures that displayed the most bioactivity against *Labyrinthula* were M14 and P1a. Based on the results of NCBI BLAST and UNITE blastn, along with their chemotype, P1a is likely *Trichoderma* and M14 is likely *Diaporthe*, both of which are well known to have bioactivity against pathogens. *Trichoderma* spp. are known to sense and overcome other potentially pathogenic fungi and also improve plant health, growth, and nutrient absorption through excreted compounds, which has made them useful in agricultural and horticultural crops such as cucumbers, chrysanthemums, tomatoes, and chickpeas (López-Bucio et al., 2015). In addition to improving plant health, *Trichoderma* spp. can also provide pathogen protection, including inhibiting the growth of several *Pythium* spp., which are known oomycete plant pathogens, both *in vitro* and *in planta* for both tobacco and cucumber plants (Liu et al., 2022). *Trichoderma* spp. also produce several secondary metabolites that can inhibit phytopathogenic fungi (reviewed in Khan et al., 2020), and in our case, the isolate P1a (*Trichoderma* spp.) produced peptaibols, which were likely the bioactive compounds against *Labyrinthula*. Peptaibols isolated from *Trichoderma* spp. that presented antipathogenic activity include trichorzins, which displayed antifungal and antibacterial properties (Goulard et al., 1995), and trichorzianines, which inhibit spore germination and hyphal elongation of phytopathogenic fungi (Schirmböck et al., 1994). Other peptaibols isolated from *Trichoderma* spp. include trichokonin VI, which exhibited activity against the oomycete plant pathogen, *Phytophthora parasitica*, in addition to pathogenic fungi and bacteria that infect plants (Shi et al., 2012; Khan et al., 2020).

Diaporthe spp. are also known to provide many benefits to the host plant, including producing a variety of antipathogenic compounds and promoting growth and nutrient uptake (reviewed in Hilário and Gonçalves, 2022). *Diaporthe* spp. (along with its asexual state previously known as *Phomopsis* spp.) can also potentially deter the oomycete pathogen, *Plasmopara viticola*, in *Endodesmia calphylloides* (Talontsi et al., 2012; Hilário and Gonçalves, 2022). This genus also produces diverse secondary metabolites that seem to mostly differ between terrestrial and marine environments (reviewed in Wei et al., 2023). From marine environments, several compounds displayed medicinal potential with antibacterial (e.g., cytochalasin H, isochromopilones, penicilazaphilone D), antifungal (e.g., diaporthelactone), cytotoxic (e.g., phomoxanthone A, diaporisoindole A, phomopsin F, fusaristatin A), anti-inflammatory (e.g., diaporindenes, diaporisoindoles, diaporpenoids), antiviral (e.g., pestalotiopsones), anti-osteoclastogenic (e.g., phomopsichins), antiangiogenic (e.g., cordysin A), and enzymatic activity (e.g., longidiacid A,

longichalasin B; reviewed in Wei et al., 2023). In our dataset, M14 produced cytosporone B, which is likely the main compound inhibiting *Labyrinthula* spp., as was shown in the assay with commercially purchased cytosporone B. In previous studies, cytosporones E and P, isolated from a marine source (i.e., mangroves), showed activity against the protist, *Plasmodium falciparum*, which causes malaria (Kornsakulkarn et al., 2015; Xu et al., 2021). Although *Plasmodium* spp. and *Labyrinthula* spp. are only distantly related protists, the fact that marine-isolated endophytes can produce anti-protistan compounds is interesting, considering that protists can cause disease in several marine macroalgae and plants (reviewed in Schwelm et al., 2018). Cytosporone B has great potential in the pharmaceutical and medicinal industries as a compound that has shown anticancer activity (i.e., colorectal cancer; Ismaiel et al., 2021), attenuation of tissue grafts (Ding et al., 2022), antiviral activity (i.e., influenza; Egarnes et al., 2017), and inhibition of bacterial pathogens, such as *Salmonella enterica* (Jianfang et al., 2013). Cytosporone B and derivatives have previously been isolated from endophytes of marine plants, such as mangroves (Huang et al., 2008; Mei et al., 2021; Dankyira et al., 2022), and shown to have antifungal activity against the plant pathogens, *Fusarium oxysporum* (Huang et al., 2008) and *Geotrichum candidum* var. *citri-aurantii* (Yin et al., 2019), as well as other fungi such as *Candida albicans* (Huang et al., 2008; Dankyira et al., 2022). Cytosporone B can now also be considered an anti-*Labyrinthula* compound and possibly be useful when investigating potential strategies to mitigate seagrass wasting disease.

The relative abundance of the two most bioactive genera (*Diaporthe* and *Trichoderma*) was relatively low in the field samples (less than 1% in both epiphytic and endophytic samples), with the exception of *Trichoderma* in six samples, where it reached up to 17% (Supplementary Table 7). Furthermore, an exact 100% ID match to the ITS sequence of our strains, M14 and P1a, was not found in our amplicons. Although the bioactive strains were not represented in the ITS amplicon datasets, we cannot say that these bioactive strains are not at all present in the field, but rather we can conclude that these two strains are either rare (relative abundance <0.01%) or moderately rare (relative abundance between 0.01% and 1%; Liang et al., 2020). Moreover, fungi can have varying lengths of ITS regions in their DNA and amplicon sequencing of these regions may be biased against longer sequences (Weig et al., 2013; Bakker, 2018). Nevertheless, low abundance microbes have been recognized as important drivers of microbiome microbial community compositions in several organisms (Benjamino et al., 2018; Compant et al., 2019), and despite the potential rarity of the two most active strains against *Labyrinthula* growth, we cannot disregard their possible importance in the endospheres of *T. testudinum*.

Although we determined that certain fungi have the potential to deter *Labyrinthula* spp. *in vitro*, it is also important to study the potential of these fungi and their secondary metabolites *in planta*. Future studies should also consider the impact of abiotic stressors, such as temperature, light, salinity, and nutrient availability, especially because *Labyrinthula* spp. are opportunistic pathogens that cause seagrass wasting disease when seagrasses are under stress (Jakobsson-Thor et al., 2020).

Conclusion

This is the first study to investigate the relationship between fungal endophytes of *T. testudinum* and *Labyrinthula* spp. Through amplicon sequencing, we identified several strains that are known to produce antipathogenic compounds that could potentially aid in the defense against seagrass wasting disease. Through culturing and metabolite extractions, we further identified two fungal strains that had strong activity and 20 strains that had moderate activity against our lab-isolated strain of *Labyrinthula*. Furthermore, we also determined that four of the top 20 abundant genera showed activity against *Labyrinthula* in the disk diffusion assays. Although we only tested 50 strains of endophytes, it is important to consider the many fungi that might be present, but were not cultured, might be involved in disease defense against *Labyrinthula*.

Data availability statement

The datasets presented in the study were deposited in GenBank, accession numbers OR355682–OR355766, OR356117, and the NCBI Sequence Read Archive, accession number PRJNA 1001931.

Author contributions

KU: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. AJ: Formal analysis, Methodology, Visualization, Writing – review & editing. CC: Data curation, Formal analysis, Methodology, Software, Supervision, Writing – review & editing. SL: Writing – review & editing, Conceptualization, Project administration, Supervision. US: Conceptualization, Project administration, Supervision, Writing – review & editing, Methodology, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2024.1359610/full#supplementary-material>

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