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Influence of seagrass traits on the diversity of endophytic fungi

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Abstract. *Kinamot VB. 2024. Influence of seagrass traits on the diversity of endophytic fungi. Biodiversitas 25: 1254-1263.* The interest in endophytic fungi has grown recently because of their potential to enhance plant growth, tolerate stresses, and modulate phytohormone synthesis. Understanding how plants influence their endophytic fungal assemblage is vital to manipulating them for biotechnological applications. In this study, Bray-Curtis analysis determined the variation of endophytic fungal assemblage among different seagrass species. Leaf/root area, leaf/root dry matter content, leaf mass per area, leaf toughness, and specific leaf area of seagrasses were measured using a standard protocol. The Folin-Ciocalteau method was utilized to quantify the phenolic and tannin contents in seagrasses using tannic acid as standard. Canonical correspondence analysis correlated the influence of seagrass traits on endophytic fungal diversity. Results showed that endophytic fungal diversity varied with seagrass hosts in which higher similarity of the fungal assemblage was observed among the same seagrass species than in different seagrass species. The seagrass traits mediated the variation of endophytic fungi. Phenolic and tannin in seagrasses significantly influenced the diversity of endophytic fungi. An antifungal assay using the methanolic crude extract of seagrasses had 80-100% mycelial growth inhibition to the five species of endophytic fungi tested, which could be accounted for the chemicals like phenol and tannin that the seagrass hosts produce.

Keywords: Diversity, fungi, phenolic, Philippines, seagrass

INTRODUCTION

Endophytic fungi have been isolated from diverse hosts ranging from lichens (Santiago et al. 2021), bryophytes (Zhou et al. 2015), gymnosperms (De Mesa et al. 2020), and angiosperms (Gautam et al. 2013; Gautam 2014; General and Guerrero 2017). In the marine environment, endophytic fungi were found in every marine plant, e.g., mangroves (Apurillo et al. 2019), algae (Sahoo et al. 2021), and seagrasses (Supaphon et al. 2017; Ettinger and Eisen 2020). These endophytic fungi protect the host plants from abiotic and biotic stresses like pests and pathogens. They also promote plant growth through phosphate solubilization, nitrogen fixation, and phytohormone synthesis. Furthermore, the close relationship of endophytes with their hosts resulted in the production of bioactive metabolites and novel natural products (Singh and Kumar 2023). Despite the immense biological importance of endophytic fungi, little is known about their ecology, particularly the host-fungal interaction in seagrasses.

Plant traits and identity were reported to influence the endophytic fungal diversity in terrestrial environments (Kembel and Mueller 2014). Oftentimes, different plant species harbor different fungal assemblages because of the variation of their traits. For instance, in *Ficus*, leaf traits such as specific leaf area, leaf N content, leaf pH, and toughness explained 32.9% of the total variation in the foliar endophytic fungi based on the canonical correspondence analysis (Liu et al. 2019). In bromeliads, C3 and CAM plants have dissimilar endophyte assemblages because of the difference in leaf traits like toughness and water content (Tellez et al. 2020). Likewise, the foliar fungal symbiont of the grasses in the Colorado Rocky Mountains varied among plant genera, with *Elymus* having the highest fungal diversity, while *Festuca* and *Loa* had the lowest fungal diversity. This is accounted for by the variation in leaf length among the host plants (Kivlin et al. 2019). Leaf thickness, tissue density, and plant defensive compounds were also reported to restrict endophyte fungal colonization in plants. Endophytic fungi can easily penetrate the plants with thinner and less denser leaves (Van Bael et al. 2017). Variations in the cell wall, flavonoids, anthocyanins, and terpenoids in ten dominant tree species in Southern Chile were associated with the differences in horizontally transmitted endophyte communities (Gonzalez-Teuber et al. 2020). Furthermore, different compounds released by the different host species into the surrounding soil favor or restrict the colonization and uptake of endophytic fungi, resulting in variation in the endophytic fungal communities between hosts (Chen et al. 2020).

In the aquatic environment, host species could also play a major role in shaping the endophytic fungal communities. For instance, most OTUs of fungal endophytes were unique in aquatic plants, *Batrachium bungei*, *Myriophyllum spicatum*, and *Hippuris vulgaris* from southwest China, probably due to the intraspecific variation in host traits (Zheng et al. 2021). However, further studies are required to validate this assumption.

In seagrass, different seagrass species were dominated by other endophytic fungal communities. For instance,

Sordariomycete dominated the endophytic community in *Enhalus acoroides*, *Halophila ovalis*, and *Thalassia hemprichii* in Thailand (Supaphon et al. 2017), while Eurotiomycete dominated in *Zostera marina* in California (Ettinger and Eisen 2020). Dothideomycete was dominant in *Posidonia oceanica* in the Mediterranean (Vohnik et al. 2019). *Penicillium* was the most abundant in *Cymodocea serrulata* in India, while *Aspergillus terreus* was the most abundant in *Halodule beaudettei* and *Thalassia* sp. (Venkatachalam et al. 2015). Raja et al. (2016b) revealed that fungal distribution in seagrasses is unique and that most seagrass species harbor different endophytic fungal populations. Despite this, studies seldom reported the link between endophytic fungal assemblage and seagrass traits. Raja et al. (2016a) assumed that wider leaf area influenced a higher colonization rate of endophytic assemblages in *C. serrulata* than in *Halodule pinifolia* and *Halophila ovalis*. However, the relationship between leaf area and fungal diversity must be investigated. Moreover, defensive chemicals in seagrasses correlated with a low diversity of endophytic fungi in seagrasses (Supaphon et al. 2014; Hurtado-McCormick et al. 2019). Yet, the effect of the chemical compounds from seagrasses on the growth of endophytic fungi was not tested.

So far, studies related to the ecology of endophytic fungi in seagrasses were on the influence of dispersal (Wainwright et al. 2018) and habitat type, such as substrate chemistry, on fungal community composition (Wainwright et al. 2019). However, these studies were only conducted in Indonesia, Singapore, and Malaysia, but none were shown in the Philippines despite the country being one of the centers of marine biodiversity. Thus, this study aimed to determine the diversity of endophytic fungi in *E. acoroides*, *C. serrulata*, and *T. hemprichii* from the coasts of Hilutungan Channel, Cebu, Philippines, and identify the influence of seagrass traits on endophytic fungal diversity.

MATERIALS AND METHODS

Isolation and identification of endophytic fungi associated with the seagrasses

The endophytic fungi were isolated from *E. acoroides*, *C. serrulata*, and *Thalassia hemprichii* from the coast of Hilutungan Channel, Cebu, Philippines (10°16'20" N, 124°0'50" E). For sterilization, each seagrass sample was immersed in 10% ethanol (EtOH) for 3 min, 3% sodium hypochlorite (NaClO) for 10 s; 10% EtOH for 3 min, and finally washed twice with sterile distilled water and blotted dry with sterile tissue paper (Supaphon et al. 2014). Sterilized explants from the seagrass were then inoculated in culture plates with cornmeal agar/CMA (cornmeal 50 g, agar 15 g, pH 6.0 ±0.2), malt extract agar/MEA (malt extract 30g, agar 15g, mycological peptone 5 g, pH 5.4 ±0.2 at 25°C) and potato dextrose agar/PDA (potato peptone 200 g, glucose 20 g, agar 15 g, pH 5.6 ±0.2) and incubated at 25±2°C for at least 14 days. Fungal colonies were then subcultured thrice in PDA and purified by the hyphal tip method and single spore sterilization method.

Morphological and molecular methods identified the species of the pure fungal culture. Morphological identification was done based on the colony color, form, texture, elevation, and margin, hyphal septation, conidia, and conidiophore. Scientific articles were used as references in the morphological identification (Houbraken et al. 2010; Chen et al. 2013; Samson et al. 2014; Visagie et al. 2014; Nyongesa et al. 2015; Campos et al. 2019; Dhar et al. 2019; Bich et al. 2021; Yee et al. 2022). For molecular identification, the mycelia of a 1-week pure culture was transferred to a 1.5 µL microcentrifuge with nuclease-free water and sent to Macrogen (Korea) for DNA extraction, amplification, and sequencing. The genomic DNA of each endophytic fungal isolate was extracted using InstaGene Matrix (Bio-Rad). The DNA fragments were amplified in DNA Engine Tetrad 2 Peltier Thermal Cycler using Internal Transcribed Region (ITS) and 18s rDNA. ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') was used as forward primer and ITS4 (5'- TCCTCCGCTT ATTGATATGC-3') as a reverse primer (White et al. 1990). NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (TCCGCAGGTTCACCTACGGA) were used as forward and reverse primers for 18s rDNA. The PCR cycle started with an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 7 min. PCR products were purified using a multiscreen filter plate and sequenced using BigDye(R) Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3730XL Analyzer following the manufacturer's protocol. Sequences were edited using BioEdit Sequence Alignment Editor 7.2 (Hall 1999) and the contig assembly of sequences was done using the cap contig assembly program. The sequences were then analyzed for the most probable closely related taxa using BLAST search. (https://blast.ncbi.nlm.nih.gov). Taxa with a 99-100% match identity were considered the closest matched taxa of our isolates.

Diversity assessment of endophytic fungi in seagrasses

The diversity of endophytic fungi in seagrasses was assessed by both alpha and beta diversity indices, For alpha diversity, species richness and evenness were computed. Species richness was determined using Chao 1 with the formula: $S_1=S_{obs} + F_1^2/ 2F_2$, where, S_{obs} is the number of species in the sample, F_1 is the number of species that occurred once in the sample, F_2 is the number of species that occurred twice in the sample. Simpson's diversity index (formula: $D_1=1- \sum P^2i$), and Shannon's diversity index (formula: $H'= -\sum P_i \ln(P_i)$ measured both richness and evenness. P_i is the proportion of the individuals belonging to the species (i) and P_{max} is the proportion of the individuals belonging to the most abundant species (i). Dominance was determined by Simpson's dominance (formula: $D_2=1/\sum P^2i$) and Berger Parker (formula BP= P*max)*. Evenness and equitability 'J values determined the evenness of endophytic fungi in the seagrass hosts and structures. The formula for equitability is $E = H'/H_{max}$, where H' is the Shannon-Wiener index and H_{max} is the ln of P_i. Evenness was calculated as $E=D_2/S$, where S is

richness. The diversity analyses were performed using the software Past version 4.03.

Beta diversity of variability in the endophytic fungal diversity between seagrass species was determined by Bray-Curtis Similarity. The formula was as follows:

$$S_{jk} = 100\left\{1 - \frac{\sum_{i=1}^{p}|y_{ij} - y_{ik}|}{\sum_{i=1}^{p}(y_{ij} + y_{ik})}\right\}$$

Where:

y_{ij} represents the entry in the ith row and jth column of the data matrix, while y_{ik} is the count for the ith species in the kth sample.

Non-metric multidimensional scaling (NMDS) was used to visualize the ordination plot of the endophytic fungal community based on the Bray-Curtis Similarity Index. The analysis was done using Primer v6.

Factors that influence the diversity of endophytic fungi in seagrasses

To determine the factors that influence the endophytic fungal community in seagrasses, ten traits of seagrasses were measured: particularly leaf area (cm^2), dry mass (g), specific leaf area (cm^2/g dry mass), leaf mass per area (g dry mass/cm^2), leaf and root dry matter (mg/g), and root area (mm^2). Defense and resistance strategies against fungal colonization, such as leaf toughness (g/mm), total phenolics, and tannin content (mg TAE/ g), were determined. A total of 40 individuals per seagrass species were examined in this study.

Leaf area was calculated using the leaf weighting method: leaf area = x/y, where x is the weight (g) of the whole leaf and y is the weight of 1 cm^2 area of the leaf (Mahmoud and Osman 2023). The dry matter content was calculated as dry mass divided by the sample's fresh mass (mg g^{-1}) (Jovanovic et al. 2023). To estimate the dry mass of the sample, seagrass leaves and roots were placed in a sealed plastic bag with deionized water and kept in the dark (4°C) for 24 h. After this, the samples were blotted dry with paper, weighed, and dried in an oven at 60°C until constant weight. The dry mass (g) of the leaves and roots was equal to the weight of the oven-dried (leaf/root material. Specific leaf area (cm^2 g^{-1}) was the ratio of leaf area to leaf dry mass (Liu et al. 2019). The reciprocal of this was the leaf mass per area (g cm^{-2}). The root surface area (mm^2) is proportional to resource acquisition and microbial habitability. It was calculated as root length multiplied by the root diameter and π. The diameter and length of the roots were measured using the *ImageJ* software.

The leaf toughness correlated with the mechanical barrier of seagrasses from microbial colonization. In this study, leaf toughness was measured indirectly by the force required to tear apart a leaf sample as adopted by Kiffer et al. (2018). It was done by securing 8-12 mm diameter leaf discs in between two pegs in the tearing device. Gradually, sand was added to the cup in the tearing device until the leaf discs broke into two. Leaf toughness (g mm^{-1}) was equal to the weight of the cup required to break the leaf discs.

The concentration of phenolics and tannin compounds in seagrasses was measured by the Folin-Ciocalteau method using tannic acid as standard based on the protocol adopted by Balogun et al. (2014) with modification. The crude extract was prepared by adding 50 mL of 80% methanol to 500 mg powdered sample and heated on a hot plate for 30 min. To quantify the total phenolic compound concentration, 500 µL of the extract was mixed with 500 µL of 10% Folin-Ciocalteau reagent, 1 mL of 7.5% sodium carbonate solution, and 8 mL distilled water in a test tube. The mixture was allowed to remain at room temperature for 30 min for color development. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Dynamica, HALO DB-20R). To quantify the tannin content, 500 mL of the extract was precipitated by 500 mg casein and 5 mL distilled water and absorbance was measured at 760 nm. The difference between the absorbance of the extract with and without casein was equal to the tannin concentration. Tannic acid was used as standard. This assay was performed in triplicate. The phenolic and tannin contents were expressed as milligrams of tannic acid equivalents per gram of extract (mg TAE g^{-1}). To investigate the effect of the total phenolic on the growth of the endophytic fungi, a mycelial growth inhibition assay using the methanolic crude extract of the seagrasses was conducted.

Mycelial growth inhibition assay of the seagrass crude extract

The crude extract from seagrasses was assayed for its inhibition of the growth of endophytic fungi. The organisms used in the assay were *Xylaria* sp., *Aspergillus ochraceopetaliformis*, *Aspergillus tamarii*, *Beauveria bassiana*, and *Penicillium citrinum*. These five species of endophytic fungi were isolated from the three seagrass hosts tested in this study.

The crude extract was prepared from approximately one kilogram of fresh seagrass samples. After collection, the samples were washed thoroughly with seawater and distilled water to remove the epiphytes and other debris. The samples were then air-dried under the shade for two weeks and oven-dried at 60°C until constant weight. One hundred milligrams of dried samples yielded from 1kg of fresh seagrass were powdered using a blender. Crude extracts were extracted from the powdered seagrass with methanol (10 g: 100 mL methanol) using a Soxhlet extraction apparatus (Pyrix). The extract was then filtered thrice with Whatman No.1 filter paper. The methanol solvent in the extract was evaporated using a rotary evaporator (Heidolph) at 50°C. A stock solution was prepared by dissolving the dried extract in methanol at a concentration of 1 g mL^{-1}.

The treatment extract was prepared from a 2 mL aliquot of the stock solution added with methanol five percent until the final concentration. This extract was then mixed with warm PDA media (50°C) at 0.5:1v/v. Three millimeters of mycelium were transferred from a working culture to extract-treated media using sterile fine-point tweezers and incubated at 27°C for 36-72 h. Five percent methanol was used as a control. It was prepared by adding the same

amount of methanol to prepare the treatment extract. This assay was conducted with three replicates. The effect of seagrass crude extract was determined by the percentage of mycelial growth inhibition according to the formula: % inhibition= (C -T)/C x100, where C is the average mycelial growth of the control, T is the average mycelial growth of the treatment (Hernandez-Ceja et al. 2021).

Statistical analysis

One-way ANOVA determined the significant difference in each functional trait between seagrass species. Canonical Correspondence Analysis (CCA) was used to correlate the host traits with the abundance of endophytic fungi. The significant effect of each trait was determined using a Generalized Linear Model (GLM). The traits that showed significant effects were plotted using a linear regression model. These analyses were done in R software. The relationship between the tannin content, total phenolic, and growth inhibition of the seagrass crude extract on endophytic fungi was determined by Pearson Moment Correlation.

RESULTS AND DISCUSSION

This study isolated and identified eight species of endophytic fungi based on morphological and molecular methods (Kinamot and Monotilla 2023). These were *A. tamarii*, *A. ochraceopetaliformis*, *A. sydowii*, *A. terreus*, *P. citrinum*, *B. bassiana*, *Eutypella* sp. and *Xylaria* sp. (Table 1). *A. tamarii* had an olive-green to brown colony in PDA and CMA media for 7 days of incubation at 25±2°C. The colony was circular, powdery, and slightly raised. This species was highly sporulating in culture. The conidiophore was biseriate and the conidia were green to brownish green in color, sub-globose to globose with a spinous surface. *A. ochraceopetaliformis* had a light brown colony in PDA media for 7 days of incubation at 25±2°C, cottony and circular form. The hyphae were septate, the conidiophore was biseriate and the conidia were globose and smooth. *Aspergillus sydowii* had a circular white colony obverse and light brown on the reverse. It had septate vegetative hyphae. It did not sporulate in culture. *Aspergillus terreus* had a brown colony with a light brown margin which became darker when matured. Its form is filamentous with a plumose margin and slightly raised elevation. *P. citrinum* had a green colony in PDA and MEA media after 7 days of incubation at 25±2°C with dispersed growth (Figure 1). It was highly sporulating with a biverticillate conidiophore with equal-length rami. The conidia were globose and smooth. *B. bassiana* had white to cream colonies with irregular edges and a powdery appearance. The conidiogenous cells had a zigzag rachis in which a chain of conidia emerged. The conidia were globose. *Eutypella* sp. had a white colony on the surface, cream on the reverse side, with a flat, velvety, and circular colony. Lastly, *Xylaria* sp. had a white colony with conspicuous radial lines that bifurcated at the edge. It had septate hyphae and the conidium was ovate. Sporulation was very low which was observed after 28 days of culture in malt extract agar.

The BLASTn analysis using ITS and 18s rDNA gene sequences showed that the endophytic fungi associated with seagrasses had >99-100% nucleotide similarity to the closest match taxa. *A. tamarii* had 99.83% nucleotide similarity with its closest matched taxa while *A. ochraceopetaliformis* had 99.67% nucleotide similarity. Aspergillus sydowii and A. terreus had 100 and 99.93% nucleotide similarity, respectively. The nucleotide similarity of *P. citrinum*, *B. bassiana*, *Eutypella* sp. and *Xylaria* sp. with their closest matched taxa was 99.64, 100, 99.84 and 99.48 %, respectively.

The alpha diversity endophytic fungi from *E. acoroides*, *T. hemprichii*, and *C. serrulata* based on Shannon-Weiner (H') and Simpson diversity indexes (D_1) were H'=1.81; D_1= 0.82, H'=1.79; D1= 0.81, H'=1.74; D_1= 0.82, respectively. This showed that *E. acoroides* and *T. hemprichii* were slightly more diverse than *C. serrulata*. Regarding species richness, Chao-1 showed that *E. acoroides* and *T. hemprichii* had more endophytic fungi species than *C. serrulata*. Values of evenness and equitability in *E. acoroides* and *T. hemprichii* were smaller at J'= 0.93; ED_1= 0.87, J'= 0.92; ED_1= 0.85, respectively compared to *C. serrulata* at J'= 0.97; ED_1= 0.94 (Table 2). This suggested that the endophytic fungi of *E. acoroides* and *T. hemprichii* were more varied relative to abundance than in *C. serrulata*. The lowest diversity of endophytic fungi *C. serrulata* was consistent with a previous report in the seagrasses of Thailand showing that *C. serrulata* had the lowest phylogenetic diversity of endophytic fungi compared with *E. acoroides*, *T. hemprichii*, and *H. ovalis*. On the other hand, the phylogenetic diversity of endophytic fungi in *E. acoroides* and *T. hemprichii* was comparable (Supaphon et al. 2017).

In terms of beta-diversity of endophytic fungi, non-multidimensional plot of the Bray-Curtis similarity index showed that fungal assemblage from the same seagrass species had 80% similarity. Meanwhile, endophytic fungi from different seagrass species were only 40% similar (Figure 1). This implied that the diversity of endophytic fungi was more varied in different seagrass species. This result is parallel with the fungal communities reported from different seagrass species in Thailand (Supaphon et al. 2017) and India (Raja et al. 2016a). For instance, *E. acoroides* and *C. serrulata* from Thailand were dominated by Sordariomycetes at 80% while Dothideomycete and Sordariomycete were equally dominant in *T. hemprichii* (Supaphon et al. 2017). On the other hand, Eurotiomycetes dominated the seagrasses of India including *E. acoroides* and *C. serrulata* (Raja et al. 2016a). Variations of endophytic fungal assemblage among host species could be due to variations in the host plant's traits (Liu et al. 2019; Yao et al. 2019; Tellez et al. 2020).

Table 1. Identification of endophytic fungi based on morphological and molecular methods

Species	Macroscopic (Colony) Features								Molecular Identification	
	Color		Form	Margin	Texture	Elevation	Conidia	Conidiophore	Match identity (%) using Blast Search	GenBank Acc ID of closest matched taxa
	Obverse	Reverse								
Aspergillus tamarii	Olive green to brown	White to light yellow	Circular	Entire, white margin	Powdery	Slightly raised	Sub-globose to globose with spinous surface	Biseriate	99.83	MK638758
Aspergillus ochraceopetaliformis	White to light brown	Light brown	Circular	Entire	Powdery to cottony	Flat to slightly raised	Globose with smooth surface	Biseriate	99.67	MH857406
Aspergillus sydowii	White	Light brown	Circular	Entire	Powdery	Flat	Not observed	Not observed	100	MH233983
Aspergillus terreus	Brown	Brown	Circular	Filamentous	Plumose	Slightly raised	Not observed	Not observed	99.93	MN995485
Penicillium citrinum	Ash green	White to orange	Circular with dispersed growth	Entire, white margin	Powdery	Flat	Globose	Biverticillate	99.64	MH858073
Beauveria bassiana	White	White	Circular with dispersed growth	Irregular	Powdery	Slightly raised	Globose	Dense cluster of conidia with zigzag rachis	100	MT530083
Eutypella sp.	White	Crean	Circular	Entire	Velvety	Flat	Not observed	Not observed	99.84	MK775825
Xylaria sp.	White with radial lines	White	Circular with conspicuous radial lines	Filiform	Plumose	Raised, slightly raised and flat	Ovate	Not observed	99.48	DQ480355

In this study, ten traits of *E. acoroides*, *C. serrulata*, and *T. hemprichii* were measured to investigate their effects on the diversity of endophytic fungi. Table 3 shows the measurement of each trait from the three seagrass species. Among the three hosts, *E.acoroides* had the highest leaf and root area ranging from 41.61-44.09 cm^2; and 156.73-193.93 mm^2, respectively. *T. hemprichii* had a leaf area of 16.73-18.41 cm^2 and a root area of 45.57-57.43 mm^2. The leaf and root area of *C. serrulata* was the smallest among the three hosts. The leaf dry mass was also highest in *E. acoroides* while the leaf dry mass of *T. hemprichii* and *C. serrulata* was comparably similar. On the other hand, the leaf mass per area of *E. acoroides* and *C. serrulata* were relatively similar among the two seagrass hosts while *T. hemprichii* had the lowest at 0.009-0.01 g cm^{-2}. Regarding leaf toughness, *E. acoroides* had the toughest leaf blades among the three hosts. The leaf dry matter content of the three hosts was relatively the same. Whereas the root dry matter content was highest in *T. hemprichii*. *T. hemprichii* had also the highest specific leaf area. In terms of the concentration of chemical compounds, *C. serrulata* had the highest total phenolics and tannin contents ranging from 132.55-173.42 mg TAE g^{-1} and 142.52 155.7 mg TAE g^{-1}, respectively. The total phenolics and tannin in *E. acoroides* and *T. hemprichii* were only at 12.61-15.08 mg TAE g^{-1}; and 7.30-7.33 mg TAE g^{-1}, respectively.

Canonical Correspondence Analysis (CCA) showed that seagrass traits mediated the variation of endophytic fungi. CCA 1 and CCA 2 together accounted for 63.5% (37.3% CCA1; 26.2% CCA 2) of the cumulative diversity of the endophytic fungi. In CCA1, three traits were related to the variation of endophytic fungi: specific leaf area (eigenvalue = 0.32), leaf mass per area (eigenvalue = -0.19), and leaf dry matter content (eigenvalue = -0.26). In CCA 2, 6 traits were associated with the variation of endophytic fungi: total phenolic (eigenvalue = 0.17), root dry matter content (eigenvalue = 0.08), root area (eigenvalue = 0.25), leaf area eigenvalue = (0.12), leaf dry mass (eigenvalue = 0.12) and leaf toughness (eigenvalue = 0.06) (Figure 2).

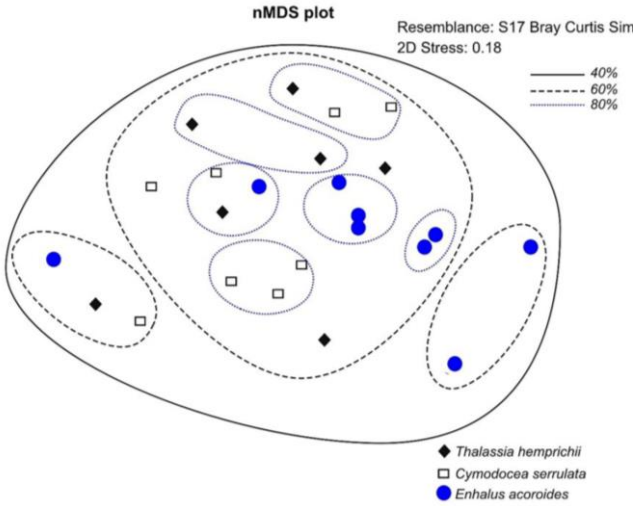

Figure 1. Non-multidimensional scaling (NMDS) shows endophytic fungi's beta diversity between the three seagrass species based on Bray-Curtis Similarity

Table 2. Alpha diversity of endophytic fungi using different diversity indices

Diversity Index	*Enhalus acoroides*	*Thalassia hemprichii*	*Cymodocea serrulata*
Taxa_S	7	7	6
Abundance (# Isolates)	105	89	72
Chao-1	7	7	6
Simpson's Index of Evenness (E_{D1})	0.8697	0.8521	0.9457
Shannon's Index of Evenness (J')	0.9282	0.9178	0.9689
Simpson's Diversity Index (D_1)	0.8194	0.8143	0.8167
Shannon Weiner's Diversity Index (H')	1.806	1.786	1.736
Simpson's Dominance (D2)	0.1806	0.1857	0.1833
Berger-Parker (BP)	0.2667	0.2472	0.2361

Table 3. Measurement of each seagrass trait in *Enhalus acoroides*, *Cymodocea serrulata* and *Thalassia hemprichii*

Seagrass traits	*Enhalus acoroides*	*Thalassia hemprichii*	*Cymodocea serrulata*	p-value
Leaf area (cm^2)	41.61-44.09±5.62	16.73-18.41±1.05	6.67—7.20±0.76	1E-05
Root area (mm^2)	156.73-193.93±36.74	45.57-57.43±12.53	66.21-87.2±24.2	1E-05
Leaf dry mass (g)	1.55-2.06±0.28	0.15-0.16±0.02	0.14-0.17±0.02	1E-05
Leaf mass per area (g cm^{-2})	0.04-0.05±0.00	0.009-0.01±0.00	0.02-0.03±0.00	1E-05
Leaf Toughness	1185.4-1271.46±84.14	408.35-432.1 ±31.12	143.6-145-96±2.11	1E-05
Specific leaf area (cm^2 g^{-1})	22.68-23.97±4.42	116.00-123.15±17.89	42.85-44.63±9.79	1E-05
Leaf dry matter content (mg g^{-1})	0.16-0.19±0.02	0.15-0.18±0.00	0.15-0.16±0.03	0.031
Root dry matter content (mg g^{-1})	0.10-0.20±0.06	0.29-0.32±0.00	0.05-0.09±0.07	1E-05
Total phenolic (mg TAE g^{-1})	15.08-17.22±0.21	12.61-16.55±3.32	132.55-173.42±6.17	1E-05
Total tannin (mg TAE g^{-1})	7.33-9.43±0.50	7.30-14.93±0.48	142.52-155.7±1.55	1E-05

Note: Range values are shown; ± is the standard deviation

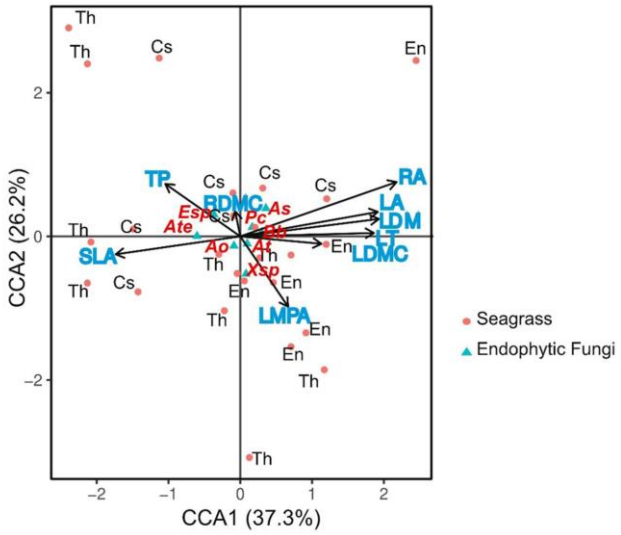

Figure 2. Canonical correspondence analysis (CCA) ordination shows that host traits correlate with the diversity of endophytic fungi. LA= leaf area, LDM- leaf dry mass, LT, leaf toughness, LMPA= leaf mass per area, LDMC= leaf dry matter content, SLA= specific leaf area, RDMC= root dry matter content, TP= total phenolic

The leaf and root areas reflect the habitability of endophytic fungi; thus, plants with large surface areas may positively correlate to high fungal diversity. In terrestrial plants, specific leaf area was one of the traits that significantly explained the variation of the foliar endophytic fungi of *Ficus* (Liu et al. 2019). In seagrasses, longer and wider *C. serrulata* leaves than *H. pinifolia* were correlated to dense endophytic hyphal assemblage in the former (Raja et al. 2016a). The findings of the current study supported the previous reports. *E. acoroides*, with the highest leaf and root area, also had the highest endophytic fungal diversity.

Based on the leaf economic spectrum, leaves with high leaf mass per area had high tissue thickness, density, and investment to defenses like leaf toughness and dry matter content. Thus, plants with high leaf mass per area have low endophytic fungal diversity because they have dense tissues that restrict the entry and proliferation of endophytes in the mesophyll layer (Van Bael et al. 2017). In woody plants of Panama, foliar endophyte abundance, diversity, and richness negatively correlated leaf toughness, leaf mass per area, and leaf dry matter content because these traits serve as a mechanical barrier that limits fungal penetration (Tellez et al. 2020). However, the current study showed that *E. acoroides* had the highest leaf toughness and, at the same time, the highest endophytic fungal diversity, suggesting that leaf toughness did not restrict the colonization of endophytic fungi inside the seagrass tissues. The finding agrees with the horizontally transmitted endophytes in the temperate rainforest of Chile, in which leaf toughness had no association with their colonization (Gonzalez-Teuber et al. 2020).

The ability of the endophytic fungi isolated in this study to synthesize the cell wall-degrading enzymes may allow them to colonize the seagrass tissues amidst the resistance mechanisms. Eight species of endophytic fungi were identified from the three seagrass hosts: *A. tamarii*, *A. ochraceopetaliformis*, *A. sydowii*, *A. terreus*, *B. bassiana*, *Eutypella* sp., *P. citrinum* and *Xylaria* sp. Though not tested in the present study, previous reports revealed enzyme production of these endophytes. For instance, *Aspergillus terreus* has multi-cellulase genes that encode for several lignocellulosic enzymes like glucanases, hydrolases, glucosidase, and xylanase (Kumar and Parikh 2015). *P. citrinum* is also reported to produce cellulose-degrading enzymes like β-glucosidases that can hydrolyze β-1-4 linkages of cellulose (da Costa et al. 2018). *Xylaria* also has high cellulase and xylanase activity (Carrion-Paladines et al. 2019). *B. bassiana* has been recorded to produce amylase, beta-glucosidase, endoglucanase, and xylanase (Amobonye et al. 2021). Hence, the production of these enzymes from our endophytic fungi could be a significant factor for successful colonization in seagrass tissues.

Specific leaf area and root dry matter content are considered superior predictors of net primary productivity. In the leaves, dry matter content measures the mass investment of photosynthesis (Smart et al. 2017). Since endophytes derive their nutrition from their host, the primary productivity of the hosts may also influence the endophytes. Santiago et al. (2021) previously reported that endophytic fungi associated with lichen may depend on their lichen photobiont for nutrition. In this context, endophytic fungi may also derive their nutrition from seagrass hosts. *T. hemprichii* has the highest amount of food for the endophytic fungi relative to its high net productivity based on the specific leaf area and dry matter content. However, further investigation has to be conducted.

Seagrasses were known to produce diverse chemicals which serve as chemical defenses against the microorganisms that will colonize their tissues. For instance, the ethanolic extract of *E. acoroides* inhibited *Candida albicans* up to 73.89mg/L. *T. hemprichii* inhibited *Fusarium acuminatum Aspergillus niger A. terreus*, *A. fumigatus* and *Penicillium expansum* (Gono et al. 2022). Among the secondary metabolites that showed potent antimicrobial activity in seagrasses are phenolic compounds (Zidorn 2016). *T. hemprichii* from the Red Sea of Arabia produced thalassiolin, a sulfated flavone that reportedly has potent antimicrobial activity (Hawas 2014). *C. serrulata* and *E. acoroides* had tannins (Baby et al. 2017). These compounds showed growth inhibition in various fungi like *Cryptococcus neoformans* (Oliveira et al. 2020), *Candida albicans* (Carvalho et al. 2018), and *Penicillium digitatun* (Zhu et al. 2019). In this study, *C. serrulata* had the highest concentration of phenolic and tannin compounds. Thus, the lowest fungal diversity in *C. serrulata* could be attributed to its very high phenolic and tannin contents. Based on the generalized linear model, the total phenolic content significantly influenced the endophytic fungal diversity. The result of the linear

regression model showed that phenolic content had a significantly negative relationship with the diversity of endophytic fungi. Thus, when the concentration of total phenolic is high, endophytic fungal diversity is low, while if the concentration of total phenolic is low, endophytic fungal diversity is high (Figure 3). This implies that the total phenolic content had an antifungal effect on endophytic fungi in seagrasses. So, the intraspecific variation of phenolics and tannin content among the seagrass hosts corresponds to the variation of endophytic fungal diversity.

The antifungal effect of phenolic and tannin on the endophytic fungi in seagrasses was supported by the result of the mycelial growth inhibition assay. Findings of this assay showed that the methanolic extract of the three seagrass species had 80-100% mycelial growth inhibition in *Xylaria sp., B. bassiana, A. tamarii, A. ochraceopetaliformis* and *P. citrinum. C. serrulata* had the highest inhibition rate across the five species of endophytic fungi tested (Figure 4). The correlation analysis between mycelial growth and total phenolic was r= -0.79 (p=0.001), suggesting a strong negative correlation. The tannin content of the extract, which was determined from the total phenolic content after removing the phenolic wastes, was also negatively correlated with mycelial growth; r=-0.80 (p=0.00).

Phenolic compounds inhibit mycelial growth by disrupting the enzymatic processes for energy production, damaging the cell membrane permeability and affecting nucleic acid synthesis. In *P. digitatum*, there was a decrease in protoplasm production when exposed to the tannic acid cell wall and membrane disruption of the fungi (Zhu et al. 2019). It was also reported that tannin and gallic acid synergistically bind to ergosterol in the fungal membrane, resulting in pore formation, macromolecule content reduction, and enzyme inhibition (Carvalho et al. 2018). These mechanisms may be the reason for the low endophytic fungal diversity in seagrasses. Further investigation is needed to validate this assumption.

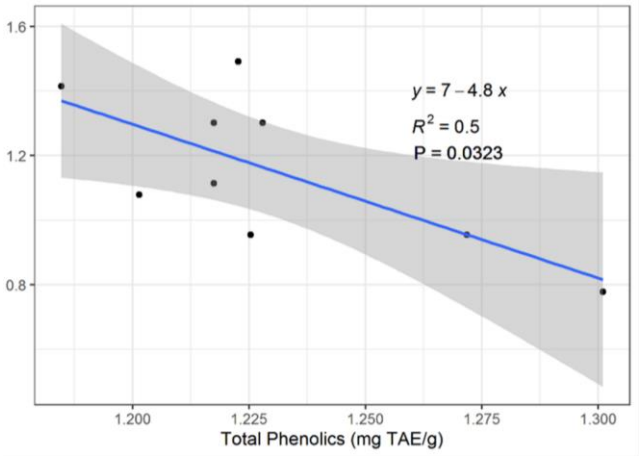

Figure 3. The scatterplot shows a significant relationship between the total phenolic content and endophytic fungi diversity based on the linear regression model

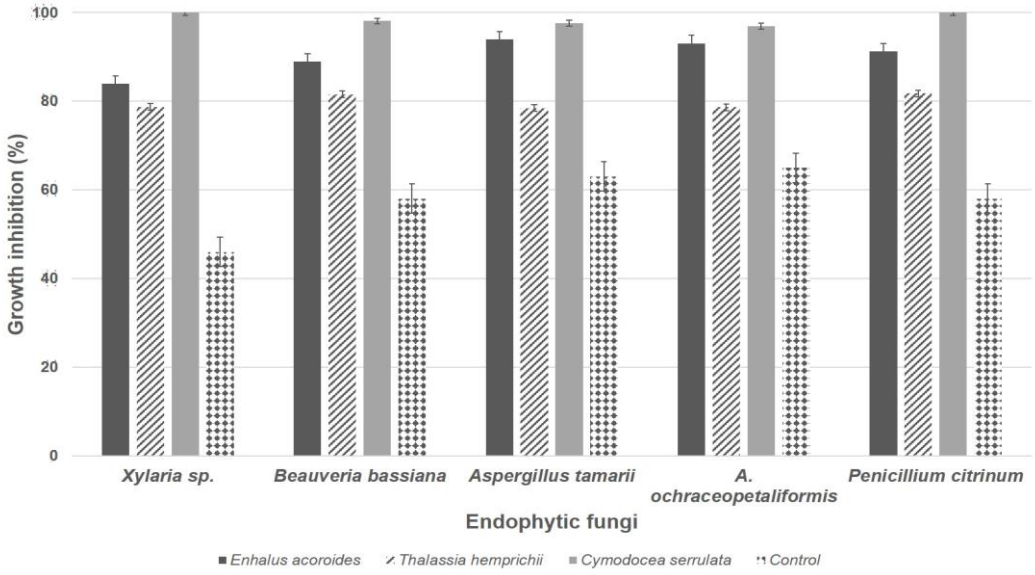

Figure 4. Mycelial growth inhibition of the seagrass crude extract on five species of endophytic fungi. Data represent the mean, error bars = standard error of the mean (SEM)

The environmental conditions in our sampling sites, like the input of domestic and industrial wastes to the sea, exposed the seagrasses to stress are possible factors for the increased production of phenolic compounds in seagrasses. For instance, nutrient enrichment in the water from anthropogenic sources encouraged macroalgal overgrowth, resulting in competition for light intensity with seagrasses and triggering the production of phenolic compounds. In Malaysia, *E. acoroides, T. hemprichii, Halophila ovalis, H. major*, and *H. spinulosa* possessed higher total phenolic and flavonoid contents in the *Ulva reticulata*-colonized sites than those of the non-colonized sites. Furthermore, sea walls constructed on the coasts of Mactan Island, Philippines, reduced the hydrodynamic forces and water current velocity, resulting in accumulated nutrients in the seagrass beds and favoring the growth of macroalgae as observed in Merambong, Malaysia (Emmclan et al. 2022).

In conclusion, findings of this study demonstrated that variation in the diversity of endophytic fungi between seagrass species is caused by the variation in their host traits. This information provides essential understanding on the mechanism of endophytic fungal assembly in seagrasses. Lastly, since phenolic compounds have protective and preventive functions against biotic and abiotic stress, metabolome analysis on how seagrass and endophytic fungi are linked together is recommended to project future seagrass management strategies and biotechnological manipulation.

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