Colonization and antagonistic activity of endophytic fungi in seagrasses: Understanding endophyte interactions

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ABSTRACT

Aims: This study assessed the colonization and antagonistic interaction of endophytic fungi isolated from the Philippine seagrasses.

Methodology and results: A total of 2690 explants from Enhalus acoroides, Thalassia hemprichii and Cymodocea serrulata were examined in this study. The colonization rate per seagrass species and tissue type were calculated from the number of explants with fungal growth after 14-day incubation on PDA culture media. The presence of fungal structures in seagrass tissues was examined by a histological method. The co-culture method and non-volatile compound assays determined the interaction between endophytic fungal species. Results showed that eight species of endophytic fungi colonized seagrasses, varying with host species and tissue types. Aspergillus ochraceopetaliformis and Penicillium citrinum were the most predominant particularly in E. acoroides leaves. Histological examination of seagrass explants revealed that endophytic fungi preferably colonize the intercellular and intracellular spaces of the cortical tissues. No fungal hyphae were detected in the vascular tissues of the three seagrass species suggesting no systemic fungal growth. The endophytic fungi in seagrasses interact antagonistically with each other in which hyphal interference, the presence of a demarcation line at the interaction zone, changes in colony colour and growth inhibition were observed during the interaction.

Conclusion, significance and impact of study: Seagrasses were colonized by a dynamic assemblage of endophytic fungi, which interact antagonistically with each other. Endophytes’ ability to restrict the growth of other fungal species during interaction suggested their importance in biocontrol and the production of inhibitory molecules.

Keywords: Antagonistic activity, colonization, endophyte interaction, seagrass

INTRODUCTION

Endophytic fungi have long coexisted and coevolved with their host plants resulting in a special mutualistic relationship (Alam et al., 2021). These organisms help plants tolerate environmental stresses, improve vigour, recycle nutrients, decrease susceptibility to pathogens and pests and modulate the synthesis of phytohormones and bioactive metabolites (Khare et al., 2018; Wolfe and Ballhorn, 2020). On the other hand, host plants provide the endophytic fungi with their needed organic nutrients for growth, protection and guaranteed transmission to other hosts (Rai and Agakar, 2016).

The interaction of endophytic fungi with their hosts was considered more effective in systemic than local colonization (Yan et al., 2015), like the enhanced plant growth (Wei et al., 2020) and insecticidal effects of Beauveria bassiana (Rajab et al., 2020; Ramakuwela et al., 2020). In terrestrial plants, colonization of endophytic fungi occurred systemically throughout the sub-niches of the host plants indicating a close interaction among them (Andrade-Linares and Franken, 2013). In seagrasses, Raja et al. (2016) reported the endophytic fungi from the leaves of Halophila ovalis, C. serrulata and Halodule pinifolia, while Torta et al. (2015) and Vohnik et al. (2017) revealed dark septate endophytes in Posidonia oceanica roots. However, it is still ambiguous if fungi grew systematically in seagrass tissues. Thus, this study examined the whole seagrass plant for the colonization of endophytic fungi in multiple parts.

Inside the host plants, antagonistic interactions among species of endophytic fungi are common (Yan et al., 2015). In most cases, the antagonistic activity of one fungal species on the other species elicits the production of inhibitory molecules, which became important in medicine (Ding et al., 2018; Wang et al., 2022) and biocontrol (Pecundo et al., 2021). Though advanced techniques like proteomics and transcriptomics were
available to understand the mechanism of interaction at the molecular level, the co-culture method is still economical and effective. The co-culture method allowed the macroscopic observation of pairwise interactions and measured the strength of interactions (Yan et al., 2015; Abdallah et al., 2017; Hamzah et al., 2018). In Casuarina equisetifolia and Pinus kesiya, co-culture between endophytes and the phytopathogenic, Fusarium spp. showed three antagonistic approaches (De Mesa et al., 2020). In Cytisus, antagonistic interactions classified as types B, C, E and F were observed on the five fungal isolates against the phytopathogenic, Diaportha and Colletotrichum using the co-culture method (Pecundo et al., 2021). In this study, the co-culture method determined the antagonistic interaction between different endophytic fungi species from tropical seagrasses. In addition, in vitro co-cultivation of endophytic fungi assessed the potential species for future bioprospecting. In the Philippines, the endophytic fungi from lichen (Galnatito, 2021; Santiago et al., 2021) and mangroves proved to be prolific sources of bioactive metabolites (Moron et al., 2018; Apurillo et al., 2019). But in seagrasses, only Aspergillus tubingensis from Enhalus acoroides was bioactive, producing malformin A1 (Notarte et al., 2017). With the diversity of endophytic fungi, many species are yet to be discovered for biotechnological application.

MATERIALS AND METHODS

Seagrass sample collection

Seagrass samples were collected along the coastal areas of Hilutungan Channel, Central Philippines (10°16’20” N, 124°05’0” E) using a sterilized razor. A total of 15 stations (each consisting of a 100 m transect laid perpendicular to the shore) were set up. Quadrats with an area of 30 cm × 30 cm were laid in every 20 m distance of the transect. Seagrasses identified as E. acoroides, Cymodocea serrulata and Thalassia hemprichii inside the quadrat were collected and brought to the University of San Carlos-Marine Research Station. The samples were rinsed with filtered seawater until the epiphytes were removed. It was then surface sterilized using 10% ethanol (EtOH) for 3 min, 3% sodium hypochlorite (NaClO) for 10 sec: 10% EtOH for 3 min and finally rinsed twice with sterile distilled water and blotted dry with sterile tissue paper (Supaphon et al., 2014).

Colonization of endophytic fungi in seagrasses

A total of 2690 surface sterilized explants were prepared from the three-seagrass species’ three tissue types (leaves, rhizomes, roots) to determine the colonization of endophytic fungi in seagrass. Two thousand ninety (2090) (750 leaves, 610 rhizomes, 730 roots) of which were inoculated in potato dextrose agar/PDA culture plates (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. The presence of fungal growth in these explants after the incubation was recorded to determine the colonization quantitatively based on the colonization rate. The colonization rate was calculated as % colonization rate (CR) = the number of explants colonized by the fungus/total number of sample explants × 100 (Supaphon et al., 2014). The colonization rate of endophytic fungi was compared between seagrass species and tissue types.

Morphological and molecular methods then identified the species of endophytic fungi. Fungal colonies that grew from the edges of the explant were subcultured and purified using the hyphal tip method. Phenotypic features of each fungal colony, like colony colour, form, texture, elevation, margin, conidiophore type, conidia and hyphal type were used in the identification. Scientific articles were used as references in the morphological identification (Chen et al., 2013; Samson et al., 2014; Visagie et al., 2014; Cañón et al., 2019; Dhar et al., 2019; Bich et al., 2021).

For molecular identification, the mycelia of a 1-week pure culture of twelve isolates were transferred to a 1.5 µL microcentrifuge with nuclease-free water using a sterile loop and sent to Macrogen (Korea) for DNA extraction, amplification and sequencing. The genomic DNA of the twelve isolates was extracted using InstaGene Matrix (Bio-Rad) and amplified using internal Transcribed Region (ITS) and small ribosomal subunit (SSU) primers. The primers for ITS were ITS5 (5'-GGAAGTAAAAGTGTAACAAAGG-3') and ITS4 (5'-TCCCTCGCTTATTGATATGC-3') (White et al., 1990) as forward and reverse primers, respectively. NS1 (5'-GATGTCAATAGTGGTCTCTC-3') and NS8 (TCCGCAAGTTACCTACCGGA) were used as forward and reverse primers for SSU. 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. 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 and spurious sequences were deleted. The DNA sequence was then assembled into a contiguous consensus sequence in 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. The sequences of these taxa were downloaded and included in multiple alignments using CrustalW in MegaX software (Thompson et al., 1994). The aligned sequences were then used to construct the phylogenetic tree by neighbor-joining with 1000 bootstrap replications using the Kimura-2 parameter model.

Meanwhile, the remaining 600 explants (300 leaves, 300 rhizomes/roots) were sectioned histologically to describe qualitatively the location of fungal hyphae in the seagrass tissues. The explants were placed in potato dextrose agar and incubated at 25 °C for 72 h (Bernardi-Wenzel et al., 2010). The explants were then fixed for 24
h with formaldehyde acetic acid (FAA) fixative solution (10 mL of 40% formaldehyde, 5 mL of 99-100% glacial acetic acid, 50 mL of 99.9% ethanol and 35 mL of sterile distilled water) and stained with calcifluor white (1%), lactophenol cotton blue (1:50 v/v HCl) and acridine orange (0.05%) (Raja et al., 2016). Stained explants were sectioned and examined under a compound light microscope with a camera attachment for the presence of fungal hyphae.

**Antagonistic activity of endophytic fungi**

To test the antagonistic activity of endophytic fungi isolated from *E. acoroides*, *C. serrulata* and *T. hemprichii*, co-culture and non-volatile compounds assays were done. In the co-culture assay, colonies of two different fungal species were incubated in the same culture plate to investigate their interactions. The non-volatile compounds assay investigated the effects of non-volatile metabolites on the growth of endophytic fungi. One-month-old culture of each isolate was used in each assay.

**Co-culture assay**

A 5 mm disc of the two different fungal species disc was inoculated on the opposite sides of the PDA plate (approximately 3 cm from the margin of the plate) and incubated for 7 days at room temperature. Plates inoculated with a 5 mm disc from one fungal species served as the control (Hamzah et al., 2018). This assay was done in five trials with three replicates per trial using fungal discs from different cultures. After the incubation period, the type of interaction for each pairwise fungal combination was examined macroscopically based on Badalyan et al. (2002). Three main types, described as types A, B, C and 4 subtypes of interaction, described as $C_{A1}$, $C_{A2}$, $C_{B1}$ and $C_{B2}$ were used to describe the type of interaction. Types A and B are deadlocks at contact and at a distance, respectively, while type C is a replacement. $C_{A1}$ and $C_{A2}$ are partial and complete replacements after a deadlock at contact, while $C_{B1}$ and $C_{B2}$, are partial and complete replacements after an initial deadlock at a distance, respectively.

To calculate the antagonism index of each fungal species, the following score for each type of interaction was adopted following the method of Badalyan et al. (2002). One (1) is the score for type A interaction, two (2) for type B and three (3) for type C. The scores for types $C_{A1}$, $C_{A2}$, $C_{B1}$ and $C_{B2}$ were 3.5, 4, 4.5 and 5, respectively. The antagonism index (AI) was then calculated for each fungal species using this formula: $AI = A(n \times 1) + B(n \times 2) + C(n \times 3) + C_{A1}(n \times 3.5) + C_{A2}(n \times 4) + C_{B1}(n \times 4.5) + C_{B2}(n \times 5)$, where $n$ is the number of interactions. Based on the AI values, the antagonistic activity of each fungal isolate was described as active (AI>15), moderately active (AI=15-10) and weakly active (AI≤10) (Badalyan et al., 2002).

The percentage of inhibition (%) was calculated as $\% = \frac{r1 - r2}{r1} \times 100$, where $r1$ is the radial growth of the control and $r2$ is the radial growth of the co-culture (Hamzah et al., 2018). Radial growth (mm) was measured from the fungal discs to the edge of the colony towards the centre using a micrometre (Supplementary Figure S1).

**Non-volatile compounds assay**

The antifungal effects of non-volatile metabolites produced by the endophytic fungi were assessed by non-volatile compounds assay following the method of Hamzah et al. (2018). A 5 mm fungal disc of each species of endophytic fungi was inoculated in 250 mL sterilized Erlen Meyer flask containing 100 mL potato dextrose broth (PDB). After 15 days at 27 °C incubation, the potato dextrose broth was filtered thrice through a sterilized Whatman filter paper #42. The culture filtrate was then poured into the Petri dish with molten potato dextrose agar (PDA) (40 ± 3 °C) at a final concentration of 20% (v/v). Once the PDA medium solidified, a 5 mm disc of the test fungal isolate was inoculated at the centre of the plate and incubated for 7 days at 27 °C. These were done in triplicates per endophytic fungal species. The control plate was prepared using PDA media without the culture filtrate. After the incubation period, the radial growth of the test fungal isolate was measured. The inhibition rate was calculated as $\% = \frac{[Dc - Dt/Dc]}{100}$, where $Dc$ is the diameter (mm) of a fungal colony of the control and $Dt$ is the diameter of a fungal colony of the treatment (Karlic et al., 2021). The illustration of the fungal growth measurement is shown in Supplementary Figure S2.

**Data analysis**

Kruskal-Wallis test determined the significant difference in inhibition rate per species of endophytic fungi during the co-culture and non-volatile compounds assays. The calculations were done using Past software version 4.03.

**RESULTS**

**Colonization of endophytic fungi in seagrass tissues**

This study isolated and identified eight species of endophytic fungi based on morphological and molecular methods (Kinamot and Monotilla, 2023). These were *Aspergillus tamarii*, *A. ochraceopetaliformis*, *A. terreus*, *A. sydowii*, *Penicillium citrinum*, *Xylaria sp.*, *B. bassiana* and *Eutypella sp.* (Table 1). The molecular phylogeny of ITS and 18s rDNA sequences complemented the identification of these species with a well-supported clade of each isolate with their closest match taxa (Figure 1 and Figure 2).

Out of 8 species, six were isolated from the leaves of *E. acoroides*, two from the rhizomes, and three from the roots. In *T. hemprichii*, five species were isolated in both leaves and rhizomes. None was isolated in the roots. In *C. serrulata*, 8 species of endophytic fungi were isolated from the leaves, while only three and two species were isolated from the rhizome and root, respectively. *A. ochraceopetaliformis*, *A. tamarii*, *B. bassiana*, *Eutypella sp.* and *P. citrinum* were common to the three seagrass
Figure 1: Phylogenetic tree of ITS sequences inferred by neighbor-joining. The phylogenetic tree was rooted with the *Agaricus albiceps* as outgroup. Isolates in black box, written in codes were isolates from the current study.

Figure 2: Phylogenetic tree of *Aspergillus terreus* using 18s rDNA sequences inferred by maximum likelihood. The phylogenetic tree was rooted with the *Ceratomyrium camillium* as outgroup. Fungal species in black box, written in codes were isolates from the current study.
Table 1: Morphological characteristics of endophytic fungi isolated from seagrasses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Macroscopic (colony) features</th>
<th>Microscopic features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color</td>
<td>Form</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em></td>
<td>Green to brown</td>
<td>Brown</td>
</tr>
<tr>
<td><em>Aspergillus ochraceopetaliformis</em></td>
<td>Yellow to brown</td>
<td>Cream to brown</td>
</tr>
<tr>
<td><em>Aspergillus sydowii</em></td>
<td>White</td>
<td>Cream to brown</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>Brown</td>
<td>Brown</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td><em>Eutypella</em></td>
<td>White</td>
<td>Cream</td>
</tr>
<tr>
<td><em>Xylaria sp.</em></td>
<td>White with radial lines</td>
<td>White</td>
</tr>
</tbody>
</table>

Table 2: Type of antagonistic interaction manifested by the antagonist on the other species of endophytic fungi during co-culture assay.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Species co-cultured with the antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. sydowii</em></td>
</tr>
<tr>
<td><em>Aspergillus ochraceopetaliformis</em></td>
<td>C</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em></td>
<td>C</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>C</td>
</tr>
<tr>
<td><em>Xylaria sp.</em></td>
<td>CB₂</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>C</td>
</tr>
</tbody>
</table>

A*: Deadlock at mycelial contact, B: Deadlock at a distance, C: Replacement without deadlock, CA₁: Partial replacement after an initial deadlock at mycelial contact, CA₂: Complete replacement after an initial deadlock at mycelial contact, CB₁: Partial replacement after an initial deadlock at a distance, CB₂: Complete replacement after an initial deadlock at a distance (Badalyan et al., 2002). * Denotes no antagonistic interaction.
hosts. *A. sydowii* was only isolated from *E. acoroides* leaves. On the other hand, *Xylaria* sp. was present in *E. acoroides* and *T. hemprichii* leaves while *Aspergillus terreus* was isolated from *T. hemprichii* rhizome and *C. serrulata* leaf. The colonization rate of endophytic fungi varied with seagrass species and tissue type. In the leaves, *A. ochraceopetaliformis* and *P. citrinum* had the highest colonization rate in *E. acoroides* while *A. tamarii* had the highest colonization rate in *T. hemprichii* and *C. serrulata*. In the rhizomes, *B. bassiana* colonized predominantly in *E. acoroides* and *C. serrulata* while *A. ochraceopetaliformis* colonized predominantly in *T. hemprichii*. In the roots, *A. tamarii* was dominant in *E. acoroides*, while *Eutypella* sp. was dominant in *C. serrulata* (Figure 3).

Histological examination of the seagrass explants revealed the presence of fungal structures inside the tissues. In the leaves of *E. acoroides*, fungal hyphae colonized both the intercellular and intracellular spaces of the epidermal and cortical cells. However, in *T. hemprichii* and *C. serrulata*, fungal hyphae were observed only in the cortical cells beneath the epidermis. Fungal hyphae were dense and formed branching and coiling in the cortex (Figure 4). In the rhizomes and roots, fungal hyphae were detected in the cortical tissues of the three seagrass species (Figures 5-8). In *C. serrulata* and *T. hemprichii*, fungal hyphae were specifically observed in the intracellular spaces of the outer and middle cortex, where the air lacunae are large. The hyphae branched laterally and extended to the neighbouring cells. It was interesting to note that the hyphae observed in the roots and rhizomes were relatively finer than in the leaves. For instance, the fungal hyphae in the *C. serrulata* leaf measured 0.26-0.519 µm in width, while the fungal hyphae in the rhizome measured 0.649-1.48 µm in width. No fungal hyphae were also detected in the vascular...
tissues of the three seagrass species. Lastly, no apparent signs of necrosis or disorganization of the host cell’s cytoplasm were observed in all the seagrass tissues.

Antagonistic activity

Seven different types of interaction were recorded during the co-culture assay. Deadlock (both type A and B) was observed in the pairwise combination using *A. ochraceopetaliformis*, *A. tamarii*, *Xylaria* sp., *P. citrinum*, *B. bassiana* and *Eutypella* sp. Replacement without deadlock (Type C) was predominant in *A. terreus* and *A. sydowii* when co-cultured with the other seven species of endophytic fungi. Whereas the subtypes of type C interaction (*CA*1, *CA*2, *CB*1 and *CB*2) were common in *Xylaria* sp., *B. bassiana* and *Eutypella* sp.
cultured with *A. ochraceopetaliformis*, *A. tamarii* and *P. citrinum* (Table 2). Different interactions resulted from the interspecific pairing using similar fungal species. Deadlock at mycelial contact (type A) was observed in the co-culture between *A. tamarii* and *B. bassiana* (Figure 9a); *A. tamarii* and *A. ochraceopetaliformis* (Figure 9b); *Xylaria* sp. and *A. ochraceopetaliformis* (Figure 9c); *Xylaria* sp. and *A. tamarii* (Figure 9d); *Eutypella* sp. and *B. bassiana* (Figure 9e). Aside from type A interaction, *Xylaria* sp. had CA1 interaction with *A. ochraceopetaliformis* (Figure 9f) and CB2 interaction with *A. tamarii* (Figure 9g). *B. bassiana* had deadlock at a distance (type B interaction) with *Xylaria* sp. (Figure 9h) and *A. ochraceopetaliformis* (Figure 9i). *P. citrinum* had type C (Figure 9j) and CB2 interactions (Figure 9k) with *B. bassiana*. Notable morphological changes such as

**Figure 7**: Endophytic fungal colonization in the rhizome of *Thalassia hemprichii*. The red arrow is pointing to the fungal hyphae. OC: Outer cortex, MC: Middle cortex, IC: Inner cortex.

**Figure 8**: Endophytic fungal colonization in the roots of *Enhalus acoroides*. The red arrow is pointing to the fungal hyphae. OC: Outer cortex, MC: Middle cortex.
Based on the antagonism index (AI) calculated during the co-culture assay, A. tamarii, A. ochraceopetaliformis, P. citrinum, B. bassiana, Eutypella sp. and Xylaria sp. were active antagonists (AI≥15). Among the 6 species, A. tamarii had the highest value while Eutypella sp. had the lowest (Figure 10). The antagonism index complemented the inhibition rate of each species on the radial growth of other endophytes during the co-culture assays (Supplementary Table S1 and Table S2). A. tamarii, inhibited at least 50% of the fungal growth of all endophytic fungi except A. ochraceopetaliformis (This species was most active against A. sydowii, A. terreus and Eutypella sp. Moreover, A. ochraceopetaliformis, P. citrinum and Xylaria sp. inhibited the radial growth of A. sydowii, A. terreus and Eutypella sp. by 60-100%. The inhibition rate of Eutypella sp. on the radial growth of the other 7 species was only <40%. A. terreus and A. sydowii did not manifest inhibition during the co-culture assay. But the crude extract of A. sydowii and A. terreus inhibited other endophytes especially Eutypella sp. during the non-volatile assay Figure 11). A significant difference in the inhibition rate was recorded in A. ochraceopetaliformis, A. tamarii, P. citrinum, B. bassiana, Xylaria sp. and Eutypella sp. during the co-culture assay (p≤0.05, CI=95%) but no significant difference in all species during the non-volatile compounds assay.

**DISCUSSION**

Colonization of endophytic fungi in seagrasses

The interaction between endophytic fungi and their host plants starts with fungal colonization in the host tissues. In this study, eight species of endophytic fungi were observed to colonize the leaves, rhizomes and roots of E.
acoroides, T. hemprichii and C. serrulata which vary with the host species and tissue types suggesting the influence of host traits. Variation in the diversity of endophytic fungi in relation to host species was also reported in Ficus spp. in the Philippine natural forest (Solis et al., 2016). According to Supaphon et al. (2014), a combination of physical and chemical factors influenced the colonization and distribution of endophytic fungi in the seagrass tissues. Seagrasses are known to produce compounds like phenols, tannin, terpenes and flavonoids which are antifungal. Kannan et al. (2013) reported the presence of phenol, tannin and flavonoid in the crude extract of seagrasses. E. acoroides had the lowest content of these compounds while C. serrulata had the highest. Thus, it is speculated that intergeneric variation of antifungal chemical compounds in seagrasses influenced the colonization of endophytic fungi.

The presence of hyphal structure in the cortical tissues through the histological examination of seagrass explants supported the fungal colonization in seagrass. Among the epidermis, cortex and vascular tissues, the fungal hyphae preferred to colonize the cortex in all
seagrass species both intracellularly and extracellularly. Fungal colonization in intercellular and intracellular spaces indicated that endophytes had close interaction with their hosts as reported in Luehea diversicata (Bernardi-Wenzel et al., 2010) and Sapindus saponaria (Garcia et al., 2012). Raja et al. (2016) also suggested close interaction between endophytes and C. serrulata, T. hemprichii and H. ovalis because the seagrass leaves were extensively colonized by hyphae. Less lignified cells in the cortex, a reliable source of nutrition and less competition for space give the endophytic fungi a favourable niche for their growth (Kulda and Bacon, 2008; Kandel et al., 2017). This study observed histologically that the cortical cells of seagrass had numerous air lacunae, which give ample space for hyphal branching and coiling. In the rhizome of T. hemprichii and C. serrulata, the cortex is composed of outer, middle and inner layers. The outer cortex is multilayered with compactly arranged parenchyma cells and small air lacunae. The middle cortex is composed of loosely and radially arranged parenchyma cells with large air lacunae. The inner cortex has compactly arranged parenchyma cells surrounding a stele with vascular tissues. Observation under the microscope revealed that fungal hyphae were most dense in the middle cortex, which could be attributed to the presence of air lacunae in this tissue. In contrast, compactly arranged cells in the epidermis could be responsible for the absence of hyphae in the epidermis of seagrass except for E. acoroides leaves.

The absence of hyphal colonization in the vascular tissues of seagrasses suggested the non-systemic distribution of endophytic fungi throughout the seagrass plant. The growth of endophytic fungi is maintained and restricted in defined areas of the plant tissues by the hosts' defence reaction (Gao and Mendgen, 2006), like strengthened cell walls and membranes and activated plant innate immune responses (Gebrei, 2016). For instance, in Pisum spp. roots, both physical and chemical barriers, resisted the progression of Fusarium oxysporum by cell wall lignification, formation of papillae-like structures, and accumulation of polyphenolic and carbohydrate compounds (Bani et al., 2018). Though such defence responses from seagrasses were not observed in this study, the restriction of most fungal hyphae from vascular tissues indicated a host defence reaction. This interaction may also reflect the balanced antagonism between endophytic fungi and seagrass to maintain an endophytic relationship. Fungal endophytes ranged from latent pathogens to mutualistic symbionts depending on the hosts' physiological condition. If the balance is distorted, endophytes may become virulent and pathogenic (Sarsaiya et al., 2020). Pathogenic fungal colonization was described as extensive hyphal development within vascular tissue, heavily branched mycelia in many cells, and extensive cell wall degradation. For instance, Lasiodiplodia theobromae, a phytopathogenic fungus of cashew, revealed massive colonization in the secondary xylem of its host tissues with the disintegration of the cell wall (Muñiz et al., 2011).

Unlike the fungal pathogens, this study observed no cell necrosis suggesting a non-pathogenic relationship.

The observation of thick hyphae in the leaves while fine-threadlike hyphae in the roots and rhizomes implied the colonization of different species in the seagrass tissues. This is also well supported by the difference in the species composition of endophytic fungi isolated from the three tissues of each seagrass species. These findings served as another indicator that the distribution of each fungal species in the seagrass tissues is non-systemic.

**Antagonistic activity**

Plants harbour diverse microbial communities, including endophytic fungi in their tissues, which interact dynamically with each other (Alam et al., 2021). Antagonistic interaction is common among endophytic fungi (Yan et al., 2015) because of resource competition (Boody, 2000). In this study, the eight species of endophytic fungi showed antagonistic activity against the other isolates. The antagonistic activity of A. terreus and A. sydowii was observed in non-volatile compounds assay in which their culture filtrate inhibited the radial growth of all endophytes, especially Eutypella sp. Hamzah et al. (2018) suggested that the culture filtrates of mangrove endophytes contained antimicrobial compounds, like non-volatile compounds, which inhibited the growth of Fusarium solani. On the other hand, the other 6 species of endophytic fungi in this study showed antagonism during both the cu-culture and non-volatile compounds assay. In these species, hyphal interference, morphological change of the colony, deadlock and replacement were observed. Hyphal interference was employed between B. bassiana and Eutypella sp. in which aggregation of their hyphae physically block each other’s colony from invading and replacing their territory. Hiscox and Boddy (2017) described this antagonistic mechanism as a constitutive defence. Aside from hyphal interference, the fungi produced inhibitory metabolites during constitutive defence. The demarcation line and colony pigmentation observed in Xylaria sp., when paired with A. ochraceopetaliformis and A. tamarit suggested high quantities of inhibitory metabolites and extracellular pigment production. Fungi reportedly produced pigments to protect their mycelia from the harmful effects of reactive oxygen species (ROS), toxins and hydrolytic enzymes produced by their antagonists. This is also observed in the study of Hamzah et al. (2018).

The production of antimicrobial compounds is the main mechanism employed in antagonism (Hamzah et al., 2018), wherein volatile organic compounds (VOCs) and diffusible compounds are attributed to antagonism at a distance (Boddy, 2000). In this regard, it is suggested that deadlock at a mycelial distance between Xylaria sp. and B. bassiana is associated with the production of volatile organic (VOCs) or diffusible compounds. To support our claim, several studies reported the production of volatile organic compounds in these species. For instance, a strain of Xylaria sp. isolated from...
Haematoxylin brasiliello produced 40 types of VOCs that inhibited the growth of Alternaria solani and Fusarium oxysporum during the antagonism bioassays (Sanchez-Ortiz et al., 2016). Xylariales sp. isolated from H. ovalis produced bioactive xylaripholine, an azaphilone derivative (Arunpanichlert et al., 2016). B. bassiana produced as many as 97 VOCs classified as aldehydes, ketones, alcohols, esters, acids and terpenes (Bojke et al., 2018; Lozano-Soria et al., 2020). According to Boddy (2000), volatile organic compounds can reach 15 mm in culture media. If this is extrapolated in seagrass tissues, it is possible that deadlock between endophytic fungal species can produce compounds whose effects can diffuse through the tissues of the plants. As Yan et al. (2015) explained, the systemic effects of endophytic fungi in plants were due to chemical movement during the antagonistic interaction with other species and not due to fungal growth.

Replacement interaction observed in this study could be a result of high growth rate, tolerance to inhibitory metabolites, and high production of potent metabolites by the active antagonists that become detrimental to the overgrown colony. In this study, A. tamarii was the most active antagonist. The ability to increase oxygen absorption and respiration as well as the production of inhibitory metabolites in this species, could be the factors for its aggressive activity against other endophytes, as observed in the study of Campos and Jacob (2021). Moreover, A. tamarii had 70% fungal growth-reducing potential and the ability to suppress the production of mycotoxin like ochratoxin A in many fungal species (de Almeida et al., 2019). P. citrinum (Sharma et al., 2021), A. ochraceopetaliformis (Liu et al., 2020) and Xylaria sp. (Hamzah et al., 2018) produced hydrolytic enzymes which break down the cell wall of their antagonists. Azaphilone, cytochalasin (de Felício et al., 2015), piliformic acid and griseofulvin (Elias et al., 2018) from Xylaria sp. had antifungal activity. A. ochraceopetaliformis secreted a new-pyrene chlorofuranocarboxylate molecule with intense antifungal activity (Asmaey et al., 2021). On the other hand, due to the high energy-requiring process of metabolite production (Hiscox and Boddy, 2017), weak antagonists like A. terreus and A. sydowii might cease to produce inhibitory metabolites during long periods of mycelial interaction as observed in H. fasciculare (El Arebi et al., 2016), resulting to replacement.

The presence of different interaction types between the co-culture of similar fungal species in this study supported Hiscox et al. (2018) that outcomes of the interaction are not always the same even if the replicates came from the same individuals and under identical conditions. This is because even with the small variation in the abiotic factors, the antagonistic interaction between two fungal isolates could be altered since each fungal isolate has a different pattern of sensitivity and responses to the abiotic factors. For instance, Coriolus versicolor was deadlocked by Daldinia concentrica when exposed to 5% O2. But when the O2 concentration was raised to 20%, C. versicolor was replaced by D. concentrica. At 1.3 MPa water potential, D. concentrica was partial to completely replaced by C. versicolor (Boody 2000). In Trametes versicolor, changes in temperature regime resulted in replacement by Hypoloma fasciculare and Phanerochaete velutina at 15 °C but deadlock at 25 °C. Moreover, the production of volatile organic compounds and extracellular enzymes during antagonistic interactions also varies with temperature with more VOCs and active enzymes detected at 15 °C (O’Leary et al., 2019). Though the effects of culture conditions on the antagonistic interaction of each fungal combination were not within the scope of this study, observations from other fungal species may suggest that abiotic factors also influenced the antagonistic interaction between the endophytic fungi in this study. Moreover, in the natural substrata, consistency in the type of interaction could be uncertain because the antagonistic ability of one species is always affected by the other species. This pattern is described by Boddy (2000) as a cyclical competition structure of intraspecific interactions. In wood saprophytic basidiomycete, intraspecific promoted species coexistence despite having strong antagonism among species because there was no clear hierarchy of superiority at the community level.

The ecological importance of endophyte interactions in terrestrial plants suggested enhanced plant growth and resistance to pathogens. In seagrass, endophytic interactions may confer beneficial effects on their host. The species of endophytic fungi isolated in this study had been reported to enhance their host plant’s growth and increase resistance to environmental stress. For instance, P. citrinum was reported to have a gibberellin-producing capacity which helps plants modulate growth and development during salinity stress (Leitao and Engita, 2016). Aspergillus sydowii aid in the absorption and solubilization of phosphorus and nitrogen in cotton plants (Diaz et al., 2021). B. bassiana increased the bioavailability of nutrients to tomato plants through phosphate solubilization and iron siderophore production (Barra-Bucarei et al., 2020). Nevertheless, a comprehensive investigation of endophyte-seagrass is vital to elucidate the mechanism of interaction. Economically, antagonistic activity manifested by the species of endophytic fungi suggested their potential as sources of bioactive metabolites or as biocontrol agents. For instance, co-culture of marine algal-derived P. citrinum and A. sydowii produced bioactive new citrin dimer seco-phenethyl A and L, 8 citrin derivatives and phenol (Yang et al., 2018). Co-culture of marine-derived P. citrinum and Beauveria feline produced an anti-pathogen, citrifulins (Meng et al., 2015). The production of lovastatin by A. terreus increased because of the strong inducing effect of Aspergillus unguis during co-culture (Wang et al., 2022). Co-culture of endophytic fungi, Aspergillus sp., Fusarium sp. and Ramularia sp. from Rumex melinii increased the yield of antifungal, chrysophenin, reseveratrol, chrysophanol, emodin and physcion by 3-4 folds than the control (Ding et al., 2018). In addition, the antagonistic ability of fungi is correlated with the presence of biosynthetic gene clusters. For instance, endophytic Eutypella sp. from the sponge had
polyketide synthase (PKS I & II) and non-ribosomal peptide synthase (NRPS). Still, these genes are suspected silent or unexpressed (Sibero et al., 2019). With antagonistic interaction, silent genes may be upregulated and produced novel metabolites.

CONCLUSION

Endophytic fungi in seagrasses are influenced by the hosts resulting in variation in the colonization rate. The fungal growth is not systemic based on the absence of hyphae in the vascular tissues and the variation of endophytic fungi colonizing every seagrass tissue. The distribution of endophytic fungi in seagrass tissues is concentrated in the cortex with numerous air lacunae that provide ample space for growth.

Antagonistic interaction in endophytic fungi of seagrasses is common. The co-culture method showed that antagonism in endophytic fungi occurred at mycelial contact and at a distance. Active antagonism exhibited by the species of endophytic fungi in seagrasses indicated that they could be promising sources of metabolites. Aspergillus tamarii, A. ochraceopetaliformis and P. citrinum which are active antagonists during deadlock at mycelial contact could be potential sources of secondary metabolites. While Xylaria sp. and B. bassiana, which showed deadlock at a mycelial distance, are promising sources of volatile organic compounds and extracellular enzymes. For future studies, the metabolites produced in every isolate combination should be characterized for biotechnological applications.

REFERENCES

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**SUPPLEMENTARY INFORMATION**

*Figure S1*: Measurement of the radial growth of each endophytic fungal colony during the co-culture assay. D: Fungal disc, Red arrow: Direction of measurement.

*Figure S2*: Measurement of the diameter of a fungal colony in the control and treatment plates during the non-volatile compound assay. D: Fungal disc, red arrow: Direction of measurement.
Table S1: Mean radial growth of each endophytic fungi in relation to the antagonists during the co-culture assay.

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Aspergillus sydowii</th>
<th>Aspergillus terreus</th>
<th>Aspergillus ochraceopetaliformis</th>
<th>Aspergillus tamarii</th>
<th>Penicillium citrinum</th>
<th>Xylaria sp.</th>
<th>Beauveria bassiana</th>
<th>Eutypella sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus ochraceopetaliformis</td>
<td>7.9</td>
<td>5.2</td>
<td>*</td>
<td>23.7</td>
<td>16.875</td>
<td>9.8</td>
<td>17.87</td>
<td>22.5</td>
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<tr>
<td>Aspergillus tamarii</td>
<td>6.9</td>
<td>3.77</td>
<td>20.95</td>
<td>*</td>
<td>12.499</td>
<td>7.7</td>
<td>14.99</td>
<td>15.65</td>
</tr>
<tr>
<td>Penicillium citrinum</td>
<td>9.1</td>
<td>7.5</td>
<td>21.44</td>
<td>27.5</td>
<td>*</td>
<td>10</td>
<td>16.8</td>
<td>27.1</td>
</tr>
<tr>
<td>Xylaria sp.</td>
<td>9.4</td>
<td>7.5</td>
<td>25.2</td>
<td>29.35</td>
<td>27</td>
<td>*</td>
<td>21.3</td>
<td>25.65</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>14.7</td>
<td>17.5</td>
<td>23.25</td>
<td>27.65</td>
<td>31.9</td>
<td>15.4</td>
<td>*</td>
<td>19.1</td>
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<tr>
<td>Eutypella sp.</td>
<td>17.8</td>
<td>21.5</td>
<td>28</td>
<td>28</td>
<td>27.5</td>
<td>25.5</td>
<td>30</td>
<td>*</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>35</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

* No antagonism.

Table S2: Mean radial growth of each endophytic fungi in relation to the antagonists during the non-volatile assay.

<table>
<thead>
<tr>
<th>Source of culture filtrate</th>
<th>A. sydowii</th>
<th>A. terreus</th>
<th>Aspergillus ochraceopetaliformis</th>
<th>Aspergillus tamarii</th>
<th>Penicillium citrinum</th>
<th>Xylaria sp.</th>
<th>Beauveria bassiana</th>
<th>Eutypella sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus sydowii</td>
<td>NT</td>
<td>38.9</td>
<td>63</td>
<td>72</td>
<td>72</td>
<td>40.3</td>
<td>71</td>
<td>0</td>
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<tr>
<td>Aspergillus terreus</td>
<td>33.3</td>
<td>NT</td>
<td>72</td>
<td>70</td>
<td>58.6</td>
<td>39.7</td>
<td>80*</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>10</td>
<td>0</td>
<td>NT</td>
<td>80*</td>
<td>80*</td>
<td>0</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Aspergillus tamarii</td>
<td>0</td>
<td>0</td>
<td>80*</td>
<td>NT</td>
<td>22</td>
<td>24</td>
<td>13.3</td>
<td>0</td>
</tr>
<tr>
<td>Penicillium citrinum</td>
<td>0</td>
<td>0</td>
<td>80*</td>
<td>69.5</td>
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<td>49.1</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>Xylaria sp.</td>
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<td>0</td>
<td>72</td>
<td>74.5</td>
<td>68.6</td>
<td>NT</td>
<td>66.6</td>
<td>0</td>
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<tr>
<td>Beauveria bassiana</td>
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<td>0</td>
<td>74.6</td>
<td>79</td>
<td>74.6</td>
<td>54.3</td>
<td>NT</td>
<td>0</td>
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<tr>
<td>Eutypella sp.</td>
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<td>38.9</td>
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<td>74.5</td>
<td>68.8</td>
<td>45.8</td>
<td>72</td>
<td>NT</td>
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<tr>
<td>Control</td>
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<td>80</td>
<td>55</td>
<td>80</td>
<td>80</td>
<td></td>
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</tbody>
</table>

* Low mycelial mass, NT: Not tested.