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

Venus Kinamot (vdbbio@yahoo.com)
Negros Oriental State University

Alvin Monotilla
University of San Carlos

Research Article

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Abstract

Endophytic fungal colonization in plants is governed by complex interactions with the defense mechanism of the host and antagonistic effects of other endophytes. In this study, endophytic fungal interaction was assessed by histological examination and co-culture methods. Results showed fungal colonization in the intercellular space of the epidermis and both intercellular and intracellular spaces of the cortical cells suggesting close interaction with their seagrass hosts. Dense colonization, hyphal branching, coiling and formation of networks were observed in the cortical cells. Less competition for space and reliable source of nutrition in the cortex may favor fungal growth. No fungal hyphae were detected in the vascular tissues of seagrasses.

All the endophytic fungi isolated from seagrasses showed antagonistic activity. Aspergillus tamarii, A. ochraceopetaliformis, Penicillium citrinum, Beauveria bassiana, Eutypella sp. and Xylaria sp were the most active antagonists. Antagonistic interaction involved deadlock and replacement. Deadlock was associated with physical blocking of the antagonist's colony by hyphal aggregation and production of inhibitory metabolites. Demarcation line and colony pigmentation in Xylaria sp. during co-culture assay indicated the production of high quantities of inhibitory molecules. Endophytic fungi in seagrasses also produced volatile organic compounds (VOC) which resulted to deadlock at mycelial distance. Thus, endophyte colonization and distribution in seagrass tissues are influenced by their interaction with the hosts and other endophytes. But interestingly, cyclical intransitivity of multispecies interaction manifested by these fungal species suggested possible co-existence in seagrass tissues.

Introduction

Endophytic fungi have long been coexisted and coevolved with its host plants resulting to a special mutualistic relationship (Alam et al. 2021). It helped plants tolerate environmental stresses, improve vigor, recycle nutrients, decrease susceptibility to pathogens and pests and modulates synthesis of phytohormones and metabolites (Khare et al. 2018; Wolfe and Ballhorn 2020). On the other hand, host plants provide them the organic nutrients, protection and guaranteed transmission (Rai and Agakar 2016).

The interaction of endophytic fungi with its plant hosts varies depending on whether the colonization is local or systemic. Diverse role of endophytic fungi is more effective in systemic than local colonization (Yan et al. 2015). For instance, enhanced antagonistic interactions which lead to antiherbivore effects was enhanced in systemic fungal growth (Wei et al. 2020). In terrestrial plants, colonization of endophytic fungi occurred systemically throughout the subniches of the host plants indicating a close interaction among them (Andrade-Linares and Franken 2013). However, in seagrasses, reports of endophytic fungal colonization were done in one organ only. Raja et al. (2016), reported the endophytic fungi from the leaves of H. ovalis, C. serrulata and Halodule pinifolia. Torta et al. (2015) and Vohnik et al. (2017), on other hand, reported the colonization of dark septate endophytes in P oceanica roots. No report on endophytic fungal colonization in the whole plant. Thus, this study described the colonization of endophytic fungi throughout all the organs of seagrasses.

Synergistic or antagonistic interactions influenced endophyte distribution inside the host tissues (Yan et al. 2015) and production of metabolites (Kusari et al. 2013). Fungal interactions in co-culture showed hyphal fusion among self-paired cultures indicating compatibility and possibility of systemic growth within the host. Physical contact and crosstalk among endophytes in the same habitat synergistically activate silent biosynthetic pathways and contributes to the synthesis of metabolites (Lahlali and Hijri 2010; Rabha et al. 2014). However, in most cases, endophytic fungi showed antagonistic interaction with other species by antibiosis, toxin synthesis and competition inhibiting the systemic distribution of endophytic fungi within its hosts (Yan et al. 2015). Though advance techniques like proteomics and transcriptomics were recently available to understand the mechanism at the molecular level, co-culture method is still economical and effective. It allowed the observation of pairwise interactions and morphological changes which could be a defense mechanism. The strength of interactions can also be measured and metabolite production is induced in co-culture method (Yan et al. 2015; Abdallah et al. 2017; Hamzah et al. 2018). Hence, this study would determine the interaction among endophytic fungi in seagrasses by co-culture method. Results of this study provided novel information on the interaction between the endophytic fungi and seagrasses in the Philippines. It also served as benchmark for exploring these endophytic fungi as source of natural products and biocontrol agents against seagrass herbivores and pathogens.
Materials And Methods

Seagrass Sample Collection

Seagrass samples were collected along Hilutungan Channel, Central Philippines (10°16′20″ N, 124° 0′ 50″ E) during low tide from October to December 2021. A total of 15 sampling stations were set up perpendicular to the shore in which each station was 100m-long. In every station, 5 quadrats (30x30cm in size) were laid. Seagrass identified as *Enhalus acoroides*, *Cymodocea serrulata* and *Thalassia hemprichii* inside the quadrat were collected. The whole plant was cut using a sterilized razor, placed in plastic container and brought to University of San Carlos- Marine Research Station. The samples were then washed with filtered sea water until the epiphytes were removed. It was then surface-sterilized using 10% ethanol (EtOH) for 3min, 3% sodium hypochlorite (NaClO) for 10s; 10% EtOH for 3min and finally washed twice with sterile distilled water and blotted dry with sterile tissue paper (Supaphon et al. 2014).

Histological Examination of Seagrass Tissues for Endophytes

A total of 600 surface-sterilized segments for each seagrass species: 300 leaf and 300 roots, were placed in potato dextrose agar and incubated at 25°C for 72 hrs. The segments with fungal growth at the edge of the seagrass segment were then fixed with formaldehyde acetic acid (FAA) for 24 hrs. The seagrass segments were then sectioned using a microtome and stained with lactophenol cotton blue, calcofluor white and acridine orange. Endophyte colonization was determined by the presence of fungal structure inside the tissues of seagrasses under the light microscope with digital camera. Distribution of fungal endophytes were ascertained through the location of fungal hyphae.

Isolation and Identification of Endophytic Fungi Associated in Seagrass

To isolate the endophytic fungi from the tissues of seagrasses, fresh 3-5mm explants were surface-sterilized and inoculated in each culture plate using potato dextrose agar/PDA (potato peptone 200g, glucose 20g, agar 15g, pH 5.6 ±0.2). All culture plates were incubated at 25±2°C for 14 days or until fungal growth were observed from the edges of the explants. Fungal colonies were sub-cultured on a new culture medium and purified by hyphal tip method. Pure isolates were identified by morphological and molecular methods.

Genomic DNA of each endophytic fungal isolate was extracted using InstaGene Matrix (Bio-Rad) and amplified using Internal Transcribed Region (ITS) and small ribosomal subunit (SSU) primers. ITS5 (5’- GGAAGTAAAAGTCGTAACAAGG-3’) as forward primer and ITS4 (5’- TCCTCCGCTTATTGATATGC-3’) as reverse primer (White et al. 1990) while NS1 (5’- GTAGTCATATGCTTGTCTC-3’), NS8 (TCCGCAGGTTCACCTACGGA) were used as forward and reverse primers for ITS and SSU, respectively. The sequences were then analyzed for most probable closely related taxa using BLAST search.

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. For each assay, 1 month-old culture of each isolate was used.

Co-culture Assay

In co-culture assay, 5mm discs of each fungal combination were inoculated on the opposite sides of PDA plate and incubated for 7 days at room temperature. The control plate had only one fungal isolate (Yan et al. 2015). This assay was done in three replicates. The type of interaction was examined macroscopically and scored based on Badalyan et al. (2002). Three main types described as type A, B, C and 4 subtypes of interaction described as C_{A1}, C_{A2}, C_{B1}, 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 replacement after deadlock at contact while C_{B1} and C_{B2} are for partial and complete replacement after deadlock at a distance, respectively. The score for this type is from 1-5, where type is equal to one, B=2, C=3, C_{A1}, C_{A2}, C_{B1}, C_{B2} = 3.5, 4, 4.5 and 5, respectively. The antagonism index (AI) was calculated as AI=\sum n x i, where n is the number of interaction and i is the interaction. The percentage of inhibition (I%) was calculated as I%=[(r1-r2)/r1] x 100, where r1 is the radial growth of the
control, r2 is the radial growth of the co-culture. Radial growth was measured from the center to the edge of the colony using a micrometer after 14 days incubation (Yan et al. 2015). Differences in the radial growth in each species was determined by Kruskal-Wallis test.

**Non-Volatile Compounds Assay**

The effects of metabolites on the growth of endophytic fungi were investigated by antifungal non-volatile compounds test. A culture filtrate was prepared from 5mm fungal disc inoculated on 100ml potato dextrose broth (PDB) and incubated for 15 days at 27°C. The culture filtrate was then mixed with molten PDA and 5mm disc of the test fungal isolate was then inoculated at the center of the plate. The control plate was prepared without the culture filtrate (Hamzah et al. 2018). There were three replicates for this assay. The inhibition rate was calculated as $I\%=[(Dc-Dt)/Dc] \times 100$, where $Dc$ is the diameter (cm) of the control and $Dt$ is diameter of the treatment (Hamzah et al. 2018). Differences in the radial growth in each species was determined by Kruskal-Wallis.

**Results**

**Colonization of endophytic fungi in seagrass tissues**

Leaves, rhizomes and roots of *C. serrulata* *E. acoroides* and *T. hemprichii* are composed of epidermis, cortex and vascular bundle. The epidermis is one-celled layer which are compactly arranged. The cortex in the leaves has mesophyll cells with numerous air lacunae. In the rhizomes and roots, cortex is divided into three layers: outer, middle and inner cortex. 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 (Fig. 1).

Hyphal structures were commonly observed in the leaf, rhizome and roots of seagrasses both intracellularly and intercellularly. They have varied width, some have septation, branching and coiling inside the tissues. In *C. serrulata* leaf, fungal hyphae ranged from 0.26–0.519 µm in width. Some hyphae had septation while others have none. It was observed that fungal hyphae penetrated the epidermal layer through the intercellular space. It then extended inward towards the mesophyll cell in the cortex where it coiled and formed networks. They branched to colonize the neighboring cells by penetrating into the cell wall even if a single cell was not yet completely colonized by hyphae. No fungal colonization was observed in the vascular tissues of the leaves (Fig. 2a-c). In rhizome, fungal hyphae measured 0.649–1.48 µm in width and morphologically different from those in the leaves. They were predominantly at the cortical cells specifically in the middle cortex with large air lacunae. It developed branching forming network inside the cell's lacuna. Colonization of hyphae into the neighboring cells occurred by direct penetration of the hyphae through the cell wall (Fig. 2d-f). On the other hand, no hyphae were observed at the epidermis and vascular bundles. No fungal hyphae were detected in *C. serrulata* roots.

In *E. acoroides*, networks of fungal hyphae were detected in the leaves, rhizomes and roots. Fungal hyphae in the leaves measured 0.632–4.808 µm in width. It was observed both within the intercellular and intracellular spaces of the epidermal and cortical cells. These hyphae extended laterally colonizing the neighboring cells by following the contour of the cell membrane. Colonization was observed dense in the mesophyll cells of the cortex where it is loosely packed inside the air lacuna. There were no fungal hyphae at the vascular bundle of the leaves (Fig. 3a-c). In rhizomes, hyphae were very fine. It was observed to form networks in the intracellular space of the middle cortex. Likewise, no fungal hyphae at the vascular bundle of the rhizomes (Fig. 3d-e). In the roots, networks of fungal hyphae were detected at the epidermis. Colonization to neighboring cells of the epidermis occurred by lateral branching of the hyphae penetrating the cell wall of the adjacent cells. However, dense network of fungal hyphae was restricted inside the air lacunae of the cortical cells (Fig. 3f-g). The vascular bundle of the seagrass roots was also free from fungal hyphae.

In *T. hemprichii*, network of fungal hyphae was detected in the intracellular space of the cortical cells in the leaves. No fungal hyphae were detected in the epidermis and vascular bundles of the leaves (Fig. 4a-b). In the rhizomes, presence of fungal hyphae was detected in the outer and middle cortex. Fungal hyphae penetrated and colonized the neighboring cells by passing through
the cell wall (Fig. 4c-d). No fungal hyphae were observed in the roots of *T. hemprichii*. In all the seagrass samples, no sign of necrosis nor disorganization of the cell’s cytoplasm was observed when colonized by fungal hyphae

**Antagonistic Activity**

This study isolated and identified 8 species of endophytic fungi based on morphological and molecular methods. These were *Aspergillus tamarii*, *A. ochraceopetaliformis*, *A. terreus*, *A. sydowii*, *Penicillium citrinum*, *Xylaria sp.*, *Beauveria bassiana* and *Euplytella sp.* (unpublished results). Co-culture assay of these 8 species revealed 7 different types of interaction (Table 1). Deadlock at mycelial contact (Type A interaction) was observed in all species except in *Aspergillus terreus* and *A. sydowii*. Co-culture between *A. tamarii* and *B. bassiana* (Fig. 5a); *A. tamarii* and *A. ochraceopetaliformis* (Fig. 5b) showed deadlock at mycelial contact. A notable morphological change was observed during deadlock at mycelial contact. For instance, demarcation line and colony color turning into yellow and black were observed in *Xylaria sp.* when it interacted with *A. ochraceopetaliformis* (Fig. 5c-d) and *A. tamarii* (Fig. 5e). The colony of *A. sydowii* and *Euplytella sp.* was also changed to yellow when co-cultured with *B. bassiana*. Another observation during deadlock at mycelial contact was hyphal aggregation as observed in *Euplytella sp.* and *Beauveria bassiana* (Fig. 5f). Deadlock at a distance (Type B) was observed in all species except in *Aspergillus sydowii*. Co-culture between *Xylaria sp.* and *B. bassiana* showed deadlock at a distance with distinct clear space around *Xylaria sp.* (Fig. 5g).

<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>, <em>A. terreus</em>, <em>A. ochraceopetaliformis</em>, <em>A. tamarii</em>, <em>P. citrinum</em>, <em>Xylaria sp.</em>, <em>B. bassiana</em>, <em>Euplytella sp.</em></td>
</tr>
<tr>
<td><em>Aspergillus sydowii</em></td>
<td>Did not inhibit the growth of other species</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>B</td>
</tr>
<tr>
<td><em>Aspergillus ochraceopetaliformis</em></td>
<td>C, CB₂, A, B, CA₁, CA₁, CA₂, CA₁, CA₂, CB₁, CB₂, B, C, CA₁</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>C, B, CA₁, CA₁, CA₁, A, B, CA₂, A, B, CB₁, CA₁, CA₂, CA₁, B, CB₁</td>
</tr>
<tr>
<td><em>Xylaria sp.</em></td>
<td>CB₂, CA₁, A, CA₁, A, A, A, B, B, A</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>C, CB₂, C, B, C, A, CB₂, C, CA₁, CA₂, A, CB₁, BA, CA₁, CA₁, CB₁</td>
</tr>
<tr>
<td><em>Euplytella</em></td>
<td>C, CA₁, C, A, CB₂, A, C, A, A, B</td>
</tr>
</tbody>
</table>

This type of interaction was also observed in co-culture between *B. bassiana* and *A. ochraceopetaliformis* (Fig. 5h). Replacement without deadlock (Type C) and partial replacement after initial deadlock at mycelial contact (CA₁) were observed in all species except *A. terreus* and *A. sydowii*. For instance, *P. citrinum* was replaced by *B. bassiana* without initial deadlock (Fig. 5i). On the other hand, partial replacement was observed in *Xylaria sp.* by *A. ochraceopetaliformis* after initial deadlock (Fig. 5j). Complete
replacement after initial deadlock with mycelial contact \((CA_2)\) was observed in \(A.\ ochraceopetaliformis, A.\ tamarii\) and \(B.\ bassiana\). Figure 5k showed \(Aspergillus\ tamarii\) was completely replaced by \(A.\ ochraceopetaliformis\) after initial deadlock at mycelial contact. Partial replacement after initial deadlock at a distance \((CB_1)\) was observed in \(A.\ ochraceopetaliformis, A.\ tamarii, B.\ bassiana\) and \(P.\ citrinum\) while complete replacement after initial deadlock at a distance \((CB_2)\) was observed in all species except \(A.\ terreus\) and \(A.\ sydowii\). Co-culture between \(Euplytella\ sp.\) and \(P.\ citrinum\) (Fig. 5l); \(P.\ citrinum\) and \(B.\ bassiana\) showed Type \(CB_1\) interaction (Fig. 5m). On the other hand, \(Euplytella\ sp.\) and \(B.\ bassiana\) (Fig. 5n); \(Xylaria\ sp.\) and \(A.\ tamarii\) (Fig. 5o) showed type \(CB_2\) interaction. Hyphal extension was also observed in \(Euplytella\ sp\) which resulted to complete replacement of \(A.\ sydowii\) (Fig. 5p).

Interestingly, a complex pattern of interactions among \(A.\ ochraceopetaliformis, A.\ tamarii, P.\ citrinum, Xylaria\ sp., B.\ bassiana\) and \(Euplytella\ sp\). For instance, \(Aspergillus\ ochraceopetaliformis\) replaced the colony of \(A.\ sydowii, A.\ terreus, A.\ tamarii, P.\ citrinum, Xylaria\ sp., B.\ bassiana\) and \(Euplytella\ sp\). But its colony was also replaced by \(A.\ tamarii, P.\ citrinum, Xylaria\ sp., B.\ bassiana\) and \(Euplytella\ sp\). On the other hand, \(A.\ tamarii\) was replaced by \(P.\ citrinum, Beauveria\ bassiana\) and \(Euplytella\ sp\). Whereas, \(P.\ citrinum\) was replaced by \(A.\ ochraceopetaliformis, A.\ tamarii, B.\ bassiana\) and \(Euplytella\ sp\). Lastly, \(Xylaria\ sp.\) and \(Euplytella\ sp\) were replaced by \(A.\ ochraceopetaliformis, A.\ tamarii, P.\ citrinum\) and \(B.\ bassiana\). Overall, the type of interaction observed during co-culture assay was combination-specific in which different interspecific pairing resulted to different interaction (Table 1).

Based on antagonism index \((AI)\), all the species showed antagonistic activity against the other endophytes. Highest antagonistic activity was recorded from \(A.\ tamarii\) (Fig. 6). \(A.\ terreus\) and \(A.\ sydowii\) were observed to have very low antagonistic activity. Based on the antagonism index, there are three categories of fungal isolates. Active with \(AI > 15\), moderately active with \(AI\) between 10–15 and weakly active with \(AI < 10\). In this study, \(A.\ tamarii, A.\ ochraceopetaliformis, P.\ citrinum, B.\ bassiana, Euplytella\ sp.\) and \(Xylaria\ sp.\) were active antagonist while \(Aspergillus\ terreus\) and \(A.\ sydowii\) were weak antagonist.

In terms of radial growth, endophytic fungi showed inhibition on their antagonist at varying level. For instance, \(A.\ sydowii\) inhibited the radial growth of \(P.\ citrinum\) and \(B.\ bassiana\) at 15 and 36%, respectively. \(A.\ terreus\) slightly inhibited the growth of \(Xylaria\ sp.\) and \(B.\ bassiana\). \(A.\ ochraceopetaliformis\). Whereas, \(A.\ tamarii, P.\ citrinum, and Xylaria\ sp.\) showed radial growth inhibition in all of their antagonists. Highest growth inhibition of these species was recorded on \(A.\ sydowii\) and \(A.\ terreus\). \(B.\ bassiana\) showed growth inhibition in all species except on \(A.\ terreus\). \(Euplytella\ sp.\) did not have inhibition on the growth of \(A.\ terreus\) and \(A.\ ochraceopetaliformis\) (Table 2).
The production of metabolites during mycelial interaction between two species was confirmed in non-volatile compounds assay. Using fungal crude extract, all the species of endophytic fungi had radial growth inhibition. Crude extract from *A. tamarii* had the highest inhibition rate. No growth in *A. sydowii*, *A. terreus* and *Euplytella sp.* was observed when exposed to the crude extract of *A. tamarii* but presence of exudates was observed indicating endophyte interaction. In other species, radial growth inhibition was manifested by low mycelial mass (Table 3).

### Table 2

Inhibition rate (% of the 8 species of endophytic fungi in seagrasses by co-culture assay.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Species co-paired with the antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. sydowii</em></td>
</tr>
<tr>
<td>Aspergillus sydowii</td>
<td>0.00</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Aspergillus ochraceope taliformis</em></td>
<td>68.5 ± 4.29</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em></td>
<td>72.50 ± 2.95</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>63.71 ± 3.81</td>
</tr>
<tr>
<td><em>Xylaria sp.</em></td>
<td>62.50 ± 2.07</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>41.11 ± 1.99</td>
</tr>
<tr>
<td><em>Euplytella sp.</em></td>
<td>28.75 ± 3.94</td>
</tr>
</tbody>
</table>

(±) standard deviation, (*) significant p = value, CI = 95%
Table 3
Inhibition rate (%) of endophytic fungi in seagrasses by non-volatile assay.

<table>
<thead>
<tr>
<th>Source of the extract-treated media</th>
<th>Inhibition rate (%) in each species of endophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. sydowii</td>
</tr>
<tr>
<td>Aspergillus sydowii</td>
<td>22.20 ± 1.53</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>16.70 ± 3.06</td>
</tr>
<tr>
<td>*Aspergillus ochraceopetaliformis</td>
<td>75.00 ± 3.28</td>
</tr>
<tr>
<td>*Aspergillus tamarii</td>
<td>100.00</td>
</tr>
<tr>
<td>*Penicillium citrinum</td>
<td>100.00</td>
</tr>
<tr>
<td>*Xylaria sp.</td>
<td>100.00</td>
</tr>
<tr>
<td>*Beauveria bassiana</td>
<td>45.50 ± 2.83</td>
</tr>
<tr>
<td>*Euplytella sp.</td>
<td>35.00 ± 0.52</td>
</tr>
</tbody>
</table>

(±) standard deviation, (*) significant p = value, CI = 95%

Discussion

Colonization of endophytic fungi in seagrasses

Histological examination of seagrass leaves, rhizomes and roots showed the colonization of endophytic fungi in hosts’ tissues. Endophytic fungi first colonize the epidermal cell layer through the intercellular spaces. It seldom colonized the intracellular space of the epidermal cells because these cells are lignified and contained suberin, tannin and cuticle which gave difficulty for fungal hyphae to penetrate intracellularly (Larkum et al. 2006). Unlike the epidermal cells, hyphal colonization in the cortical cells were dense because the cells were less lignified. Moreover, there is little competition for space in the cortical cells because it has large air lacuna. There is also reliable source of nutrition in the intracellular space of cortical cells because it has rich in organic and inorganic nutrients (Kulda and Bacon 2008; Kandel et al. 2017).

Endophytic colonization in both intercellular and intracellular spaces in the leaves were also reported in C. serrulata, T. hemprichii and Halophila ovalis indicating endophytes’ close interaction with their hosts (Raja et al. 2016). The penetration of fungal hyphae to neighboring cells through the cell wall indicated carbohydrate enzymatic digestion. However, some findings of our study were different from the report of Raja et al. (2016). For instance, fungal hyphae colonized the neighboring cells even a single cell was not yet completely filled up. No fungal spores were detected in the leaves and vascular tissues were free from fungal hyphae.
In rhizomes and roots, fungal hyphae were dense in the cortex both intracellularly and intercellularly. There were also scanty fungal hyphae at the epidermal cells and absent in the vascular tissues as observed in *Posidonia oceanica*. But contrary to the dark septate endophytes in *P. oceanica* roots, endophytes in this study reached and densely colonized the cortical cells (Vohnik et al. 2017). Hydrolytic enzymes produced by endophytic fungi like *Xylaria, Aspergillus* and *Penicillium* may help lyse the cell wall of the cortical cells allowing them fungi to penetrate and colonize the cortex (Hamzah et al. 2018; Al Shibli et al. 2019; Sharma et al. 2021).

Variation in endophytic colonization and distribution in seagrass species could be attributed to the intergeneric hosts’ defense variation such as antifungal compounds. According to Supaphon et al. (2014), seagrasses produce antifungal compounds such as alkaloids, phenolic acids, terpenes, tannins, flavonoids, alipathic and aromatic compounds which influence the colonization and distribution of endophytic fungi. Amount and types of these compounds vary with the species of seagrass, age, as well as tissues examined. Phytochemical analysis of crude extract showed that *C. serrulata* contained the highest amount of phenol, tannin and flavonoids compared to *Enhalus acoroides* and *Thalassia hemprichii*. Among the three *E. acoroides* had the lowest (Ragupathi Raja Kannan et al. 2013). Based on the type of phenol and phenylpropanoid derivatives, the three seagrass species also vary. *E. acoroides* had coumaric acid but *Cymodocea spp.* and *Thalassia hemprichii* had none instead it had caffeic acid. Both *E. acoroides* and *T. hemprichii* had protocatechic acid while *Cymodocea spp.* had vanillic acid. Furthermore, the flavonoids of *E. acoroides* and *T. hemprichii* were sulfated (Subhashini et al. 2013).

In this study, absence of hyphal colonization in the vascular tissues of seagrasses suggested non-systemic fungal colonization. According to Gao and Mendgen, 2006, the growth of endophytic fungi is maintained and restricted in defined areas of the plant tissues by the hosts’ defense reaction. For instance, cell wall and membrane are strengthened and plant innate immune responses are activated resulting to reactive oxygen species generation; defense genes expression and hormones synthesis (Gebrei 2016). In *Pisum spp.* resistance to progression *Fusarium oxysporum* in the roots was established by both physical and chemical barriers. The fungi were restricted at the root surface by cell wall lignification and formation of papillae-like structures blocking hyphal penetration. Polyphenolic and carbohydrate compounds accumulated at the intercellular space especially around the vessel elements (Bani et al. 2018). In this study, histological sections using light microscopy were not able to observe such defense responses from seagrass tissues when colonized by fungal hyphae but restriction of most fungal hyphae to cortical cells indicated the presence of host defense reaction.

The fungal-seagrass interaction in this study showed an endophytic relationship and not pathogenesis. Pathogenic colonization was described as extensive hyphal development within vascular tissue, invasion of heavily branched mycelia in many cells, extensive cell wall degradation. Optical microscopy on the longitudinal section of *Lasiodiplodia theobromae*, a phytopathogenic fungus of cashew revealed extensive colonization in the secondary xylem of its host tissues with disintegration of the cell wall (Muniz et al. 2011). Unlike the pathogens, fungal hyphae in this study did not colonize the vascular tissues of seagrasses. Moreover, no sign of necrosis nor disorganization of the cytoplasmic contents was observed suggesting that these fungi grew in a non-pathogenic interaction with seagrasses.

Differences in the structure of fungal hyphae found in the tissues of the three seagrass species implied several species colonizing the host. These results would appear to corroborate the conclusions of Raja et al. (2017) that endophytes within leaves represent a mosaic of localized colonization. These fungal species interact with each other which may influence their colonization and distribution inside host.

**Antagonistic activity**

Plants harbor diverse microbial communities including endophytic fungi in its tissues which interact dynamically with each other (Alam et al. 2021). In this study, all the species of endophytic fungi showed antagonistic interactions by inhibition of radial growth in one colony by the other by during co-culture and non-volatile assays. Morphological change of the colony and production of inhibitory metabolites, enzymes and pigments during mycelial interaction are responsible for antagonistic activity (Hiscox and Boddy 2017). Hyphal aggregation during co-culture of *Beauveria bassiana* and *Euplytella sp.* suggested physical blocking of each other's colony from invading and replacing their individual territory. Without interaction, colonies of these species were dispersed. Secondary metabolites like tannin, phenols, flavonoids and hydrolytic enzymes produced by *Aspergillus tamarii* (Campos et al. 2019) *Eutypella sp.* (Sibero et al. 2019) *Penicillium citrinum* (Rahaman et al. 2020, Sharma et al. 2021)
bassiana (Deb et al. 2017) could be responsible to deadlock at mycelial contact by co-culture. Inhibition of radial growth by non-volatile compounds assay supported the production of inhibitory metabolites by these species during mycelial interaction.

The demarcation line observed in Xylaria sp. when paired with Aspergillus ochraceopetaliformis is probably the result of production of high quantities of inhibitory metabolites. Xylaria sp. is known to produce inhibitory compounds like cytochalasin, pilifomic acid and griseofulvin (Elias et al. 2018). Moreover, pigmentation observed in the colony of Xylaria sp. when paired with Aspergillus ochraceopetaliformis, Aspergillus tamarii and Euplytella sp. could be an indication of extracellular pigment production like carotenoid, melanin, flavins, phenazines and quinones to protect its mycelia from the harmful effect of reactive oxygen species (ROS), toxins and hydrolytic enzymes. This is also observed in the study of Hamzah et al. (2018).

Deadlock at mycelial distance observed between two colonies like Xylaria sp. and Beauveria bassiana suggesting the production of volatile organic compounds (VOC). 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 Halophila ovalis produced bioactive xylariphilone, an azaphilone derivative (Arunpanicklert et al. 2018). On the other hand, 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), the effects of volatile organic compounds can reach until 15mm in culture media. This could be significant enough to inhibit replacement. Other species of endophytic fungi in this study have been also reported to produce variety of VOCs. Ditryptophenaline was produced by A. ochraceopetaliformis from the coral reef of Red Sea (Abd El Rahman et al. 2020). Bioactive eutypellenoids from Eutypella sp. (Yu et al. 2018), sesquiterpenes eutyscoparin, steroid eutyscoparene, and terpenoid were isolated from endophytic Eutypella scoparia (Zhang et al. 2021).

Replacement interaction observed in this study could be a result of potent metabolite production by the active antagonists that is detrimental to the overgrown colony. For instance, P. citrinum (Sharma et al. 2021) A. ochraceopetaliformis (Liu et al. 2020), and Xylaria sp. (Hamzah et al. 2018) produced hydroptic enzymes which break down the cell wall of its antagonists. This species also produced antifungal compounds such azaphilone, cytochalasin (de Felicio et al. 2015), pilifomic acid and griseofulvin (Elias et al. 2018). A. ochraceopetaliformis secreted a new-pyrone clyxofuranocide molecule observed to have strong antifungal activity (Asmaey et al. 2021). A. tamarii had 70% fungal growth-reducing potential and ability to suppress the production of mycotoxin like ochratoxin A in many fungal species (de Almeida et al. 2019). On the other hand, due to high energy requiring process of metabolite production (Hiscox and Boddy 2017), weak antagonists like A. terreus and A. sydowii might ceased to produce inhibitory metabolites during mycelial interaction as observed in H. fasciculare (El Ariebi et al. 2016), resulting to replacement.

This study presented that the outcome of interaction is not always the same even with the same combination of species. For instance, co-culture of A. tamarii and Xylaria sp. resulted to deadlock, partial replacement and complete replacement. Different sensitivity to abiotic factors like water potential, gaseous exchange and temperature are among the reasons for different outcomes of interaction. For instance, Xylariaceous ascomycete are less sensitive to low water potential (Boddy 2000) while Aspergillus and Penicillium have the ability to tolerate wide a range of salinity (Cha et al. 2021). Moreover, the interactions among endophytic fungi in this study were not linear instead, following a circular pattern. For instance, it was observed in this study that even though A. tamarii was the most active antagonist having the highest antagonism index value, but its colonies were replaced by P. citrinum. On the other hand, P. citrinum, was replaced by Euplytella sp. which was then replaced by A. tamarii. This pattern is described by Boddy (2000) as cyclical competition structure of intransitive interactions in which species A is more combative than B, but B is more combative than species C which was more combative than species A. In wood saprophytic basidiomycete, intransitivity promoted species coexistence despite having strong antagonism among species because there was no clear hierarchy of superiority at the community level. So, complex and dynamic interaction between endophytic fungi in seagrasses determined their composition and distribution.

The ecological importance of endophyte-endophyte interaction in terrestrial plants suggested enhanced plant growth and resistance to pathogens. In seagrasses, increase vigor and pathogen resistance brought about by the production of novel metabolites from endophyte-endophyte interaction could be possible. Nevertheless, a comprehensive investigation at the molecular level is vital to elucidate the mechanism of this 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 citrinin dimer seco-penicitrinol A and L, 8 citrinin derivatives and phenol (Yang et al. 2018). Co-culture of marine-derived *P. citrinum* and *Beauveria feline* produced an anti-pathogen, citrifelins (Meng et al. 2015). The production of lovastatin by *A. terreus* increased because of the strong inducing effect of *Aspegillus unguis* during co-culture (Wang et al. 2022). Co-culture of endophytic fungi, *Aspergillus* sp., *Fusarium* sp., and *Ramularia* sp. from *Rumex gmelini* increased the yield of antifungal, chrysophanein, resveratrol, 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 cluster. For instance, endophytic *Eutypella* sp. from the sponge had polylketide synthase (PKS I & II) and non-ribosomal peptide synthase (NRPS) but these are suspected silent or unexpressed (Sibero et al. 2019). Thus, the endophytic fungi from seagrasses offered vast opportunities and co-culture of different species should be considered to take advantage of their antagonistic activity in upregulating silent genes for novel metabolite production.

### Conclusion And Recommendations

Endophytic fungi have close interaction with their seagrass hosts. Dense fungal colonization in the intracellular space of the cortex suggested as the most favorable space for the endophytic fungi because of less competition for space and availability of nutrients to support their growth. The absence of endophytic fungal colonization in the vascular tissues indicated restriction by the hosts’ defense mechanism. However, this needs to be elucidated at the molecular level for comprehensive understanding.

Endophyte-endophyte interaction in seagrasses was documented through antagonistic activity manifested by all the species. Antagonism was done by deadlock and replacement. This suggested that each colony produced inhibitory metabolites which restrict each other’s growth both upon mycelial contact and at a distance. If one colony ceased to produce inhibitory molecules, it is outcompeted and replaced by the more active colony. Therefore, when endophytic fungi colonized the tissues of seagrasses, there growth and distribution are determined by their interaction with other endophytes. Further, the results of the study showed intransitive interactions where each species vary its susceptibility to antagonistic potential of other species allowing co-existence among different species.

A multiple species interaction is recommended to make the understanding of endophyte-endophyte interaction ecologically meaningful since the endophytic community in seagrasses is composed of mixed species. Outcome of interaction involving several species may also elicit different responses and set of secondary metabolites. The role of genes and proteins in understanding the intricate and complex antagonistic interactions should be investigated.

### Declarations

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#### Competing Interests

The authors have no relevant financial and non-financial interests to disclose.

#### Data Availability

The datasets generated during this study are available from the corresponding author on a reasonable request. DNA sequences of fungi isolated from this study were deposited in GenBank.

#### Code Availability

Not Applicable

#### Authors’ Contribution
All authors contribute to the study conception and design. Material preparation and data collection were performed by Venus B. Kinamot. Data analyses and preparation of manuscript were performed by Venus B. Kinamot and Alvin P. Monotilla. All authors read and approved the final manuscript.

**Ethics Approval**

Not Applicable. This study did not involve human or animals.

**Consent to Participate**

Not Applicable. This study did not involve human or animals.

**Consent for Publication**

Not Applicable. This study did not involve human or animals.

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Figures
Figure 1

Cross-section of seagrass (a) leaves, (b) rhizomes and (c) roots. e= epidermis, l= air lacuna, m= mesophyll cells, v= vascular cambium, oc= outer cortex, mc= middle cortex, ic= inner cortex.

Figure 2
Endophytic fungal colonization in the (a-c) leaves and (d-f) rhizomes of *Cymodocea serrulata*. Red arrow is pointing the fungal hyphae.

**Figure 3**

Endophytic fungal colonization in the (a-c) leaves, (d-e) rhizome and (f-g) roots of *Enhalus acoroides*. Red arrow is pointing the fungal hyphae.
Figure 4

Endophytic fungal colonization in the (a-b) leaves and (d-e) rhizome of *Thalassia hemprichii*. Red arrow is pointing the fungal hyphae.
Figure 5

Interactions of the 8 species of endophytic fungi by co-culture assay. (a-f) Deadlock at mycelial contact, (g-h) Deadlock at a distance, (i) Replacement with no deadlock, (j) partial replacement after initial deadlock at mycelial contact, (k) complete replacement after initial deadlock at mycelial contact, (l-m) partial replacement after initial deadlock at a distance, (n-p) complete replacement after initial deadlock at a distance. At= Aspergillus tamarii, Ao= Aspergillus ochraceopetaliformis, Bb= Beauveria bassiana, E= Euplytella sp, Pc= Penicillium citrinum, X= Xylaria sp.
Antagonistic index of seagrass fungal isolates by co-culture assay. Active = AI > 15, moderately active = AI between 10-15, weakly active = AI < 10.

**Figure 6**