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Short communication

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Fungal endophytes of the seagrasses *Halodule wrightii* and *Thalassia testudinum* in the north-central Gulf of Mexico

Abstract: The purpose of this note is to communicate the diversity of endophytic fungi isolated from living leaves of *Halodule wrightii* and *Thalassia testudinum*, two ubiquitous seagrasses in the north-central Gulf of Mexico. Fungi were isolated from surface-sterilized leaf fragments of the two plants that had been inoculated onto seawater malt extract agar. Leaves of *T. testudinum* harbored slightly more endophyte taxa than those of *H. wrightii*. *Trichocladium alopallonellum* and 10 other fungal taxa are new records for *H. wrightii*. No distinct seasonal pattern in fungal filamentous growth was observed; however, our evidence suggests peak activity may take place in spring.

Keywords: abundance; ecology; filamentous fungi; seagrass.

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Filamentous fungi have been recorded in many freshwater, estuarine, and marine ecosystems worldwide (Kohlmeyer and Kohlmeyer 1979). Together with bacteria, filamentous fungi, mainly members of the Ascomycota, play an important role in the decomposition and nutrient recycling of certain freshwater and marine detritus, such as detritus from marshes and mangroves (Newell 1996, Gessner et al. 1997, Walker and Campbell 2010). Filamentous fungi can also grow as endophytes within tissues of marine plants such as seagrasses without causing any harm to the hosts (Devarajan et al. 2002, Sakayaroj et al. 2010). However, much work remains to be done in order to establish how prominent filamentous fungi are in seagrass beds, particularly for some seagrass species where fungal occurrence has been little studied (such as *Halodule wrightii* Asch.). This is an important step toward an understanding of the role of filamentous fungi in the ecology of seagrass beds.

Seagrass beds are ubiquitous in shallow waters of the Gulf Coast region of the United States (Duke and Kruczynski 1992), where they play a number of crucial ecological roles for the well-being of marine life and humankind (Duarte 2002). However, seagrass beds are suffering from anthropogenic impacts, such as reduced water clarity by dredging and siltation, leading to a decline in their numbers worldwide (Orth et al. 2006). In temperate and subtropical latitudes, seagrass beds typically show a seasonal cycle in the production of leaf biomass and detritus. Leaf growth is stimulated in early spring as temperature and photoperiod increase, which results in high accumulation of leaf biomass throughout the summer. In late summer/early fall, as temperature and photoperiods drop, most of the leaf biomass accumulated throughout the summer senesces and sheds, which leads to the large accumulation of leaf detritus on the bottom of the bed (Sauers 1981, Cebrián et al. 1997). Whether this seasonal cycle is related to oscillations in the activity of filamentous fungi is not well known (Sathe and Raghukumar 1991).

The purpose of this note is twofold. First, we communicate the diversity of endophytic filamentous fungi in the leaves of two dominant seagrass species, *Thalassia testudinum* Kon. and *H. wrightii*, in the north-central Gulf of Mexico. Second, we examine whether the abundance of endophytic fungi on leaf-inoculated Petri plates varies seasonally in an attempt to elucidate potential correlations between the seasonal cycle of seagrass growth and fungal activity.

Of a total of 758 plates inoculated with leaf fragments, fungal filamentous growth occurred on 236 (31.1%), whereas 143 (18.9%) were contaminated with bacteria and 379 (50%) displayed no growth (Table 1). Most plates were inoculated with *H. wrightii* (73.4%), as most of the samples collected in the field corresponded to this species, and consequently more isolates in terms of absolute number of plates were cultured from this species. However, a

Table 1 Number of plates with filamentous growth, with no growth, or that were contaminated with bacteria out of the total number of plates inoculated with leaf fragments.

	Plates	Plates with filamentous fungi	Plates with bacteria	Plates with no growth
Thalassia testudinum	202 (26.6%)	73	34	95
Halodule wrightii	556 (73.4%)	163	109	284
Total	758 (100%)	236 (31.1%)	143 (18.9%)	379 (50.0%)

Our study site was the East Cove lagoon located in Big Lagoon State Park, Florida. The lagoon (30.308° N, 87.403° W) is in pristine condition, surrounded by salt marshes, and populated by shoalgrass (*Halodule wrightii*) (Stutes et al. 2007). Patches of turtlegrass (*Thalassia testudinum*) are located on the sandy outer banks of the lagoon. Living seagrass leaves were collected once monthly between November 2008 and August 2009 at four random locations within the lagoon (*H. wrightii*) and at one location outside the lagoon (*T. testudinum*). The leaves collected were attached to the shoots and were mostly green. Samples were placed in clean quart-sized ziploc bags and transported in a cooler with ice packs to the laboratory for immediate processing.

Methods for isolation, culture, and observation of fungi were modified from Vrijmoed (2000). Blades were gently scraped, when covered with periphyton, to exclude any epiphytic fungal presence. Seagrass blades were surface sterilized by placing them in 0.5% bleach solution and then in sterile saltwater solution (instant ocean 35 g l^{-1}) for 2 min each. Fragments 2–3 cm in length were cut from the mid-section of the sterilized blades. Two to three leaf fragments were placed onto 60×10 mm disposable Petri plates containing saltwater malt extract agar (swMEA) and antibiotics (5% penicillin, 5% streptomycin). A total of 15–20 plates were inoculated for each sampling location per sampling date, for a grand total of 758 plates. Plates were kept at room temperature and observed every other day for up to 4 weeks for fungal growth emerging from the leaf fragments under a stereomicroscope.

slightly higher isolation frequency was observed in *T. testudinum* (73/202=36%) when compared with *H. wrightii* (163/556=29%).

We isolated 127 fungal colonies out of the 236 plates where we observed filamentous growth, with 69 plates corresponding to fungi isolated from *H. wrightii* leaf fragments and 58 plates to *T. testudinum* (Table 2). The proportion of isolated fungal colonies in relation to plates with fungal filamentous growth was higher for *T. testudinum* (58/73=79%) than for *H. wrightii* (69/163=42%). About 64% of the total occurrences were fungus-like organisms (mainly Labyrinthulomycetes) and non-sporulating fungi (termed "sterile mycelia") in both seagrasses (Table 2). Except for *Penicillium* sp., no other taxa reached values higher than 10% of occurrence on either seagrass. *Trichocladium alopallonellum* (Meyers & R.T. Moore) Kohlm. & Volkm.-Kohlm., *Halenospora varia* (Anastasiou) E.B.G. Jones, *Penicillium* sp., *Fusarium* sp., *Cladosporium* sp., and *Pestalotiopsis* sp. were present on both seagrasses. *Dendryphiella arenaria* Nicot, *Exserohilum halodes* (Drechsler) K.J. Leonard & Suggs and *Lindra thalassiae* Orpurt, Meyers, Boral & Simms, *Trichoderma* sp., and *Phoma* sp. only occurred on *T. testudinum*. *Arthrobotrys* sp., *Phialophora* sp., and *Aspergillus* sp. were only found on *H. wrightii*. The number of isolated fungal taxa (excluding sterile mycelia and fungus-like organisms) colonies was slightly higher in *T. testudinum* (11) than in *H. wrightii* (9).

The frequency with which plates inoculated with *H. wrightii* leaves showed filamentous fungal growth varied largely among the different sampling times (Figure 1). For instance, in January, only ca. 8% of the inoculated plates displayed growth, whereas ca. 70% of the plates displayed growth in April. The frequency of filamentous fungal growth on *H. wrightii* leaves displayed significantly higher values in the spring months (March and April) than during most of the rest of the year, as shown by the 95% confidence intervals around the frequency values (Figure 1). The frequency with which plates inoculated with *T. testudinum* leaves showed filamentous fungal growth also varied largely among the different sampling times, from a minimum 8% in March to a maximum 100% in April. Unlike for *H. wrightii*, the frequency for *T. testudinum* leaves was not higher in the spring months than during most other months, but instead it displayed alternately high and low values through successive months.

Our survey has identified nine taxa of endophytic filamentous fungi cultured from green leaves of the seagrass *H. wrightii*, excluding non-sporulating fungi grouped as "sterile mycelia." This represents an unprecedented account of fungal diversity reported from this seagrass species. Our results also build upon previous reports of fungal occurrence on the seagrass *T. testudinum* (Meyers et al. 1965, Wilson 1998). The common marine species *Dendryphiella arenaria*, *Halensopora varia*, and *Lindra thalassiae* and other so-called facultative marine species such as *Exersohilum halodes*, *Phoma*, *Fusarium*, *Penicillium*, *Pestalotiopsis*, *Arthrobotrys*, and *Cladosporium* have been reported on diverse marine substrates, including *T. testudinum* (Kohlmeyer and Kohlmeyer 1979, Kohlmeyer 1980, Kohlmeyer and Volkmann-Kohlmeyer 1991, Jones and Puglisi 2006, Jones et al. 2009, Sakayaroj et al. 2010).

Table 2 List of endophytic filamentous fungi and their occurrence observed on leaves of *Halodule wrightii* and *Thalassia testudinum*.

	Halodule wrightii			*Thalassia testudinum*			Total no. of occurrence	% Occurrence
	No. of occurrence	% Occurrence	Isolated fungal colonies	No. of occurrence	% Occurrence	Isolated fungal colonies		
Sterile mycelia	52	31.9	26	40	54.8	29	92	39.0
Fungus-like organisms	56	34.4	10	3	4.1	3	59	25.0
Penicillium sp.	20	12.3	6	2	2.7	1	22	9.3
Trichocladium alopallonellum	12	7.4	10	4	5.5	4	16	6.8
Fusarium sp.	10	6.1	8	4	5.5	5	14	5.9
Halenospora varia	4	2.5	3	5	6.8	2	9	3.8
Pestalotiopsis sp.	3	1.8	1	1	1.4	1	4	1.7
Arthrobotrys sp.	3	1.8	2	0	0.0	0	3	1.3
Phialophora sp.	1	0.6	1	0	0.0	0	1	0.4
Aspergillus sp.	1	0.6	1	0	0.0	0	1	0.4
Cladosporium sp.	1	0.6	1	1	1.4	1	2	0.8
Exserohilum halodes	0	0.0	0	1	1.4	1	1	0.4
Dendryphiella arenaria	0	0.0	0	1	1.4	1	1	0.4
Trichoderma sp.	0	0.0	0	3	4.1	3	3	1.3
Phoma sp.	0	0.0	0	4	5.5	4	4	1.7
Lindra thalassiae	0	0.0	0	4	5.5	3	4	1.7
Total	163		69	73		58	236	

The term "number of occurrence" represents the total number of hyphal emergences from the leaf fragments on swMEA for each fungal taxon. Occurrence (%) equals the number of plates in which filamentous growth of each fungus on swMEA was observed divided by total number showing filamentous growth for each seagrass ×100. Isolated fungal colonies were obtained by axenically subculturing hyphae growing out of leaf fragments onto new swMEA plates. Mycelia and reproductive structures of all isolated fungal cultures were mounted on a slide with 5% potassium hydroxide and observed under an Olympus CX41 light microscope (Pittsburgh, PA, USA). Dichotomous and pictorial keys in Kohlmeyer and Kohlmeyer (1971), Kohlmeyer and Volkmann-Kohlmeyer (1991), and Jones et al. (2009) were employed for taxonomic identification.

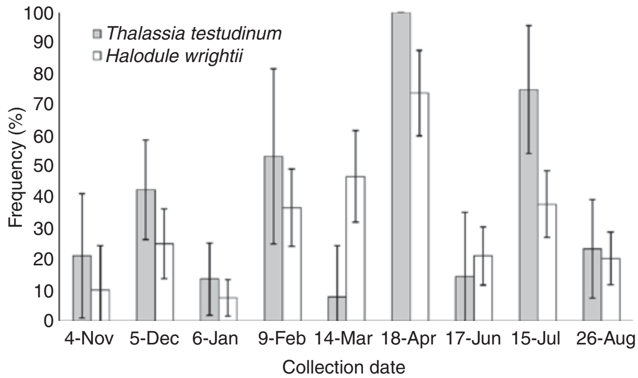

Figure 1 Frequency of fungal filamentous growth on *Thalassia testudinum* (solid bars) and *Halodule wrightii* (open bars) collected in different months.
Frequency was calculated by dividing the number of plates with fungal filamentous growth by the total number of inoculated plates for each seagrass for each collection date. Error bars correspond to 95% confidence intervals.

Newell and Fell (1980) reported fungus-like organisms and mycelia sterilia in their work with *T. testudinum*. *Trichocladium alopallonellum*, a fungus previously reported on woody substrates (Kohlmeyer and Kohlmeyer 1971), is a new record on *T. testudinum* and *H. wrightii*. Our previous work has also reported for the first time the occurrence of the obligate marine fungi *Trichocladium achrasporum* (Meyers and R.T. Moore) Dixon and *Cumulospora marina* I. Schmidt on the seagrass *Ruppia maritima* L. (Paradis et al. 2009).

It is possible that our technique favored isolation of certain fungi while failing to notice those either with a slower growth or not able to produce any filaments in the malt extract medium used here. Thus, the inventory of fungal taxa in this study is likely conservative. However, our results show that filamentous fungi can be diverse and common on leaves of *H. wrightii* and *T. testudinum* and, thus, may play important ecological roles for these seagrass species.

The apparent difference in the number of endophyte taxa between these two seagrasses was small. Yet, they could at least suggest that, although endophytic filamentous fungi are common and diverse on the leaves of both species, *T. testudinum* leaves may be a better substrate for fungi than *H. wrightii* leaves, possibly due to larger leaf surface area and higher dry matter (Siegal-Willott et al. 2010). Additionally, *T. testudinum* leaves have a longer life span than *H. wrightii* leaves (Duarte 1991), which may favor fungal establishment.

Despite substantial month-to-month variability, the frequency with which plates inoculated with *H. wrightii* leaves displayed fungal filamentous growth was generally higher in spring than in other seasons. These differences must be interpreted with caution as we only sampled for a 10-month period (from November 2008 to August 2009) and all samples collected in May 2009 were lost due to mold contamination. The higher occurrence of fungal growth on the leaves of this species in spring might be associated with the stimulation of leaf metabolic and growth rates that typically occur in this season as temperature and photoperiod increase (Marba et al. 1996, Cebrián et al. 1997). Enhanced leaf metabolic activity in spring could increase the availability of metabolites for fungi, thereby stimulating their growth and resulting in the higher frequency of plates with grown fungal filaments observed in this season. Leaf metabolic activity may be reduced in the summer as a result of intense self-shading imposed by thick leaf canopies, and in the fall and winter with declining temperatures and photoperiod (Enríquez and Pantoja-Reyes 2005, Ralph et al. 2007), which would in turn depress fungal growth. However, this apparent seasonality in fungal growth was not observed for *T. testudinum*, despite the fact that this species follows similar seasonal patterns of leaf growth and metabolic rates to *H. wrightii* (Patriquin 1973, Anton et al. 2009). More work is needed to determine the nature and controls of temporal oscillations of fungal growth and abundance in seagrass beds.

In conclusion, this is a first report of endophytic fungi associated with the ubiquitous seagrasses *H. wrightii* and *T. testudinum* in the north-central Gulf of Mexico. Endophytic fungi occurred either on both or on only one seagrass species, although our results suggest that *T. testudinum* leaves are hosts to a larger number of taxa. *Trichocladium alopallonellum* is a new fungal record on both seagrasses. Different monthly growth frequencies suggest that seasonal patterns in fungal abundance may exist, with higher growth possibly occurring in spring. Overall, this study points to a number of potentially important avenues of research, such as the ecological roles of the diverse communities of fungi in seagrass beds, whether fungal diversity and abundance on seagrass leaves vary with leaf structural attributes (such as surface area, lignocellulose content, and life span), and the existence and controls of seasonal cycles of fungal abundance and growth in seagrass beds.

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