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Antifungal defenses of seagrasses from the Indian River Lagoon, Florida

Cliff Ross *, Melany P. Puglisi, Valerie J. Paul

Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, FL 34949, United States

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Abstract

We investigated the antifungal chemical defenses and physiological responses of five seagrasses collected from nearshore seagrass beds from the Indian River Lagoon, Florida, against a panel of co-occurring marine fungi isolated from nearby coastal communities. Whole plant tissues from *Thalassia testudinum*, *Halodule wrightii* and *Syringodium filiforme* prevented overgrowth by three of the seven fungi used in this study. Organic extracts from four of the five seagrasses inhibited the growth of at least one fungal strain. The extract from *Ruppia maritima* exhibited the highest antifungal activity, inhibiting the growth of three fungi including the pathogen *Lindra thalassiae*. Among the fungal panel, *Fusarium* sp. 2 was the most susceptible to seagrass extracts, whereas none of the extracts disrupted the growth of *Dendryphiella salina* and *Fusarium* sp. 3. Under laboratory conditions fungal inoculation elicited hydrogen peroxide production in all specimens within 25 min post-inoculation as measured with a redox sensitive dichlorodihydrofluorescein diacetate (DCFH-DA) assay. The concentration of H_2O_2 released into the immediate vicinity of infected seagrasses varied between 0.10 and 0.85 μmol g^{-1} FW min^{-1} depending on seagrass species and pathogen combination. Longer term incubation (days) of *T. testudinum* with homogenates of *D. salina* or *L. thallasiae* resulted in the induction of caspase activity, a known proteolytic activator of apoptotic and inflammatory activities. The application of micromolar concentrations of H_2O_2 to blades of *T. testudinum* induced caspase activity suggesting that fungal detection, H_2O_2 production, and caspase activation occur in a consecutive order. The seagrasses examined in this study appear to use a combined strategy to combat fungal infection, including microbial chemical defenses and signaling pathways observed in terrestrial plants.
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Keywords: Antifungal chemical defense; Caspase; Indian River Lagoon; Oxidative burst; Programmed cell death; Seagrass

1. Introduction

Seagrass stands are susceptible to periodic outbreaks of disease in which marine pathogens can cause devastating localized effects, resulting in the loss of valuable habitat (Porter, 1986; Robblee et al., 1991). The ‘‘eelgrass wasting disease’’ epidemic of *Zostera marina* in the 1930s was the most notorious outbreak resulting in massive destruction of eelgrass beds on both coasts of the Atlantic Ocean (Milne and Milne, 1951; Cottam and Munro, 1954). The marine slime mold *Labyrinthula zosterae* was the suspected pathogen, but was not clearly identified until a more localized and less severe outbreak developed in the 1990s along the coasts of North America and Europe (Short et al., 1986; Muehlstein, 1992). Outbreaks of disease in seagrass beds of *Thalassia testudinum* are most often attributed to the protist pathogen *Labyrinthula* sp. (Muehlstein, 1992), but have also been caused by fungal pathogen *Lindra thalassiae* (Porter, 1986). Etiological studies suggest that these organisms are secondary decomposers of senescent or stressed seagrasses, yet there is some evidence to suggest they may be also opportunistically pathogenic (Muehlstein, 1992). Environmental stressors such as low light or high temperatures may compromise the resistance of seagrasses resulting in higher levels of infection. An interesting observation of seagrass disease outbreaks, as well as many other disease outbreaks on tropical reefs, is that infections usually only occur in a single or related species suggesting that neighboring species may maintain a physical or chemical defense against infection (Engel et al., 2002).

Pathogenically induced chemical defense responses in marine plants are not well understood. It has been suggested

* Corresponding author. Present address: Department of Biology, University of North Florida, 1 UNF Drive, Jacksonville, FL 32224, United States. Tel.: +1 904 620 2830; fax: +1 904 620 3885.
E-mail address: cliff.ross@unf.edu (C. Ross).

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that seagrasses produce secondary metabolites that have a defensive role against marine pathogens (Jensen et al., 1998; Puglisi et al., 2007). For example, flavone glycosides isolated from *T. testudinum* were shown to inhibit the growth of the thraustochytrid (zoosporic fungus) *Schizochytrium aggregatum* in laboratory assays (Jensen et al., 1998). In other studies, the production of phenolic acids increased in *Z. marina* and *T. testudinum* during times of pathogenic infection (Vergeer and Develi, 1997; Steele et al., 2005). Steele et al. (2005) showed that infection of *T. testudinum* with Labyrinthula sp. caused phenolic acids to accumulate above, but not below, infection sites. They termed this response ''pseudo-induction'', which they attributed to the accumulation of carbon-based compounds in tissues above wound sites.

In addition to the synthesis of secondary metabolites that may have anti-pathogenic properties, the production of reactive oxygen species (ROS) has also been shown to significantly contribute towards the survival of many plant species. Fungal pathogens have been demonstrated to elicit the production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) (Huckelhoven et al., 2001; Huckelhoven and Kogel, 2003). Aside from functioning as a direct toxic agent against invading microbes, ROS have been demonstrated to be a critical component of the plant–pathogen hypersensitive response involved in cell wall strengthening (Otte and Barz, 1996), the activation of defense genes (Jabs et al., 1997), caspase activation (Ge et al., 2005) and the establishment of programmed cell death, which results in the limitation of pathogen penetration and propagation (Levine et al., 1994; Lamb and Dixon, 1997).

To date, little information exists describing the antifungal defense systems in seagrass species, both on the biochemical level and physiological response level. The goals of this study were to: (1) determine if plant tissues and organic extracts from common Florida seagrasses exhibit antifungal activity against co-occurring strains of potentially harmful marine fungi; (2) identify if seagrasses have the ability to produce ROS in response to fungal recognition and (3) determine if these ROS have a signaling role such as in the activation of caspase activity as observed in terrestrial plants.

2. Materials and methods

2.1. Seagrass collection and extraction

Specimens of five seagrasses were collected from the Indian River Lagoon in Fort Pierce, Florida in June 2005 and immediately transported to the Smithsonian Marine Station at Fort Pierce for analysis. *T. testudinum*, *Syringodium filiforme* and *Halodule wrightii* were collected from the north side of the Link Port Jetty of Harbor Branch Oceanographic Institution (27°32.218′N, 80°20.948″W). *Halophila decipiens* and *Ruppia maritima* were collected from Causeway Island (27°27.457′N, 80°18.645″W). Live intact seagrasses were cleaned of any epiphytic growth and maintained in aerated seawater tanks for no more than 24 h.

Crude extracts were prepared from whole seagrass plants. Twenty milliliters each of the five seagrasses (1.826 g of *T. testudinum*; 1.902 g of *S. filiforme*; 1.931 g of *H. wrightii*; 1.194 g of *Halophila decipiens*; 3.678 g of *R. maritima*) was measured by volumetric displacement in a graduated cylinder and extracted for 24 h by soaking in a 1:1 solution of ethyl acetate and methanol. Extracts were filtered, reduced *in vacuo* and stored at −20 °C or assayed immediately.

2.2. Fungal panel

Ten co-occurring strains of marine fungi belonging to the Ascomycota were isolated by Jones and Puglisi (2006) from a variety of substrata in Fort Pierce and the Florida Keys. Cultures were maintained on YPM P/S media (2 g yeast, 2 g peptone, 4 g D-mannitol, 16 g agar, 250 mg each of penicillin G and streptomycin sulfate in 1 L seawater). The fungal panel included: two strains of *Dendryphiella salina*, a common saprophyte on decomposing marine plants, isolated from sand (DsS) and the red alga *Gracilaria* sp. (DsG) (Kohlmeyer and Kohlmeyer, 1979); *L. thalassiae* (Lt), an indiscriminate pathogen in the ocean known to cause 'raisin disease' in the brown algae *Sargassum* spp. and '*Thalassia* disease' in the seagrass *T. testudinum*, isolated from blades of *T. testudinum* (Andrews, 1976; Porter, 1986); three species of *Fusarium*, a group known to cause disease in terrestrial plants, opportunistic infections in humans and other terrestrial mammals (Nelson et al., 1994), marine mammals (Cabanes et al., 1997) and marine invertebrates (Hyde et al., 1998) isolated from sand (Fsp1), the cordgrass *Spartina* sp. (Fsp2) and the brown alga *Sargassum* sp. (Fsp3) and four additional strains of common mangrove fungi (*Periconis prolifera* (Pp), *Aigialus parvus* (Ap), *Kalichromis tehys* (Kt) and *Quintaria lignobilis* (Ql)) were isolated from submerged dead mangroves in Florida.

Fungal homogenates (consisting of a mixture of hyphae and spores) were prepared by grinding a 2 cm^2 square section of the respective fungal culture in 0.22 μm filtered, autoclaved seawater with a sterile mortar and pestle. Homogenates were directly transferred to 50 mL sterile centrifuge tubes and used immediately.

2.3. Antifungal assays

2.3.1. Crude extract assays

To determine if the crude extracts from common seagrasses exhibited antifungal activities against co-occurring strains of marine fungi, seagrass extracts were assayed against 6 of the 10 strains from the panel that grew uniformly in sterile 24-well microtiter plates. Assay organisms included *D. salina* (DsS, DsG), *L. thalassiae* (Lt) and *Fusarium* spp. (Fsp1, 2 and 3). Assays were conducted with three replicate wells for each seagrass extract. Also included were three solvent controls, and no solvent controls as described by Engel et al. (2006). Aliquots of the crude extracts (equivalent to extract obtained from 2 mL of seagrass) were dissolved in methanol (final concentration 5% of the total volume) and incorporated into 2 mL of warm (50 °C) YPM P/S media in which the methanol quickly

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evaporated. Solvent controls contained an equivalent volume of methanol added to the media. Six hundred microliters of extract-treated YPM media and controls (5% MeOH) were dispensed into wells of the 24-well microtiter plate. A small amount of mycelium on approximately 1–3 mm of YPM media was transferred from a working culture to each well using sterile fine point tweezers. The microtiter plates were sealed with parafilm and subsequently incubated at 27 °C for 36–72 h. Assays were monitored and the experiments were ended when the fungus in each of the control wells completely covered the surface of the well. Plates were inverted, and the area of mycelium growth in each well was determined by counting the number of squares of a window screen grid that the mycelium covered. Growth inhibition was calculated as the percentage of mycelium coverage on the treatment wells relative to the control wells $(C - T)/C \times 100$.

2.3.2. Whole tissue assays

To determine if seagrasses maintained antifungal defenses at the surface of the plant, live seagrass tissues were assayed against all 10 strains of the fungal panel. Live blades were cut into three 10 cm pieces and the surfaces were carefully rinsed with ethanol using a squirt bottle. All assays were conducted in 100 mm × 15 mm sterile Petri dishes with YPM P/S media. The rinsed tissue pieces were allowed to dry for 10 min and then plated using sterile fine point tweezers. Small amounts of fungal mycelium were transferred from a working culture to each plate using sterile fine point tweezers and placed toward the edge of the plate on either side of the plant tissue. Control plates contained only fungal mycelium. Triplicate treatment (three individual plants) and triplicate control plates without seagrass were prepared for each assay. The cultures were sealed with parafilm and the experiment was terminated when the fungal mycelium covered the entire surface of the control plates, approximately 24–72 h. Plates were scored as either growth to the edge of the plant (*) or plant tissue overgrown (–). Seagrasses were reported as exhibiting antifungal activity if fungal growth was observed on at least two of the three treatment plates for each species.

2.4. Fungal elicitation of seagrass ROS production

2.4.1. Laser scanning confocal microscopy

In order to determine if the presence of fungal homogenates (mixture of spores and hyphae) could elicit ROS release in seagrasses, homogenates of *L. thalassiae* or *D. salina* (DsS) were applied to specimens of *S. filiforme* and *H. wrightii*. ROS levels were quantified as equivalents of H_2O_2. H_2O_2 production was detected by the oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen Corp., Carlsbad, CA, USA) as previously described by Ross et al. (2005).

Fully intact specimens (3–5 g) of *S. filiforme* and *H. wrightii* were placed in Petri dishes containing 10 mL of seawater and 10 μL of stock DCFH-DA (prepared as described below). Samples were incubated on a rotary shaker in the dark for 15 min and subsequently washed in filtered seawater to remove any unbound probe. To assess the localized release of H_2O_2 upon fungal challenge, a time study was conducted in which the same area of seagrass leaf was examined via confocal microscopy at selected time points post-inoculation. A 2 cm^2 square of fungal hyphae/spore culture was isolated and homogenized, as described above, and added to the seagrass sample. Confocal laser scanning microscopy (CLSM) was performed using a Nikon Eclipse E800 compound microscope (Nikon Instruments, Kanagawa, Japan) equipped with a Bio-Rad Radiance 2000 laser system (Bio-Rad, Hercules, CA, USA). Laser power was set at 20% with an excitation of 488 nm and an emission of 525 nm (channel 1) or 580 nm (channel 2). Series of 0.2 μm optical sections with maximum intensity projection along the *z*-axis were made into one 2D image with greater focal depth. Bio-Rad images were imported into Confocal Assistant 4.02 and converted into TIF files.

2.4.2. Fluorometric quantification of H_2O_2

Upon the addition of fungal hyphae/spore homogenate the concentration of H_2O_2 present in the seawater medium surrounding each of the five plant specimens was quantified by a protocol previously reported by Ross et al. (2005). DCFH-DA was dissolved in DMSO in 10 mM aliquot stocks (stored at −80 °C). Esterase (E.C 3.1.1.1, Sigma, 41 U mL^{-1}) was prepared in 0.22 μm filtered seawater.

For experimental analyses, 0.4–0.5 g of each seagrass was placed in a beaker of 50 mL of 0.22 μm filtered seawater ($n = 3$). Homogenates of 10 different fungal strains were added to the beakers and allowed to mix on a rotary table for 1 h. A 1 mL aliquot of the inoculated seawater was collected and assayed for H_2O_2 production as described below. As controls, a 1 mL aliquot of fungal homogenate was added to 50 mL of seawater without seagrass. These control values were subtracted from the experimental values. Data were log transformed to achieve homogeneity of variances and analyzed by two-way ANOVA (seagrass species and fungal strains were the two factors) followed by the Tukey test for comparison of means.

For time course analysis, 1 mL aliquots of the inoculated seawater were collected every 10 min to assay for H_2O_2 release from the challenged seagrass. The wavelengths of excitation and emission were 488 and 525 nm, respectively. Reaction mixtures included 1 mL of seagrass mixture, 0.82 U esterase and 25 μM DCFH-DA for a total volume of 2 mL. The fluorometric quantification of H_2O_2 was analyzed for a 200 min time interval on a Bio-Rad VersaFluor fluorometer (Bio-Rad). For calculating the concentration of H_2O_2 present in the samples, calibration with a standard curve was carried out at least once during any series of experiments. Standard curves were composed with known amounts of H_2O_2 in addition to 0.82 U esterase and 25 μM DCFH-DA for a total reaction volume of 2 mL. Data were analyzed by Kruskal–Wallis Test after transformation of data failed to achieve homogeneity of variances.

2.5. Induction of caspase activity

To evaluate the relationship between fungal-elicited oxidative stress and caspase activity in seagrasses three separate analyses were conducted. Three grams of intact

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T. testudinum were incubated in 200 mL of 0.22 μm filtered, autoclaved seawater in addition to (A) a homogenate of *D. salina*, (B) a homogenate of *L. thalassia* or (C) the exogenous addition of 100 μM H_2O_2 ($n = 3$). As a control 3.0 g of *T. testudinum* were incubated in 200 mL of 0.22 μm filtered, autoclaved seawater without any treatment. Sterile plant tissue culture containers were used for the incubations (ICN Biomedicals Inc., Aurora, OH, USA).

Following incubation with a fungal hyphae/spore homogenate or H_2O_2, blades of *T. testudinum* were gently towel dried, flash frozen with liquid N_2 and soluble proteins were extracted in 20 mL of 100 mM phosphate buffer (pH 7.8). The extract was centrifuged at 6600 × *g* for 5 min at 4 °C on a Beckman TJ-6 centrifuge. The supernatant was collected and protein concentration was quantified with a Quick Start™ Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions.

The Enzchek® Caspase-3 Assay Kit #2 (Invitrogen) was utilized to quantify proteolytic activity in *T. testudinum*. Caspases have been previously used as markers of programmed cell death or inflammatory activity in many different eukaryotes in responses to a variety of biotic stressors (Lamkanfi et al., 2007; Sanmartin et al., 2005). This assay exploits the specific proteolytic cleavage of the amino acid sequence Asp-Glu-Val-Asp (DEVD). Aliquots (1 mL) of the supernatant were combined with 990 μL of 1× reaction buffer and 10 μL Z-DEVD-R110 substrate (final substrate concentration, 25 μM). Samples were incubated at room temperature for 25 min and subsequently assayed for the appearance of the caspase-catalyzed fluorescent cleavage product Rhodamine-110 on a Bio-Rad VersaFluor fluorometer (E_x/E_m: 496/520 nm; $N = 3$). The reversible aldehyde caspase inhibitor Ac-DEVD-CHO (Invitrogen) was used as a negative control according to the manufacturer's methods. *T. testudinum* samples were pre-incubated with 100 μM Ac-DEVD-CHO for 20 min prior to the addition of fungal homogenate or H_2O_2. Background fluorescence was subtracted for no-enzyme controls.

The putative role of H_2O_2 as a molecule involved in the activation of caspases was further investigated by loss of function experiments using the NADPH oxidase inhibitor diphenylene iodonium (DPI; Sigma, St. Loius, MO, USA) as previously described by Küpper et al. (2002). In these experiments 3 g of *T. testudinum* were preincubated for 10 min with 10 μM DPI (from a 1 mM stock solution in DMSO) as previously described by Küpper et al. (2002). Incubations with fungal homogenates were conducted as described above and caspase activity was monitored for up to 6 days post-inoculation.

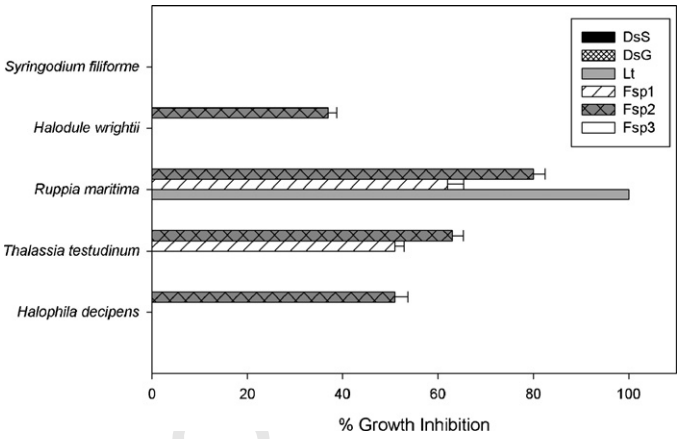

Fig. 1. Growth inhibition (%) relative to control of marine fungi by crude extracts of common seagrasses from Fort Pierce, FL. Assay organisms included *Dendryphiella salina* (DsS, DsG), *Lindra thalassiae* (Lt) and *Fusarium* spp. (Fsp1, 2 and 3). Data points represent the mean ± 1S.E.M. ($n = 3$ individuals).

3. Results

3.1. Antifungal assays

Crude extracts from four seagrasses inhibited the growth of at least one fungal strain by 35% (Fig. 1). The strains of *D. salina* or *Fusarium* sp. 3 used in this study were not inhibited by seagrass tissues or crude extracts, whereas *Fusarium* sp. 2 appeared to be the most susceptible to both. Broad-spectrum activity against three of the six fungal strains was observed in the extract of *R. maritima* (Fig. 1). In contrast, the extracts from *S. filiforme* did not inhibit growth of any of the fungi tested. Extracts from *R. maritima*, *H. decipiens*, *T. testudinum* and *H. wrightii* were active against *Fusarium* sp. 2, whereas, only extracts from *R. maritima* and *T. testudinum* inhibited the growth of *Fusarium* sp. 1. The pathogen *L. thalassiae* was only susceptible to the extracts from *R. maritima*.

Whole blade segments of three species of seagrasses from the Indian River Lagoon halted the growth of fungal mycelium at the edge of the plant for a least one fungal strain (Table 1). *T. testudinum* exhibited the most activity in the whole tissue assays by inhibiting the growth of *L. thalassiae* and *Fusarium* spp. 2 and 3 (Table 1). *S. filiforme* stopped the growth of *L. thalassiae* and *Fusarium* sp. 2, while *H. wrightii* only stopped

Table 1
Growth inhibition of marine fungi by intact seagrasses from Fort Pierce, FL

Seagrass	DsS	DsG	Lt	Fsp1	Fsp2	Fsp3	Pp
Halophila decipiens	–	–	–	–	–	–	–
Thalassia testudinum	–	–	*	–	*	*	–
Ruppia maritima	–	–	–	–	–	–	–
Halodule wrightii	–	–	–	–	–	*	–
Syringodium filiforme	–	–	*	–	*	–	–

The plates were scored subjectively as growth to edge of seagrass (*) or overgrown (–). Seagrasses were reported as exhibiting antifungal activity if fungal growth was observed on at least two of the three treatment plates for each species.

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the growth of *Fusarium* sp. 3. None of the seagrasses had an effect on the growth of *D. salina*, *Fusarium* sp. 1 or *P. prolifera*. The assay results against *A. parvus*, *K. tehys* and *Q. lignobilis* were inconclusive for these slow growing cultures became contaminated within 2 weeks.

3.2. Fungal elicitation of seagrass ROS production

H_2O_2 was released by *S. filiforme* within 10 min when inoculated with a cellular homogenate of *L. thallassiae* (Fig. 2A). As a function of time, increasing concentrations of H_2O_2 were detected over the surface of the plant blade (Fig. 2B and C). *H. wrightii*, infected with a hyphae/spore homogenate of *D. salina*, demonstrated a similar response showing a pronounced release of H_2O_2 at the site of host–spore interaction by 25 min post inoculation (Fig. 2D). A well known limitation of DCFH-DA, when detecting pathogen-induced ROS generation by confocal laser microscopy, is the concomitant generation of ROS in chloroplasts via Mehler's reaction (Apel and Hirt, 2004). This was noted on control (non-inoculated) specimens of seagrass (data not shown). However, when fungal homogenates were added to seagrass specimens, ROS production was localized to the apoplastic region of the plant (Fig. 2D inset), thus ruling out any chloroplast-generated ROS interference.

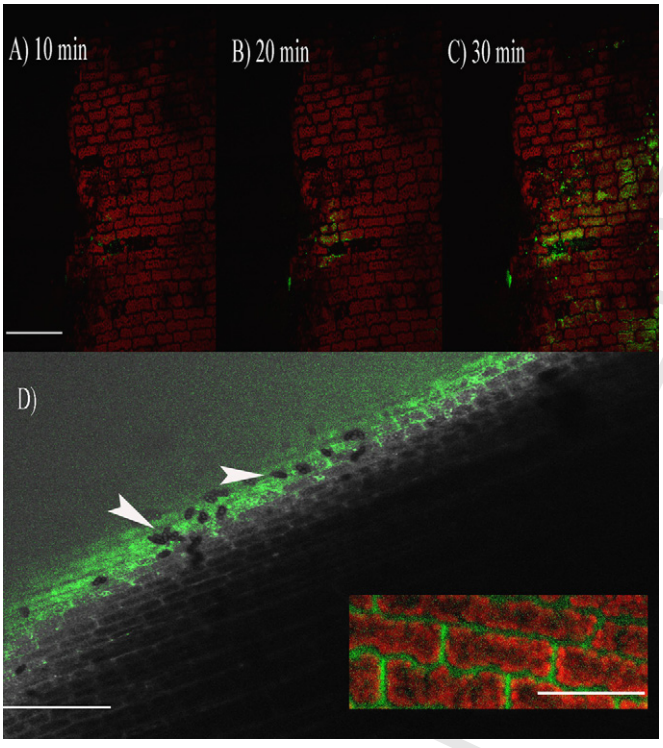

Fig. 2. Laser scanning confocal imaging of marine fungal-elicited release of H_2O_2 in seagrass specimens. Seagrass blades were loaded with the redox-sensitive fluorescent probe DCFH-DA and examined by laser scanning confocal microscopy. (A–C) Time course of H_2O_2 release when *Syringodium filiforme* was inoculated with a cellular hyphae/spore homogenate of *Lindra thallassiae*. Images (A–C) represent 10, 20 and 30 min images post-inoculation, respectively (scale bar = 500 μm). (D) Specimen of *Halodule wrightii* infected with a fungal hyphae/spore homogenate of *Dendryphiella salina*, showing a localized release of H_2O_2. Spores are identified with arrows. Image captured at 25 min post-inoculation (scale bar = 320 μm). Inset demonstrates apoplastic localization of H_2O_2 production (scale bar = 50 μm). The green fluorescence of DCFH-DA was monitored on channel 1 concomitant with the red fluorescence of the chloroplasts or an overall grey transmission image on channel 2. The two channels were merged to illustrate the localized H_2O_2 response.

Uninfected specimens chronically released low levels of ROS most likely as a byproduct of respiration or photosynthesis. This background signal was subtracted from all experimental values for all corresponding seagrass species when quantifying ROS concentrations. Upon introduction of a variety of crude fungal homogenates, all seagrass species were capable of producing ROS above basal levels as shown in Fig. 3. Catalase was capable of significantly inhibiting ROS detection, implicating H_2O_2 as the major oxidative molecule for all seagrass species (data not shown). Two-way ANOVA on log-transformed data showed that there was a significant difference in the responses to fungal exposure among the seagrass species ($F_{4,100} = 85.96$, $p < 0.0001$), but no significant difference in response of seagrasses to the various fungal strains ($F_{9,100} = 1.11$, $p = 0.36$) and no significant interaction between seagrass species and fungal strain ($F_{36,100} = 1.12$, $p = 0.32$). *T. testudinum* released the highest concentration of H_2O_2 with an average value of 0.62 ± 0.06 μmol H_2O_2 g^{-1} FW min^{-1} when comparing all fungal homogenates used in this study (Fig. 3). *S. filiforme* and *R. maritima* were statistically indistinguishable in concentrations of H_2O_2 released. *S. filiforme* had an average release of 0.32 ± 0.02 μmol H_2O_2 g^{-1} FW min^{-1} and *R. maritima* released an average of 0.20 ± 0.02 μmol H_2O_2 g^{-1} FW min^{-1}. *H. decipiens* released an average of 0.11 ± 0.02 μmol H_2O_2 g^{-1} FW min^{-1}. *H. wrightii* released the lowest concentration of H_2O_2 with an average release of 0.03 ± 0.02 μmol H_2O_2 g^{-1} FW min^{-1}.

To better quantify the kinetics of H_2O_2 production involved in the response to fungal elicitation, *T. testudinum* was used as a seagrass model due to its ability to generate high concentrations of H_2O_2. Kruskal–Wallis Test ($p < 0.001$) followed by comparison of mean ranks indicated that three species of fungi were capable of eliciting a similar response in H_2O_2 release from *T. testudinum* (Fig. 4). By 10 min post inoculation *Fusarium* sp. 3, *D. salina* and *L. thalassiae* were all capable of eliciting approximately 8 μmol H_2O_2 g^{-1} FW. A steady increase in H_2O_2 release was observed as a function of time. By 200 min post-inoculation *Fusarium* sp. 3, *D. salina* and *L. thalassiae* were all capable of eliciting responses of approximately 30 μmol H_2O_2 g^{-1} FW. *P. prolifera* did not induce as strong of a response as the other fungi used in this particular study and could not be distinguished from the control. By 200 min post-inoculation, H_2O_2 levels were just above control (no fungi added) values (Fig. 4). This suggests a certain degree of specificity is involved in the elicitation of H_2O_2 production in *T. testudinum*.

3.3. Induction of cellular programmed cell death

Samples of *T. testudinum* were exposed to a fungal hyphae/spore homogenate of *L. thalassiae*, *D. salina* or exogenously applied H_2O_2 (100 μM). Specimens of *T. testudinum* exposed

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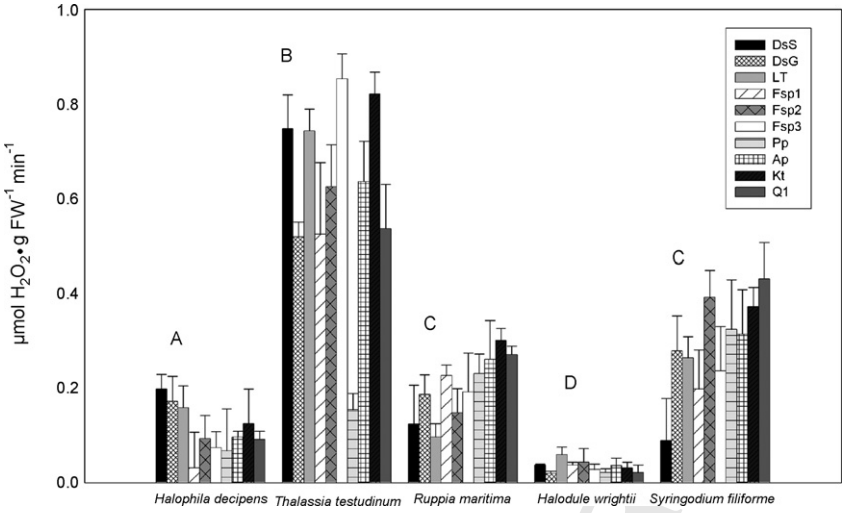

Fig. 3. Comparative analysis of H_2O_2 production in five species of seagrass from the Indian River Lagoon. Seagrass samples were incubated with homogenates of co-occuring strains of marine fungi and were incubated for 60 min. Fungal homogenates included *Dendryphiella salina* (DsS, DsG), *Lindra thallassiae* (Lt), *Fusarium* species 1–3 (Fsp1–3), *Periconis prolifera* (Pp), *Aigialus parvus* (Ap), *Kalichromis tehys* (Kt) and *Quintaria lignobilis* (Ql). Data points represent the mean ± 1S.E.M. ($n = 3$ individuals). Letters (A–D) indicate significant differences (Tukey's post hoc analysis) among seagrass species in response to fungal elicitation.

to homogenates of *D. salina* or *L. thalassiae* showed caspase activity within 24 h after inoculation. Activity increased significantly as a function of time and averaged approximately 45 nmol rhodamine mg^{-1} protein by day 6 (Fig. 5). Repeated measures ANOVA of log-transformed data (log(data + 0.1)) indicated that there were significant effects of both fungal treatment ($F_{6,70} = 36.41$, $p < 0.001$) and time ($F_{5,70} = 201.40$, $p < 0.001$) as well as a significant interaction between treatment and time ($F_{30,70} = 3.73$, $p < 0.001$). The highest caspase activity was seen in the treatment with exogenously applied H_2O_2, followed by the treatments with *D. salina* and *L. thalassiae*. Other treatments did not differ significantly from the control.

Control specimens that were not treated with fungal homogenate or exogenous amounts of H_2O_2 did not show any significant level of caspase activity (Fig. 5). The direct addition of exogenously applied H_2O_2 resulted in an expedited increase in caspase activity (Fig. 5). Caspase activity was detected by 24 h after introduction of H_2O_2 and corresponded to 30 nmol rhodamine mg^{-1} protein. By 48 h after incubation, caspase activity reached above 80 nmol rhodamine mg^{-1} protein. Caspase activity reached a plateau by 5 days after H_2O_2 addition with values reaching greater than 125 nmol rhodamine mg^{-1} protein. When cells were preincubated with the reversible caspase inhibitor Ac-DEVD-CHO prior to the addition of 100 µM H_2O_2, capase activity was drastically reduced (data not shown).

To further characterize the association between fungal induced oxidative stress and caspase activity, specimens of *T. testudinum* were subjected to a loss of function experiment. The

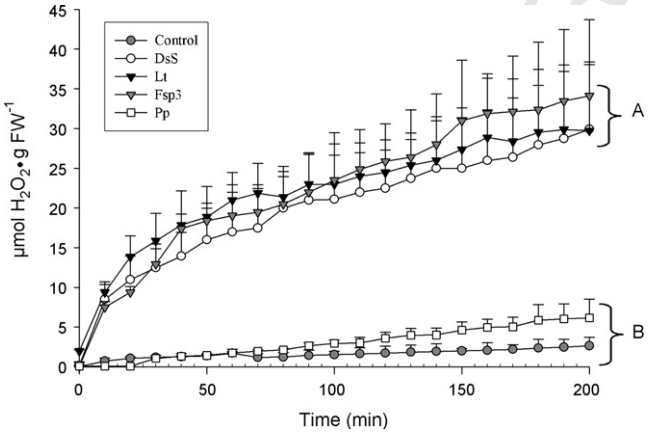

Fig. 4. Kinetics of H_2O_2 release in *T. testudinum*. Seagrass samples were incubated with homogenates of selected co-occuring strains of marine fungi. Fungal samples included *Dendryphiella salina* (DsS), *Lindra thallassiae* (Lt), *Fusarium* species 3 (Fsp3) and *Periconis prolifera* (Pp). Data points represent the mean ± 1S.E.M. ($n = 5$ individuals). At 200 min the letters (A and B) indicate significant groupings associated with fungal treatments as analyzed by Kruskal–Wallis Test followed by comparison of mean ranks.

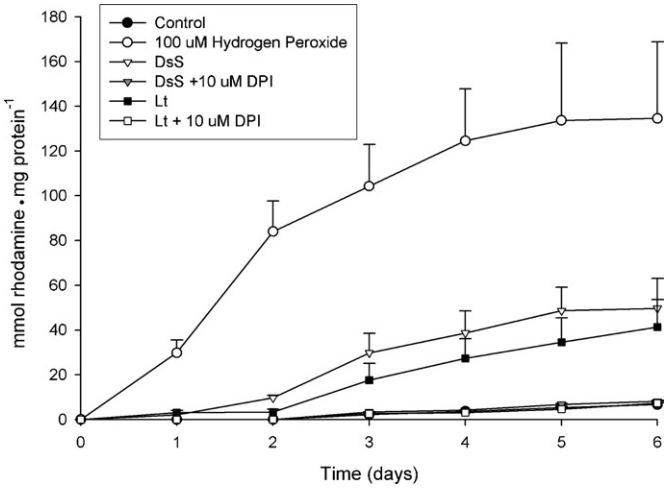

Fig. 5. Time course of caspase activity (DEVD cleavage) in the seagrass *T. testudinum*. Samples were incubated with a fungal homogenate of *Dendryphiella salina* (DsS) or *Lindra thallassiae* (Lt) as described in Section 2. Data points represent the mean ± 1S.E.M. ($n = 5$ individuals).

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ability to produce H_2O_2 was inhibited by preincubating seagrass samples with the NADPH oxidase inhibitor diphenylene iodonium (DPI) prior to fungal treatment. Samples that were pre-incubated with DPI and subsequently inoculated with a fungal homogenate (either *L. thalassiae* or *D. salina*) did not show any increase in caspase activity when compared to untreated controls (Fig. 5) thus implicating an NADPH oxidase complex as the putative enzymatic source of ROS.

4. Discussion

There have been numerous studies demonstrating that terrestrial plant defense systems involve an assortment of sustained and inducible responses against invading pathogens (Osbourne, 1996; Mellersh et al., 2002). In striking contrast, there are virtually no reports describing the physiological and biochemical responses of marine angiosperms to pathogenic invasion. Considering marine seagrasses evolved from vascular terrestrial plants and colonized the marine environment relatively recently (Hemminga and Duarte, 2000), it would be anticipated that similar chemical and physiological defense strategies would be employed to combat microbial invasion. The results of this study demonstrate that some seagrasses not only contain the ability to chemically defend themselves from pathogens but have physiological response mechanisms (production of ROS and induction of caspase activity) analogous to those reported in terrestrial plants.

Seagrasses are continuously at risk of being completely overgrown by a wide range of epiphytic organisms including fungi. The first line of defense for a marine plant to prevent infection by a marine microorganism can include a strategy to interfere with swarming, attachment, colonization and growth (Maximilien et al., 1998). We expected that in the case of chemically mediated interactions, chemical defenses at the surface of the plant should prevent overgrowth by the fungal mycelium. Our results show that in the case of the seagrasses from the Indian River Lagoon, the intact tissues did not prevent overgrowth from the majority of co-occurring marine fungi used in this study (Table 1). However, positive results for *T. testudinum*, *S. filiforme* and *H. wrightii* against *L. thalassiae*, *Fusarium* spp. 2 and 3 suggest that these seagrasses may maintain species-specific chemical abilities to combat invasive marine fungi.

Extracts from *H. decipiens*, *T. testudinum*, *R. maritima* and *H. wrightii* all appear to have inhibitory effects on co-occuring strains of *L. thalassiae*, *Fusarium* sp. 2 and 3 (Fig. 1). However, none of the fungi were susceptible to the crude extract from *S. filiforme*. Overall, our results are similar to those reported for seagrasses from the tropical Atlantic and Pacific, where almost all of the seagrass extracts failed to inhibit the growth of either strain of *D. salina* or *L. thalassiae* (Engel et al., 2006; Puglisi et al., 2007). However, an exception was the extract from *R. maritima*, which completely inhibited growth of *L. thalassiae* (Fig. 1). The *Fusarium* spp. appeared to be the most susceptible to seagrass crude extracts. While we must be cautious in interpreting the data obtained with crude extracts, our results provide further support that antimicrobial chemical defenses are prevalent in marine plants (Puglisi et al., 2007).

Fungal pathogens can elicit the production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) in a variety of terrestrial plants (Huckelhoven et al., 2001; Huckelhoven and Kogel, 2003). This rapid production of ROS, termed the oxidative burst, is triggered within minutes of infection (Apel and Hirt, 2004). The production of ROS is initiated after pathogen recognition or pathogen attack and is regulated by a series of signal transduction events involving an initial elicitation event where putative receptors are activated. In turn, a variety of downstream signaling events occur involving G proteins, adenylate cyclases, phospholipases, protein kinases, phosphatases, ion channel activation and the final activation of an ROS enzymatic source (Neill et al., 2002). Aside from functioning as a direct toxin against invading microbes, ROS have been demonstrated to be a critical component of the plant–pathogen hypersensitive response (Vranova et al., 2002).

The hypersensitive response is associated with restricted pathogen growth and represents a form of programmed cell death (Levine et al., 1994; Lamb and Dixon, 1997). Recent pharmacological and molecular studies have provided functional evidence for the conservation of some of the basic regulatory mechanisms underlying the response to pathogens and the activation of PCD in higher terrestrial plant systems. However, evidence associating the production and function of ROS in the context of host–pathogen interaction in marine seagrasses has remained undocumented. Our results demonstrate that seagrasses have the ability to release H_2O_2 as an immediate response to fungal elicitation (Figs. 2 and 4). Our study failed to produce evidence that the production of H_2O_2 may have a direct antifungal role. Fungal isolates were capable of completely overgrowing sterile discs containing millimolar concentrations of commercially available H_2O_2 (data not shown). However, evidence is presented that demonstrates that the production of H_2O_2, by a DPI-sensitive NADPH oxidase source, serves as a secondary signal activating downstream processes such as caspase activity. The recognition of fungal pathogens, subsequently triggering an oxidative burst leading to caspase activity, may be conserved as a defensive strategy between terrestrial and marine plants.

The results of this study demonstrate that some seagrasses not only contain the ability to chemically defend themselves from pathogens but have physiological response mechanisms (production of ROS and induction of caspase activity) that can feasibly confer resistance to fungal infection. Caspases have been found to be ubiquitous mediators and subsequently indicators of cellular death and inflammatory signaling pathways (Sanmartin et al., 2005; Lamkanfi et al., 2007). Recent discoveries, highlighting the involvement of caspase-like proteases as indicators of compromised chlorophyte cells, prompted this current study (Lam and Del Pozo, 2000; Segovia and Berges, 2005; Watanabe and Lam, 2004). Work by Ge et al. (2005) demonstrated that when cultured *Taxus cuspidata* cells were stressed via cerium supplementation, a biphasic burst of

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superoxide anion was triggered. This in turn promoted a downstream activation of caspase-3 and apoptosis. Although caspase activity is associated with seagrass stress, it is important to note that elevated caspase activity does not necessarily imply PCD has been initiated. Current studies in our laboratories are now focusing on this specific relationship.

Q2 Uncited references

del Pozo and Lam (1998) and den Hartog (1987, 1996).

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