2013

Plant-Pathogen Interactions Associated with Wasting Disease in the Tropical Seagrass Thalassia testudinum.

Kyle Loucks
University of North Florida, k.loucks.138999@unf.edu

Plant-Pathogen Interactions Associated with Wasting Disease in the Tropical Seagrass *Thalassia testudinum.*

by

Kyle Loucks

A thesis submitted to the Department of Biology

in fulfillment of the requirements for the degree of

Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

May, 2013

CERTIFICATE OF APPROVAL

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Approved by the thesis committee: Date

Signature Deleted

Cliff Ross
Committee Chair Person

4/12/13

Signature Deleted

David Waddell

4/12/13

Signature Deleted

Judith Ochrietor

04/12/13

Accepted for the Department of Biology:

Signature Deleted

Dan Moon
Chairperson

12 April 2013

Accepted for the College of Arts and Sciences:

Signature Deleted

Barbara A. Hetrick
Dean

13 April 2013

Accepted for the University:

Len Roberson
Dean of the Graduate School

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Abstract

Lipopolysaccharide (LPS) was shown to serve as a strong elicitor of the early defense response in the subtropical seagrass *Thalassia testudinum* Banks ex König and was capable of inducing an oxidative burst identified at the single cell level. The formation of reactive oxygen species (ROS) included a diphenylene iodonium sensitive response, suggesting the involvement of an NADPH oxidase. A 900 bp fragment of this enzyme was sequenced and found to encode a NAD binding pocket domain with extensive homology to the *Arabidopsis thaliana rbohF* (respiratory burst oxidase homolog) gene. Pharmacological dissection of the early events preceding ROS emission revealed that seagrasses contain ROS-generating machinery and signal transduction components that appear to be evolutionarily conserved with the defense response systems of terrestrial plants. It is undetermined whether or not the increased ROS associated with the oxidative burst is simply an antimicrobial agent or a signaling molecule that will initiate programmed cell death (PCD) and lead to the hypersensitive response (HR), a process not yet characterized in seagrasses. ROS accumulation was found to increase around the lesion as the duration of infection increased. The only PCD characteristic observed following infection was a slight increase in caspase-like protease activity around the lesion. Immunohistochemistry revealed inconsistent activity of proteases. Detection of nuclear condensation by TUNEL and Hoechst staining were also inconclusive and showed diffuse genetic material throughout the cytoplasm. It appears as though lesions from *Labyrinthula* spp. infection are likely to be a direct result of pathogen-based damage as opposed to host PCD.

1.

Introduction

1.1. Seagrasses

Seagrasses belong to a polyphyletic group of hydrophilous marine angiosperms that diverged in the cretaceous first from terrestrial plants then from halotolerant freshwater species (Les et al., 1997). While all members can be grouped within the subclass Alismatanae (division Tracheophyta), they are taxonomically disparate and are thus typically regarded as a single "ecological" group. These photoautotrophs can be broadly characterized by several unique characteristics: 1) exclusively inhabit estuarine and marine systems; 2) reproduction using hydrophily; 3) seeds are produced underwater and are dispersed by abiotic and biotic vectors; 4) roots are necessary for nutrient acquisition and transfer and are also accustomed to periods of anoxia; oxygen is delivered below ground from leaves and rhizomes; 5) vegetative anchorage via rhizome network; 6) Leaves lack stomata and the cuticle is reduced (Dawes et al., 2004).

Seagrass beds are an extremely valuable component of coastal ecosystems and regional economies that, aside from serving as a nursery habitat and refuge for numerous commercially important organisms, serve other primary roles including organic matter production for nearby reef and estuarine habitats and biogeochemical cycling. Furthermore, the above ground leaves act as a sediment catch which aids in biofiltration processes and the root and rhizome network serve to stabilize the sediment and prevent coastal erosion (Duarte, 2002; Orth et al., 2006). Florida has 1260 miles of coastline containing 2 million acres of seagrass, the most abundant species being *Thalassia testudinum* (Dawes et al., 2004). These seagrass beds support a wide variety of fish and invertebrate species including two of Florida's most lucrative fisheries, pink

shrimp and blue crab (Dawes, 2004). In 2012 alone these two fisheries grossed approximately 27.5 million dollars (Florida Fish and Wildlife 2012). *Thalassia testudinum* has other major supporting roles throughout the state that are important for the sustainment of a healthy coastal ecosystem. For example, seagrasses reduce wave energy and allow the gametes of broadcast spawning invertebrates to settle on and between the above ground blades. A study in St. Joseph Bay, FL showed higher fertilization success of sea urchins that spawned within the grass beds than those that spawned over adjacent sand flats (Simon and Levitan, 2011). Loss of seagrass along Florida's west coast caused a severe reduction in the broad cast spawning bay scallop, *Argopecten irradians*, populations which led to the closing of the commercial bay scallop fishery in Florida in 1994 as well as a reduction in the season and bag limit of the recreational scallop fishery (Arnold, 2009). Scallops not only live in the seagrass beds as adults but require seagrass to attach to as settling spat. As a second example, nearby coral reef habitats are protected by seagrass meadows. Corals are sensitive to nutrient enrichment, in that coral is easily overtaken by macroalgae when there is high nutrient availability (Dawes, 1998). Seagrasses serve as biological filters reducing deleterious levels of phosphorus and nitrogen. The declining reef habitats around the Florida Keys and Biscayne Bay are examples of a lack of seagrass habitat; with the decline of seagrass density in the area and nutrient rich runoff from septic tanks and canals the algae has flourished and overtaken much of the reef habitat (Lewis et al., 2002; Jaap and Hallock, 1990). Finally, above ground seagrass tissue can harbor a seasonal variety of epiphytic algae. The majority of the primary productivity associated with seagrass beds is in fact from epiphytes. Seagrasses serve as the scaffolding that allows epiphytes to be the major food source of invertebrates living in seagrass beds.

Over the past 30 years quantitative global assessments have documented that seagrass meadows have been decreasing at an alarming rate of 110 km^2yr^{-1} (Waycott et al., 2009). This reduction has been directly attributed to coastal development, population expansion, and destructive recreational boating practices, as well as harmful indirect effects such as eutrophication and increased turbidity.

The declining environmental conditions surrounding seagrass habitats have led to reductions in overall plant health. Changes in light intensity, turbidity, temperature, salinity, and dissolved oxygen and the additive stress of multiple stressors occurring at the same time are all factors that have been shown to reduce seagrass health and play a role in the sharp declines in biomass that have been reported (Koch and Erskine, 2001; Eldridge et al., 2004).

1.2. Wasting Disease and *Labyrinthula* spp.

A less common, but equally important, threat to seagrass meadows is the onset of disease. One of the most devastating diseases for seagrass is wasting disease caused by the unicellular pathogenic slime mold *Labyrinthula* spp. (Figure 1.1; Muehlstein and Porter, 1991; Muehlstein, 1992). Labyrinthulids or labyrinthulomycetes are grouped in the Stramenopile lineage of protista because of lack of chloroplasts, presence of scales covering the cell wall, and flagellated zoospores (Raghukumar, 2002). Recently labyrinthulomycota have received attention for being new sources of fatty acids that can be used as nutritional supplements and as biofuels (Raghukumar, 2008). Labyrithulids secrete an ectoplasmic network from an organelle called the bothrosome. The network links cells to one another forming a colony. The network also secretes the digestive enzymes responsible for nutrient uptake, classifying them as osmoheterotrophs.

These ubiquitous marine microbes can function as saprobes, mutualists, commensalists, and parasites (Raghukumar, 2002). As pathogens, they have the capability of degrading cell walls of their hosts with extracellular enzymes causing characteristic black lesions, an indicator of wasting disease (Figure 1.2; Tsui et al., 2002; Raghukumar, 2002). *Labyrinthula* spp. cells occupy living green tissue on the periphery of lesions. Once the leaf blade has been bisected the plant can no longer transport nutrients and oxygen to below ground tissue leading to sulfur toxicity in roots (Durako and Kuss, 1994).

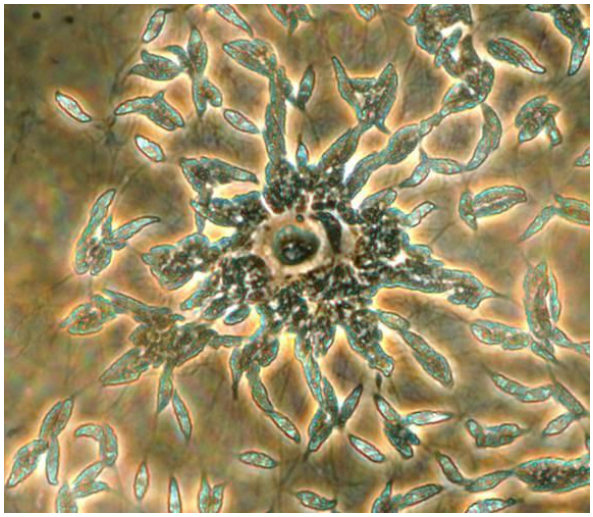


Figure 1.5 *Labyrinthula* spp. colony isolated from *Thalassia testudinum* (400x magnification).

Figure 1.6 *Thalassia testudinum* displaying symptoms of disease.

One of the most well-known die-off events believed to involve *Labyrinthula* spp. was the 1930's pandemic decline of *Zostera marina* populations along the Atlantic coasts of North America and Europe. Within two years, 90 percent of all *Z. marina* populations in the Western North Atlantic (from Canada to North Carolina) were eradicated. Wasting disease can spread quickly and decimate large areas because it is transmitted by blade to blade contact. Even when the blade has broken off the pathogen is still present on detrital material and can easily infect other plants. In Chesapeake Bay, this led to the collapse of several important fisheries and caused near extinction of local waterfowl, a drastic decline in scallop populations and an overall change in benthic composition of the bay (Orth et al., 2006). A smaller outbreak of wasting disease also occurred in the late 1980s in Florida Bay affecting *Thalassia testudinum* (Fourqurean and Robblee, 1999). It was speculated that infection, in conjunction with other abiotic stressors such as elevated sediment sulfide content and hypersalinity, prompted this regional die-off event (Robblee et al., 1991).

1.3. Plant Host-Pathogen Interactions

Plants possess a remarkable array of strategies used to combat herbivory and pathogenic invasion. These mechanisms can be subdivided into two broad categories, active and passive defenses. Passive defenses are structural modifications such as thick waxy cuticles; whereas active defenses are inducible, either by pathogen interaction or by various abiotic stressors (Hutcheson, 1998). Activated defenses can be further subdivided into primary, secondary and tertiary responses (Hutcheson, 1998). Primary responses are induced by elicitors and are produced by the cells in closest proximity to the point of pathogen attack. Secondary responses

are induced by the whole plant and affect cells further from the pathogen; tertiary responses are systematically required responses that are hormonally induced (Hutcheson, 1998). Activated defenses of agriculturally important plants such as corn, tobacco, strawberry and tomato have been well studied in order to produce disease resistant crops (Pan et al., 2012; He et al, 2012; Iannone et al., 2012). Conversely, very little is known about defense responses of marine plants and how this may influence disease transmission in coastal environments.

Plants produce Reactive Oxygen Species (ROS) as a form of both primary and secondary activated defense responses to both abiotic and biotic stress. These molecules include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Figure 1.3). ROS are continuously produced as byproducts of various metabolic pathways, namely photosynthesis and aerobic respiration and are capable of interconversion depending on the local redox environment (Apel and Hirt, 2004); they can also be produced enzymatically upon pathogen attack via apoplastic and cell wall peroxidases or membrane bound NADPH oxidases (Apel and Hirt, 2004; Torres and Dangl, 2005). When a plant is invaded by a pathogen the earliest response is ROS accumulation (Apel and Hirt, 2004). ROS, in particular H_2O_2, serve as antimicrobial agents and are up regulated during the plant defense response cascade (Legendre et al., 1993). The rapid transient production of ROS is termed the oxidative burst; this reaction is not unlike the method that phagocytes employ in mammalian systems. The oxidative burst contains O_2^- and H_2O_2, and was first demonstrated in 1985 using potato cell culture (Doke, 1985). Since then the oxidative burst has been demonstrated in a myriad of terrestrial plants.

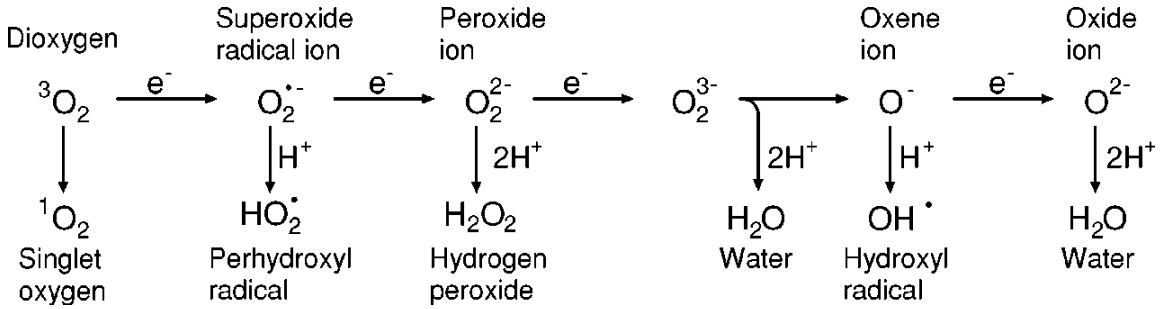


Figure 1.7 Generation of ROS by electron transfer (Apel and Hirt, 2004). As oxygen is reduced to water selected forms of ROS can form via partial reduction. Reactive oxygen species are shown along the bottom.

The toxicity of the ROS produced is largely dependent on the pathogen's sensitivity to amount of ROS produced. ROS, namely hydrogen peroxide, also serve as signaling molecules or secondary messengers which activate a wide variety of defense responses throughout the entire plant. The relative stability and ease which H_2O_2 can travel through cell membranes makes it the perfect molecule for activating a broad range of defenses throughout the entire plant (Wojtaszek, 1997). H_2O_2 plays an important role in signaling defense related functions in nearby cells; functions include stomatal closure, excess light and water stress acclimatization responses, cell wall strengthening, antioxidant enzyme activation, a transcription factor for related defense genes, calcium channel activator, and as an initiator of programmed cell death (Pei et al., 2000; Gadjev et al., 2008; Alvarez et al., 1998, Gegchev et al., 2002; Brisson et al., 1994; Mori and Schroeder, 2004). Each differentiated cell will interpret the signal in accordance to the cell's specific function.

Another activated defense response in plants is the hypersensitive response (HR). Triggered by ROS, this is an area of cell death at the point of pathogen attack and represents a form of programmed cell death (PCD) (Mur et al., 2007). PCD is cell death resulting from a series of orderly molecular and biochemical reactions; including plasma membrane degradation, DNA laddering and the presence of specific proteases (Van Breusegem and Dat, 2006). The hypersensitive response is a major element of plant disease resistance that benefits the plant by depriving pathogens access to nutrient sources and limits pathogen proliferation (Zaninotto et al., 2006). Pharmacological and genomic studies have provided strong evidence for the conservation of some of the basic regulatory mechanisms underlying the response to pathogens in both animal and plant systems. However, the ability for marine plants to engage in an HR response has not been thoroughly evaluated.

The objective of this thesis was to investigate two selected components of the defense response system of *T. testudinum*. Part one focused on the characterization, kinetics and signal transduction events associated with ROS production in *T. testudinum* when challenged with pathogen-based elicitors. Part two explored the capacity for *T. testudinum* to initiate a HR when challenged with *Labyrinthula* spp.

The rationale for examining this topic is based upon the notion that a considerable amount of effort has been invested to better understand activated defense responses in agriculturally-relevant terrestrial plants. While this is important, our knowledge of the impacts of diseases in the marine environment is severely lagging. It is important to understand the biochemical interactions between *T. testudinum* and *Labyrinthula* spp. and the physiological tipping points that may lead to diseased states in seagrasses.

2.

Lipopolysaccharides elicit an oxidative burst as a component of the innate immune system in the seagrass *Thalassia testudinum*

Abstract

This study represents the first report characterizing the biological effects of a lipopolysaccharide (LPS) immune modulator on a marine vascular plant. LPS was shown to serve as a strong elicitor of the early defense response in the subtropical seagrass *Thalassia testudinum* Banks ex König

and was capable of inducing an oxidative burst identified at the single cell level. The formation of reactive oxygen species (ROS), detected by a redox-sensitive fluorescent probe and luminol-based chemiluminescence, included a diphenylene iodonium sensitive response, suggesting the involvement of an NADPH oxidase. A 900 bp fragment of this enzyme was sequenced and found to encode a NAD^+ binding pocket domain with extensive homology to the *Arabidopsis thaliana rbohF* (respiratory burst oxidase homolog) gene. The triggered release of ROS occurred at 20 min post-elicitation and was dose-dependent, requiring a minimal threshold of 50ug/ml LPS. Pharmacological dissection of the early events preceding the emission of ROS indicated that the signal transduction chain of events involved extracellular alkalinization, G-proteins, phospholipase A_2, as well as K^+, Ca^{2+}, and anion channels. Despite exclusively thriving in a marine environment, seagrasses contain ROS-generating machinery and signal transduction

components that appear to be evolutionarily conserved with the well-characterized defense response systems found in terrestrial plants.

2.1. Introduction

Seagrasses are widely distributed marine vascular plants that not only serve as critical sources of coastal primary production and habitat, but also contribute significantly towards biogeochemical cycling and near shore sediment stabilization (Waycott et al., 2009). Seagrass meadows have experienced significant declines in recent decades due to persistent threats from both local and global scale sources (Orth et al., 2006). One such threat is the incidence of disease in which pathogenic protists of the genus *Labyrinthula* spp. (Division Stramenopila) cause local and regional-scale seagrass die-off events; a phenomenology termed wasting disease (Short et al., 1987; Muehlstein, 1989; Robblee et al., 1991). Wasting disease outbreaks have had long-lasting effects on dominant seagrass species (e.g. *Thalassia testudinum* Banks ex König, which is found in tropics and subtropics and *Zostera marina* Linnaeus, which is found in temperate regions) as some populations affected by the disease have not fully recovered, and other populations have been driven to local extinction (Young, 1943).While there is a recent increasing awareness of seagrass wasting disease in the marine environment (Bergmann et al., 2011; Garcias-Bonet et al., 2011; Trevathan et al., 2011, Bockelmann et al., 2012) the biochemical basis of pathogen recognition and defense responses in seagrasses are virtually unexplored. Considering seagrasses are descendants of terrestrial plants that evolved into a distinct lineage ~75 million years ago (Janssen and Bremmer, 2004), it would be anticipated that there would be a selected degree of homology in the principles of activated innate immunity.

A substantial amount of evidence has accumulated highlighting the importance of pathogen-associated molecular patterns (PAMPs) in plant defense systems (Ingle et al., 2006; Jones and Dangle, 2006; Boller and He, 2009; Zhang and Zhou, 2010). PAMPs are expressed by a wide range of microorganisms and include well-characterized elicitors such as chitin, eubacterial flagellin, peptidoglycans, lipoteichoic acid (LTA) of Gram-positive bacteria and the lipopolysaccharides (LPS) of Gram-negative bacteria, which often serve in many experimental systems as the prototypical model PAMP (Dow et al., 2000). LPS is a structurally conserved lipoglycan capable of inducing both systemic acquired resistance (SAR) and induced systemic resistance (ISR) in terrestrial plant studies (Coventry and Dubery, 2001; Mishina and Zeier, 2007; Van Wees et al., 2008). In addition, LPS has been implicated in the elicitation of immune responses modulating extracellular alkalinization (K^+/H^+ exchange), nitric oxide (NO) production, calcium influx, the induction of pathogenesis related (*PR*) gene expression and cell wall alterations that include the deposition of callose and phenolics (reviewed in Erbs and Newman, 2012).

The production of reactive oxygen species (ROS) in response to LPS elicitation is another early innate immune response that has been well documented chiefly in plant cell suspension cultures (Albus et al., 2001; Meyer et al., 2001; Gerber et al., 2004; Braun et al., 2005; Desaki et al., 2006). Since the discovery of the elicited production of superoxide (O_2^-) in potato in response to an avirulent isolate of *Phytophthora infestans* (Doke, 1983), superoxide and other ROS such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^{\cdot}$) have been implicated as key elements in eukaryotic defense (Wojtasek, 1997). While the enzymatic sources of ROS may include polyamine, oxalate or other apoplastic oxidases (Zhang et al., 1995; Allan and Fluhr, 1997; Yoda et al., 2006), plasma membrane-bound NADPH

oxidases (Rbohs, for respiratory burst oxidase homologs) and cell wall peroxidases are considered to be the main sources in terms of plant innate immunity (Bolwell et al., 1995, 1998; Torres and Dangle, 2005; Sagi and Fluhr, 2006).

In contrast to the growing body of terrestrial plant literature describing the onset of oxidative bursts and the preceding coordinated signal transduction events, there is only limited knowledge on the biochemical-based induced defense responses of aquatic photoautotrophs. Furthermore, the available data is restricted to algal taxa, as there have been no reports on aquatic vascular plants. An oxidative burst was described in the brown algal kelp *Laminaria digitata* and was shown to play a critical role in the regulation of epiphytic, putatively pathogenic, bacteria (Kupper et al., 2001, 2002). In addition, the same algae could recognize host-derived oligoguluronate fragments as well as exogenous prostaglandins, both capable of triggering the sudden release of ROS (Kupper, 2001; Zambounis et al., 2012). Weinberger et al. (1999, 2005) demonstrated that the Rhodophyte (red algae) *Gracilaria conferta* could respond to microbial degradation of its agar cell wall matrix with a DPI-sensitive oxidative burst, suggesting the involvement of an NADPH oxidase. Despite these efforts demonstrating the capability for marine algae to respond to both endogenous and exogenous cues, there has only been one report highlighting the involvement of an LPS-induced oxidative burst in a marine macroalgae. This was observed in the Phaeophyceae (brown algae) member *L. digitata* (Kupper et al., 2006). The lack of evidence characterizing the effects of exogenous elicitors, such as LPS, on the innate immunity of marine photoautotrophs is probably not due to the lack of biochemical machinery in these taxa, but more so due to the lack of investigations on marine species overall (Weinberger, 2007). In order to explore the early defense responses in marine vascular photoautotrophs the tropical/subtropical seagrass *Thalassia testudinum* was used as a representative model species. In

light of the historical die-off events associated with wasting disease, this study was undertaken to better understand the cellular factors that regulate seagrass innate immunity. The objectives of this work were to determine: 1) if *T. testudinum* has the capability of responding to exogenous elicitation, using purified LPS, and engage in a signaling cascade that results in an oxidative burst; and 2) if *T. testudinum* has the ability to recognize biochemical extracts of *Labyrinthula* spp. to offer insight into this important yet understudied host-pathogen interaction system.

2.2. Materials and Methods

2.2.1. Seagrass Collection and Maintenance

Thalassia testudinum shoots were collected from the Indian River Lagoon in Ft. Pierce, Florida (27°47'N, 80°31'W and 27°58'N, 80°31'W) and cleaned of epiphytes. Short shoots included the blades, sheath and a 2-inch section of rhizome. Following collection, all specimens were allowed to acclimate for no less than 1 week under greenhouse conditions at the University of North Florida, Jacksonville, FL prior to use in any experiment. Plants were maintained in aquaria at a salinity of 30 under shade cloth allowing a maximal penetration of 15% photosynthetic active radiation (< 300 μmol $m^{-2}s^{-1}$). Diel temperature values ranged between 25-27 °C.

2.2.2. Labyrinthula Culture

A known virulent strain of *Labyrinthula* spp., previously isolated from *T. testudinum*, was used for all *Labyrinthula* elicitation experiments. *Labyrinthula* spp. cultures were maintained in serum-seawater agar (SSA) as previously described in Trevathan et al. (2011).

2.2.3. Preparation of Elicitors

Labyrinthula extract was obtained by removing 5mm diameter plugs of *Labyrinthula* spp. from SSA agar plates and transferred to 8'' x 10'' acrylic sheets covered in 1L Serum-Seawater liquid media for 48 hours. The liquid media was the same as the SSA *Labyrinthula* spp. culture media minus the agar. After 48 hours of growth all *Labyrinthula* spp. that covered the surface of the plate was removed via razor blade, placed in microcentrifuge tubes and pelleted by centrifugation (3000 x g for 5 minutes). The pelleted samples were subsequently lyophilized and the resulting material was resuspended and vortexed in 0.22 μm filtered seawater (salinity of 30) to create known concentrations of *Labyrinthula* elicitor.

Lipopolysaccharides extracted from *Escherichia coli* serotype O127:B8 (Sigma Aldrich, St. Louis, MO, USA) were used for all LPS induction assays. Stock solutions of 200mg/ml LPS were prepared in DI water.

2.2.4. Hydrogen Peroxide Detection

The concentration of hydrogen peroxide released into the medium surrounding the plants was quantified using a chemiluminescence luminol assay (Glazner et al., 1991; Kupper et al., 2001) with minor modification using a Synergy 2 SL Luminescence Microplate Reader (BioTek,

Winooski, VT, USA). Selected volumes of *Labyrinthula* spp. extract or LPS stock solution were brought up to a final volume of 10mL using filtered seawater to achieve the desired final concentrations of each elicitor. Every 10 mL replicate contained 0.5 grams fresh weight of second rank plant leaf tissue. At each time point 144 uL of sample was combined with the following reaction components directly into the microwell plate: 20 uL of 248 u mL^{-1} horseradish peroxidase (HRP) (Sigma, dissolved in pH 7.8 phosphate buffer); 36 uL of 300mM luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione, Sigma). Both the HRP and luminol were autoinjected and chemiluminescence was recorded with a signal integration time of 10 s. Calibration with a standard curve of H_2O_2 was conducted at least once during a set of any experiments. All controls were run using the experimental setup described above without the addition of any elicitor.

2.2.5. Fluorescence Microscopy

To identify the cellular origin of ROS production in challenged *T. testudinum sections*, ROS accumulation was qualitatively monitored using the probe 2′,7′-dichlorodihydrofluoresceindiacetate (H_2DCF-DA; Invitrogen, Carlsbad, CA, USA), a nonfluorescing, nonpolar compound. When H_2DCF-DA reacts with cellular esterases, the diacetate group is cleaved off to yield the polar compound H_2DCF. Oxidation of H_2DCF by ROS yields the fluorescent product DCF. Cross sections were made from second rank leaf tissue of *T. testudinum* using a razor blade and incubated in 5 mL of filtered seawater (0.22 μm) containing 5 μL of 10 mM H_2DCF-DA (dissolved in DMSO) for 30 minutes. Following the incubation, samples were washed 3 times in 5 mL filtered seawater to remove any unbound probe. Individual

plant sections were placed on 35 mm glass bottom microwell dishes (MatTek Corp., Ashland, MA, USA) in a drop of filtered seawater. To start the experiment, seawater was wicked off and replaced with a 200 µg/mL solution of LPS. Fluorescent imaging (FITC: ex 488, em 525nm; Texas Red: ex 496, em 615nm) was performed using an Olympus IX 81 inverted epifluorescence microsope (Olympus Center Valley, PA, USA) in conjunction with a DP21 digital camera (Olympus).

2.2.6. Respiration and pH Measurements

To evaluate the impact of LPS addition on dark-adapted respiration, 2-cm sections of *T. testudinum (*total of 0.5g per replicate*)*, were analyzed for oxygen uptake. Respiration studies were conducted using an Oxygraph system outfitted with a DW3 liquid-phase electrode chamber (Hansatech Instruments©, Norfolk, UK). Samples were dark-adapted for at least 1 h prior to any measurement. The system was calibrated by eliminating oxygen with the addition of sodium hydrosulfite. An O_2 maximum was created by bubbling air into filtered seawater. In the reaction chamber, the leaf sections were submerged in 10 mL of O_2-saturated filtered seawater (0.22 µm). One mL of 5000 µg mL^{-1} LPS was injected into the reaction chamber to achieve a final concentration of 500 µg mL^{-1} LPS. Following injection, oxygen uptake was measured over the course of 15 min for each experiment. Respiration was calculated as nanomoles of oxygen consumed per minute per gram of tissue. To assay for alkalization of extracellular media in response to LPS addition, 0.5 g of plant tissue was combined with 500 µg mL^{-1} LPS in 10 ml of seawater. The pH was monitored using an Oaktron pH meter and probe (Oakton Instruments, Vernon Hills, IL, USA) over the course of 80 minutes.

2.2.7 Pharmacological Inhibitors and Activators of ROS production

Pharmacological inhibitors and activators of select cellular targets were utilized to characterize the signaling pathways involved in ROS generation in *T. testudinum* following LPS elicitation. Concentrations used were based upon previous studies reported in the literature. Compounds, final concentrations used and mechanisms of action/target were as follows: KCN (1mM; target, heme-dependent enzymes; Davies et al. 2006); NaN_3 (1mM; target, redox enzymes; Davies et al. 2006); Catalase (100 U/ml; mechanism, H_2O_2 decomposition; Davies et al. 2006); EGTA (1mM; mechanism, calcium chelator; Davies et al. 2006); $CaCl_2$ (2mM; mechanism, calcium supplement; Davies et al. 2006); and Pertussis toxin (1 μg/ml; target, G-protein inhibitor specifically used in the study of adenylate cyclase regulation and the role of G_i proteins; Ross et al. 2006) were dissolved in H_2O. Diphenyliodonium (DPI, 50 μM; target, suicide inhibitor of NADPH oxidases that binds irreversibly to the flavonoid group of the membrane-associated $gp91^{phox}$ subunit; Davies et al. 2006); and Phorbol 12-myristate 13-acetate (PMA, 15 μM; mechanism, protein kinase c activator; Ross et al. 2006) were dissolved in DMSO. Quinacrine (500 μM; target, flavin-dependent redox enzymes; Kupper et al. 2001); 5-nitro-2-[3-phenylpropylamino]-benzoic acid (NPPB, 100 μM; target, anion channel blocker; Kupper et al. 2001); staurosporine (5 μM; target, protein kinases; Kupper et al. 2001); chlorpromazine-HCl (100 μM; target, phospholipase A2; Kupper et al. 2001); methoxyverapamil (100 μM; target, Ca^{2+} channel blocker; Ross et al. 2006); monensin (100 μM; mechanism, Na^+ ionophore; Ross et al. 2006); A23187 (100 μM; mechanism, Ca^{2+} ionophore; Ross et al. 2006) and valinomycin (100 μM; mechanism, K^+ ionophore; Ross et al. 2006) were dissolved in

ethanol. Seagrass samples were preincubated with selected compounds for 20 min prior to the introduction of 200 µg mL^{-1} LPS. All chemicals were purchased from Sigma-Aldrich.

2.2.8. Cloning of a NADPH Oxidase from *T. testudinum*

Primer Design

Degenerate primers were designed by aligning respiratory burst oxidase homologue (rboh) gene sequences downloaded from the Pubmed database for *Hordeum vulgare* (Accession number AM265370), *Oryza sativa* (Accession number AY603975), *Arabidopsis thaliana* (Accession number NM_105079), *Nicotiana tabacum* (Accession number AJ309006), and *Medicago truncatula* (Accession number AM494844) using the ClustalW2 sequence alignment tool available on the European Bioinformatics Institute webpage (http://www.ebi.ac.uk/). Highly conserved sequences in the 5' and 3' regions of the rboh alignment were identified and two pairs of degenerate primers were designed. The primer sequences are:

Rboh 5' Forward 5'-ATGGTGGAYAAGRACGCSGACGGCC-3',

Rboh 5' Reverse 5'-CCAAGTRATGGTGTTRCGGCATACWGG-3'.

Rboh 3' Forward 5'- GGCATCCCTTCTCRATWACTTCRGCACC-3',

Rboh 3' Reverse 5'- CCAGAASACWAYATCRACWCCATTCTTGGC-3'.

Amplification and Cloning

RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) from *T. testudinum* according the manufacturer's protocol. Reverse transcription was performed with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) according

the manufacturer's protocol. Briefly, 100 ng of oligo dT primer was mixed with 500 ng of total RNA and dNTPs and heated to 65°C for 5 minutes and quick chilled on ice. First-Strand Buffer, DTT, RNase Inhibitor and M-MLV RT was then added to the reaction and incubated at 37°C for 50 minutes, followed by a 15 minute incubation at 70°C to inactivate the reaction. Polymerase chain reaction (PCR) was performed using 10% for the RT reaction, mixed with PCR buffer, $MgCl_2$, dNTP mix, Rboh degenerate forward and reverse primers and *Taq* DNA polymerase (Invitrogen). The PCR reaction was performed in an Eppendorf Mastercycler Pro at 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 60 seconds and cycled 35 times. PCR products were agarose gel purified using a QIAquick Gel Extraction Kit (Qiagen), ligated into the pGEMT-Easy plasmid (Promega, Fitchburg, WI, USA) according the manufacturer's protocol and transformed into DH5α competent *E. coli* cells (Invitrogen). Colonies were then selected, mini-prepped using the QIAprep Spin Miniprep Kit (Qiagen), restriction digested with EcoRI (New England Biolabs, Ipswich, MA, USA) to confirm the presence of an insert and sequenced using T7 and SP6 sequencing primers (Operon, Huntsville, AL, USA). Finally, the sequence from a PCR fragment generated using the Rboh 3' region degenerate primer pair was blasted against the *A. thaliana* genome and found to share sequence homology with the RbohF gene.

2.2.9. Statistical Analysis

Data for hydrogen peroxide accumulation was analyzed using a one-way ANOVA. A Tukey's post hoc test was performed to determine significant groupings. Data for respiration was analyzed using a student's t-test. Data for media alkalization was natural log transformed and subsequently analyzed using a repeated measures ANOVA with time as the repeated factor. The

data were normally distributed as was determined by the Shapiro–Wilk test. All statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

2.3. Results

2.3.1. Induction of an oxidative burst in *T. testudinum*

The addition of LPS induced an oxidative burst in *T. testudinum* with a maximal release of H_2O_2 observed at 20 min post-elicitation (Figure 2.1a). The magnitude of the H_2O_2 burst varied significantly as a function of LPS concentration (Figure 2.1b, one-way ANOVA, F = 14.891, p = 0.000). The addition of 25 µg/ml LPS caused a 16% increase in the ROS produced when compared to non-elicited controls yet this effect was not statistically significant. The minimum threshold of LPS required to elicit a response was 50µg/ml (Figure 2.1b). The addition of two elevated concentrations of LPS (100 and 200 µg/ml) resulted in a 56 and 86 percent increase, respectively, in the H_2O_2 released into the surrounding media when compared to controls. Hydrogen peroxide levels did not reach saturation in the range of concentrations tested. Extracts of *Labyrinthula* spp. failed to induce any oxidative burst in *T. testudinum*, using concentrations up to 5mg/ml (data not shown).

Sections of *T. testudinum* were challenged with LPS and monitored for ROS release using fluorescence microscopy in conjunction with the redox-sensitive probe H_2DCF-DA. The production of ROS could be localized to epidermal cells and was not detected in mesophyll cells bordering the air lacunae (Figure 2.2). The ROS signal could be detected as early as 10 min post-elicitation and continued to intensify up through 30 min.

Following LPS addition, specimens of *T. testudinum* underwent a significant increase in oxygen consumption compared to untreated controls (Figure 2.3, Student's t test, $p < 0.050$). While a distinct respiratory burst was not evident, there was a steady increase in oxygen consumption as a function of time. By the end of the experiment challenged plants had an average oxygen consumption rate of 64.2 (±6.0) nanomoles of O_2 g^{-1} minute $^{-1}$ while control plants had values roughly 3 times lower (22.4 (±5.4)). Extracellular pH also showed a steady increase following LPS addition and was significantly different than the controls (Figure 2.4, repeated measures ANOVA, $F= 155.046$, $p = <0.005$). The seawater underwent an alkalinization of approximately 0.3 pH units over the course of 80 min. There was no significant interaction between time and LPS treatment (Wilks' Lambda: $F= 88.695$, $p= 0.079$).

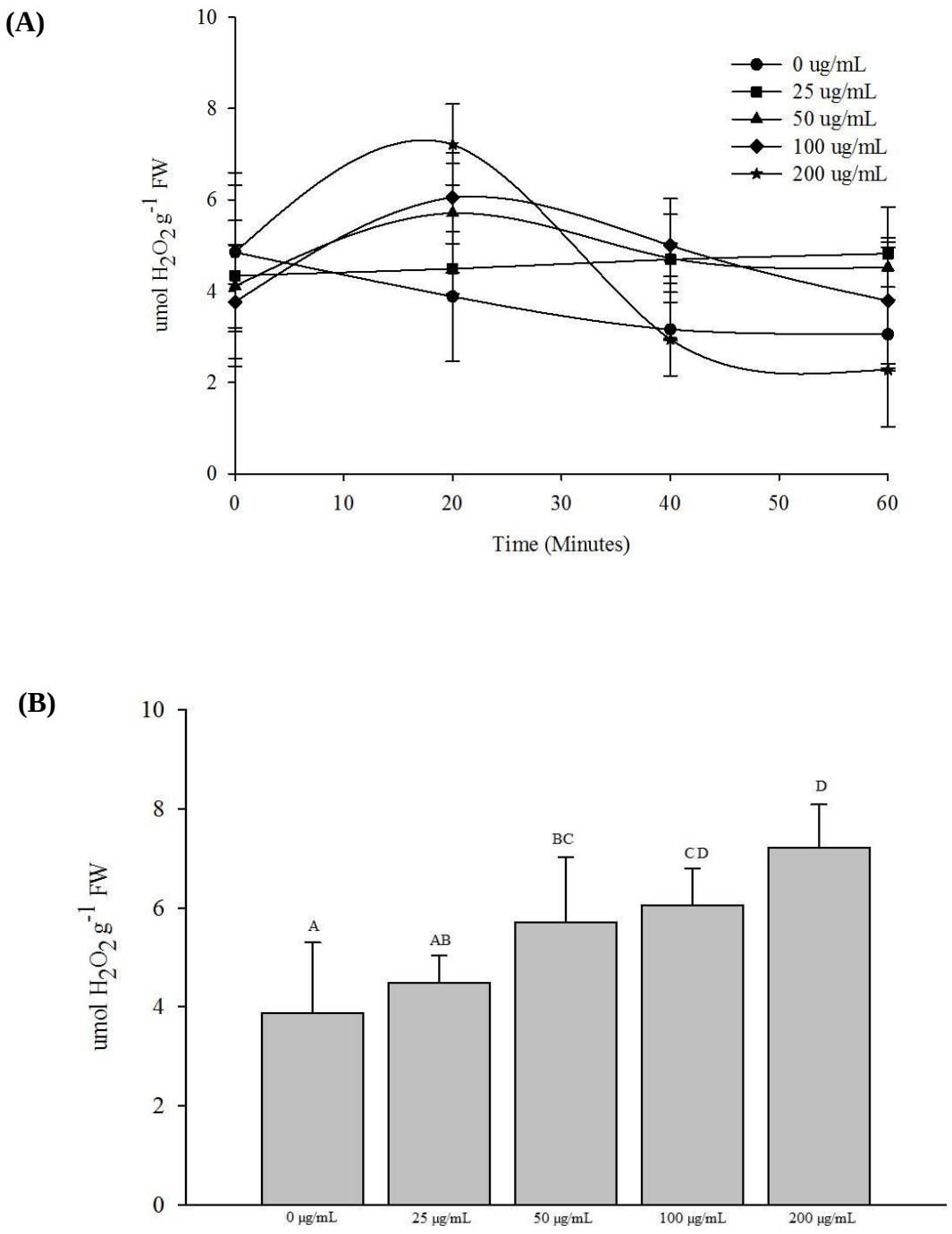


Figure 2.1 (A) Kinetics of ROS release in response to LPS elicitation in *T. testudinum*. Data points are the mean of 5 replicates (± 1 SEM). (B) H_2O_2 produced at the oxidative burst maxima (20 min time point of Fig. 1A) in response to selected concentrations of LPS. Data points are the mean of 5 replicates (± 1 SEM). Letters above bars indicate significantly different groups (p = 0.000).

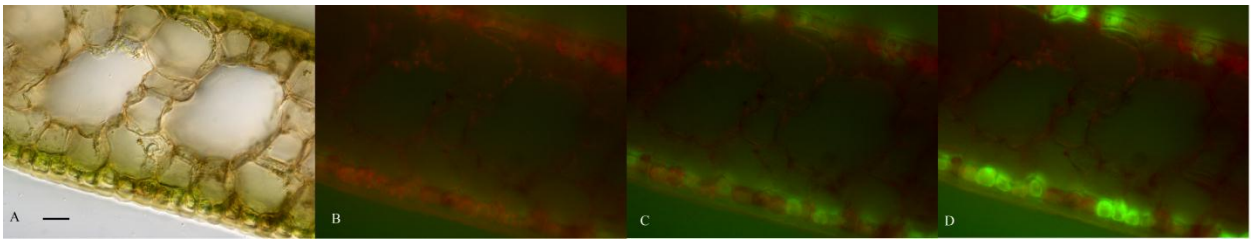


Figure 2.2 Detection of reactive oxygen species (ROS) production in *T. testudinum* with H_2DCF-DA using fluorescence microscopy. Samples were treated with a 200 µg/mL solution of LPS. (A) Brightfield image of blade cross section. (B) Fluorescent image of sample prior to LPS addition with chloroplast autofluorescence shown in red. (C) Image of sample at 10-min post elicitation showing ROS localization in green. (D) Image of sample at 30-min post elicitation. Scale bar = 20µm.

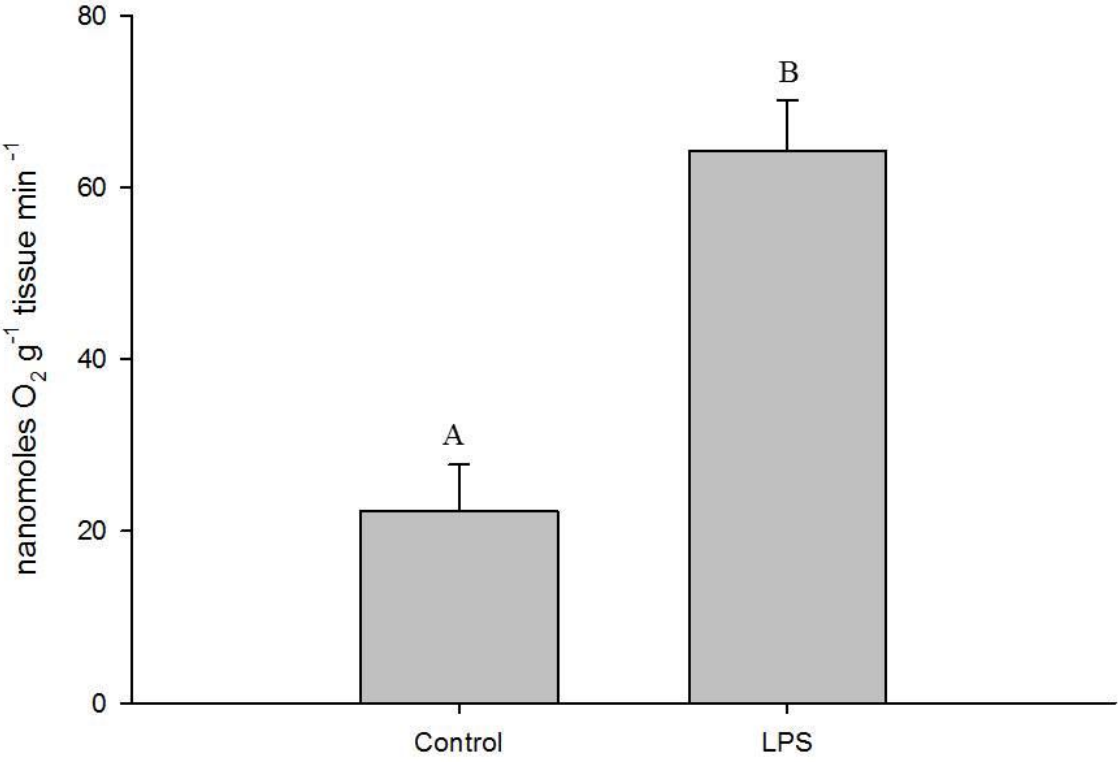


Figure 2.3 Oxygen consumption of *T. testudinum* in response to LPS elicitation. Data points represent the mean respiration values of 3 replicates (± 1 SEM). After 15 minutes respiratory rates were quantified as nanomoles of O_2 consumed g^{-1} minute $^{-1}$. Statistical difference was calculated using a Student's t test ($p < .050$). Letters indicate difference between groups.

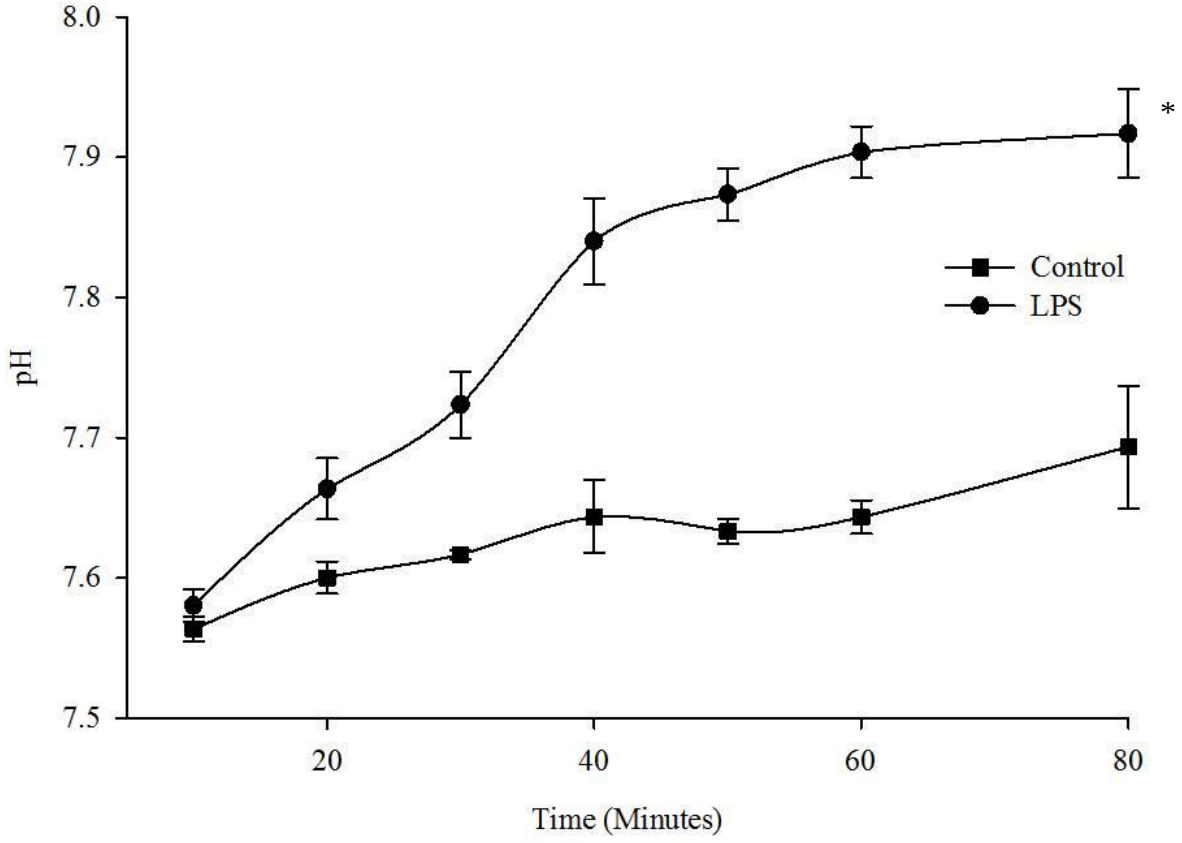


Figure 2.4 Alkalization of extracellular media in LPS treated *T. testudinum*. Data points are the mean of 3 replicates (± 1 SEM). Elicitation by LPS caused a significant increase in media pH as a function of time ($p < .0005$), * denotes significant difference between group effects.

2.3.2. Signal transduction events associated with the oxidative burst in *T. testudinum*

The signal transduction pathway underlying the oxidative burst in *T. testudinum* was examined by pharmacological inhibition/activation of specific cellular targets. The results are summarized in Table 2.1 and compare the percent inhibition or enhancement of ROS formation at the 20 min time point. Quinacrine, an inhibitor of flavin-dependent redox enzymes, was capable of inhibiting the emission of H_2O_2 by 58% at 100μM.This may indicate specificity toward the flavenoid group of a membrane-associated gp91-phox subunit of the NADPH oxidase complex. To further verify the activity of an NADPH oxidase, the suicide inhibitor DPI was used. This compound showed a moderate level of inhibition of H_2O_2 production when used at 50 uM. As ROS can be generated by a peroxidase-dependent process, the cytochrome inhibitors sodium azide (NaN_3) and potassium cyanide (KCN) were utilized and showed a 52% and 46% inhibitory response, respectively. The exogenous addition of catalase (100 U/ml) was capable of eliminating ROS production by 33%, suggesting that H_2O_2 represents a component of the ROS pool. Ion flux regulation, in response to LPS elicitation, was investigated using a series of specific ion channel blockers, as well as ionophores. Inhibitors such as the anion channel inhibitor NPPB and the calcium channel blocker methoxyverapamil were capable of blocking the oxidative burst by 51% and 66%, respectively. All the specific ionophores tested, monensin (Na^+), A23187 (Ca^{2+}) and valinomycin (K^+), enhanced the oxidative response when *T. testudinum* was challenged with LPS. Staurosporine was utilized to examine the possible involvement of protein phosphorylation in the process of LPS elicitation and subsequent signal transduction. This compound is a potent inhibitor of serine and threonine kinases (Lawrie et al., 1997) and was capable of blocking H_2O_2 production by over 55% at a concentration of 5μm. Chlorpromazine-HCl, an antagonist of phospholipase A_2 in higher plants (Chandra et al., 1996),

blocked the induction response by 94%. The heterotrimeric G-protein inhibitor pertussis toxin (Fujisawa et al., 2001) was capable of blocking the ROS production by 56%. The chelation of calcium with EGTA inhibited ROS formation, while Ca^{2+}, in the form of $CaCl_2$, slightly amplified the oxidative burst. Lastly, the protein kinase-c activator phorbol 12-myristate 13-acetate (PMA) caused a moderate inhibition of ROS production at the 20 min time point yet caused enhanced ROS production over the course of an ensuing 55 minutes (data not shown).

Table 2.1 Effect of Modulators on the Oxidative Burst in *Thalassia testudinum*

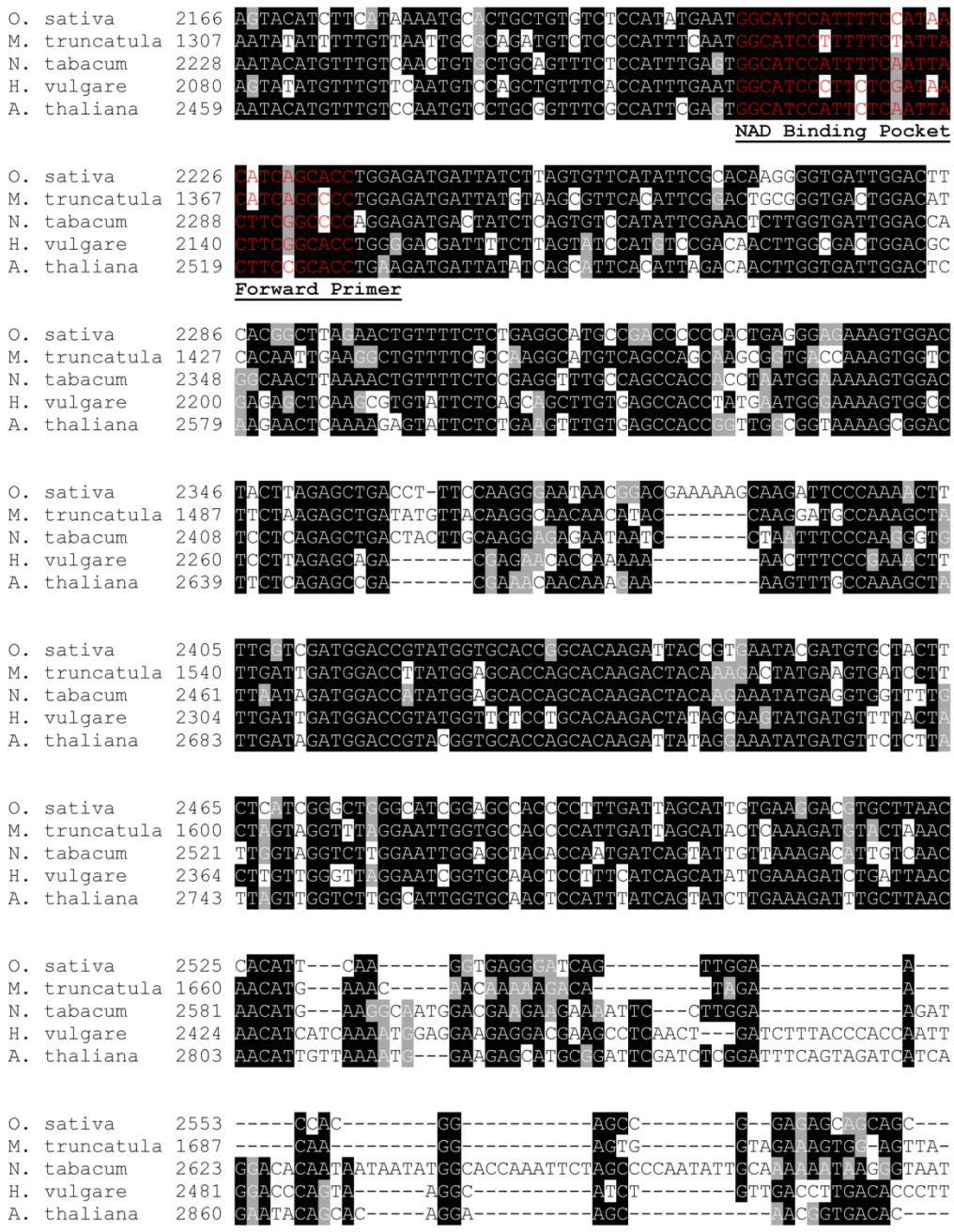
Data shown is the average value obtained from 5 independent experiments (± 1SE). Details of the experiment are provided in the Materials and methods.

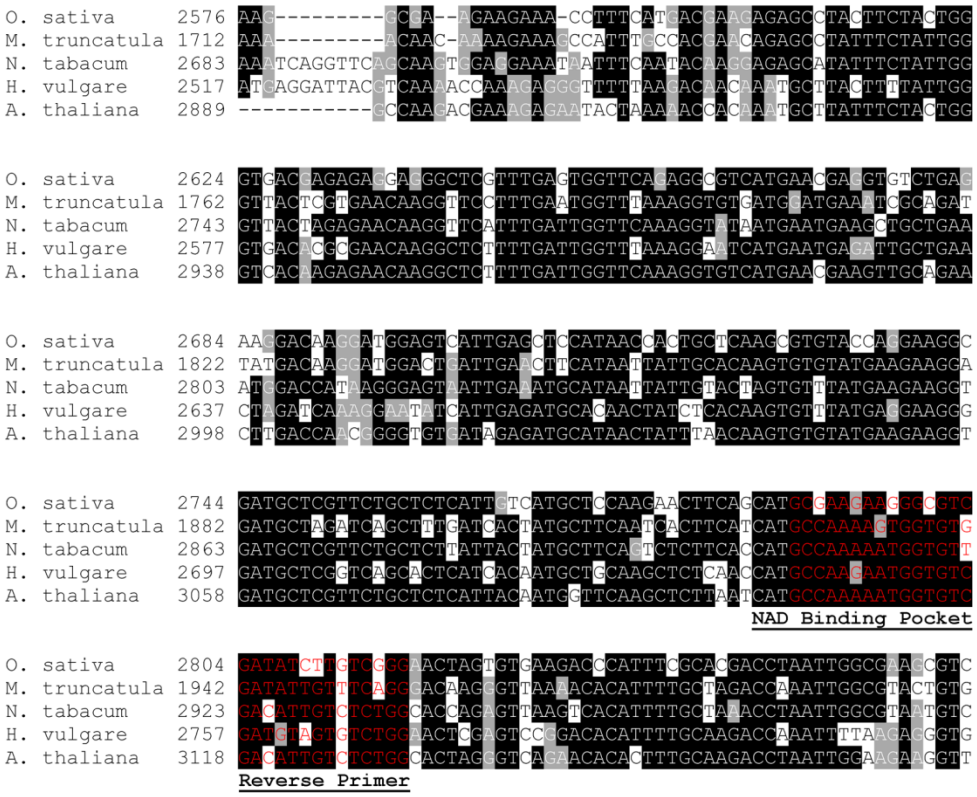
Modulator	**Concentration**	**H_2O_2 formed (% Control)**	**Mechanism of action**
Elicitor			
Lipopolysaccharide (*E. coli)*	200 µg/mL	100	N/A
Inhibitors			
Quinacrine	100 µM	42 (± 5.7)	Flavoprotein Inhibitor
DPI	50 µM	67 (± 7.6)	NADPH Oxidase Inhibitor
KCN	1 mM	54 (± 3.1)	Peroxidase Inhibitor
NaN_3	1 mM	48 (± 11.1)	Peroxidase Inhibitor
Catalase	100 U/mL	67 (± 5.8)	H_2O_2 Decomposition
NPPB	100 µM	49 (± 3.8)	Anion Channel Blocker
Staurosporine	5 µM	56 (± 2.4)	Protein Kinase Inhibitor
Chlorpromazine-HCl	100 µM	6 (±1.3)	Phospholipase A_2 Inhibitor
Methoxyverapamil	200 µM	34 (±13)	Ca^{2+} Channel Blocker
Pertussis Toxin	1 µg/mL	44 (±6.7)	G Protein Inhibitor
EGTA	1 mM	64 (+3.7)	Calcium Chelator
Ionophores			
Monensin	100 µM	104 (±1.9)	Na^+ Ionophore
A23187	100 µM	145 (±7.9)	Ca^{2+} Ionophore
Valinomycin	100 µM	155 (±8.2)	K^+ Ionophore
Activators			
PMA	15 µM	59 (±15)	Protein Kinase Activator
$CaCl_2$	2 mM	102 (±5.4)	Calcium Supplement

2.3.3. Partial Cloning of an NADPH oxidase from *T. testudinum*

Rboh gene sequences from multiple plant species were aligned and highly conserved regions were identified and used for the creation of degenerate primers designed to amplify a rboh ortholog from *T. testudinum* (Fig. 2.5A). A primer pair designed to amplify a 3' region, corresponding to the NAD binding pocket of a candidate NADPH oxidase gene, produced a 900 base pair fragment which contained a ~ 280 bp region with significant homology to the NAD binding pocket of the *A. thaliana* RbohF gene (Fig. 2.5B).

A


O. sativa 2166
M. truncatula 1307
N. tabacum 2228
H. vulgare 2080
A. thaliana 2459
NAD Binding Pocket
O. sativa 2226
M. truncatula 1367
N. tabacum 2288
H. vulgare 2140
A. thaliana 2519
Forward Primer
O. sativa 2286
M. truncatula 1427
N. tabacum 2348
H. vulgare 2200
A. thaliana 2579
O. sativa 2346
M. truncatula 1487
N. tabacum 2408
H. vulgare 2260
A. thaliana 2639
O. sativa 2405
M. truncatula 1540
N. tabacum 2461
H. vulgare 2304
A. thaliana 2683
O. sativa 2465
M. truncatula 1600
N. tabacum 2521
H. vulgare 2364
A. thaliana 2743
O. sativa 2525
M. truncatula 1660
N. tabacum 2581
H. vulgare 2424
A. thaliana 2803
O. sativa 2553
M. truncatula 1687
N. tabacum 2623
H. vulgare 2481
A. thaliana 2860


O. sativa 2576
M. truncatula 1712
N. tabacum 2683
H. vulgare 2517
A. thaliana 2889
O. sativa 2624
M. truncatula 1762
N. tabacum 2743
H. vulgare 2577
A. thaliana 2938
O. sativa 2684
M. truncatula 1822
N. tabacum 2803
H. vulgare 2637
A. thaliana 2998
O. sativa 2744
M. truncatula 1882
N. tabacum 2863
H. vulgare 2697
A. thaliana 3058
NAD Binding Pocket
O. sativa 2804
M. truncatula 1942
N. tabacum 2923
H. vulgare 2757
A. thaliana 3118
Reverse Primer

B

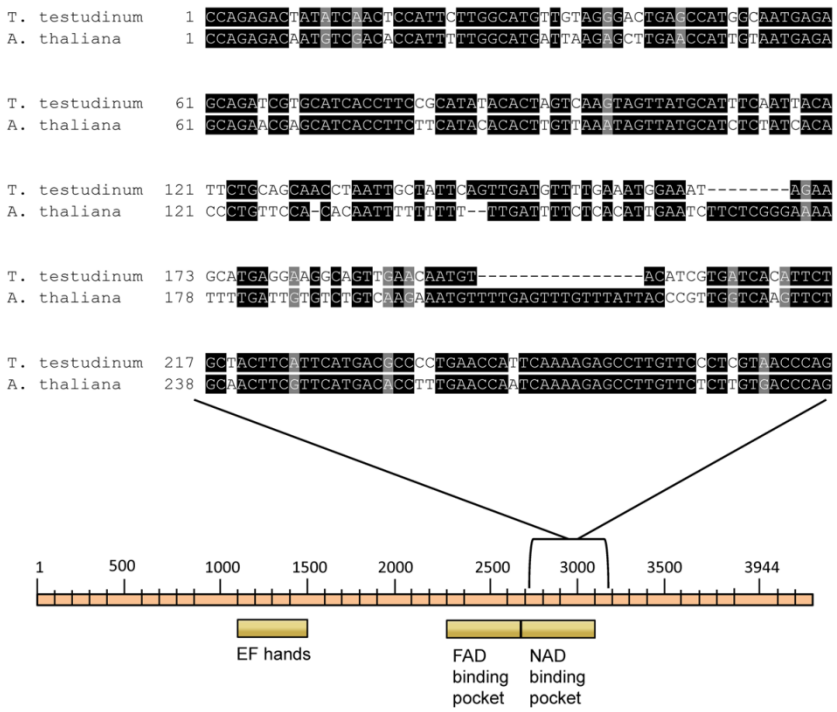


Figure 2.5 Partial sequence of a NADPH oxidase from *T. testudinum*. A) Alignment of the nucleotide sequence corresponding to the NAD binding pocket domain of rboh genes from *O. sativa, M. truncatula, N. tabacum, H. vulgare,* and *A. thaliana*. The locations of the sequence used to design the forward and reverse degenerate primers are highlighted (red), labeled and underlined. B) Alignment of a segment of the cloned *T. testudinum* NADPH oxidase fragment that shows significant sequence homology with the NAD binding pocket region of the rbohF gene from *A. thaliana*. The region corresponding to the aligned sequence is indicated on the graphical depiction of the rbohF gene.

2.4 Discussion

2.4.1. Induction of an Oxidative burst in *T. testudinum*

Despite the principal roles of ROS in terrestrial plant physiology, reports of its occurrence in marine vascular plants, especially in the context of disease resistance, are extremely limited. To our knowledge, this study represents the first finding of an LPS-induced oxidative burst in a seagrass and more broadly demonstrates that pathogen perception in aquatic angiosperms parallels the innate immune systems found in terrestrial plants and lower algae.

The concentration of LPS required to elicit an oxidative burst in *T. testudinum* was 50 μg/mL, which is comparable to results found in other plant studies where the LPS threshold ranged from 5–100 μg/mL (Erbs and Newman, 2012). While plants can detect other prokaryotic PAMPs such as flagellin and Ef-Tu elongation factor at subnanomolar levels, it has been suggested that plants only possess low-affinity membrane-associated receptors to detect LPS (Zeidler et al., 2004; Erbs and Newman, 2012). The time frame of maximal H_2O_2 emission was similar to results obtained in tobacco cell suspensions when challenged with LPS from the phytopathic bacteria *Xanthomonas campestris* (Meyer et al., 2001). In both studies, a delayed oxidative burst occurred which could be attributed to the decreased availability of LPS in aqueous solutions, as LPS can form micellar vesicles (Seydal, 1993; Schromm et al., 1995). Alternatively, the lag phase could have been due to the biochemical conversion of metabolites or induction of enzymes upstream of hydrogen peroxide production as suggested by Kupper et al. (2006).

Attempts to detect an oxidative burst in *T. testudinum* in response to extracts of *Labyrinthula* spp. were unsuccessful. One explanation for the lack of ROS production could be due to *T. testudinum*'s inability to recognize *Labyrinthula*-based PAMPs. Lipopolysaccharides

are not cell wall constituents in members of the Labyrinthulomycetes. The cell walls of *Labyrinthula* spp. are characterized by the presence of dictyosome-derived carbohydrate scales (Bahnweg and Jäckle, 1986), of which the precise composition is not known. Evidence for the presence of sulphated polysaccharides (predominantly galactose or fucose-based) and proteins in the cell walls of closely related taxa, such as thraustochytrids has been reported (Chamberlain, 1980; Bahnweg and Jäckle, 1986; Raghukumar, 2002); thus, it seems plausible that similar constituents may be found in *Labyrinthul*a spp. External to the cell wall, *Labyrinthula* spp. possess an ectoplasmic network (EN) that has been characterized as plasma-membrane bound cytoplasm rich in actin and myosin (Hoh,l 1966; Preston and King, 2005). It is possible that *T. testudinum* does not have the capability to recognize any of these cell wall or EN-based components when presented in the form of an aqueous crude extract. However, it should be noted that the use of crude extracts has been successful in eliciting defense responses in *A. thaliana* (at much lower concentrations used in our study) with fungal- (200 μg/mL; Ndimba et al., 2003) and bacterial-derived elicitors (45 μg/mL; Pfund et al., 2004).

Our O_2 consumption data for *T. testudinum* indicated that there was a notable uptake of O_2 from the ambient seawater. This uptake would clearly provide an adequate level of oxygen to use as a substrate for the enzymatically based turnover of O_2 to H_2O_2. We did not detect an immediate respiratory burst as has been characterized in other plant systems (Wojtaszek, 1997). However, there was a steady-state increase in O_2 consumption as a function of time following LPS elicitation. A similar response has been detected in potato using heat killed *Pseudomonas syringae* as the elicitor (Baker et al., 2001).

Extracellular alkalinization is one of the earliest events occurring in elicitor-treated plant cell suspension cultures. This alkalinization results from elicitor-induced depolarization of the

plasma membrane and subsequent K^+/H^+ exchange, with Ca^{2+} influx/Cl^- efflux (Boller, 1995). There is typically a simultaneous acidification of the cytoplasm that is believed to be an essential component of the signal transduction pathway leading to an oxidative burst (Zhao et al., 2005). The intensity of the pH shift in plants is clearly dependent upon the type of host cells used and the structure of the elicitor provided (Toppan and Esquerre-Tugaye, 1984; Clarke et al., 2005; Davies et al., 2006). The ability for *T. testudinum* to undergo extracellular alkalinization in response to LPS elicitation suggests this phenomenology is conserved in marine vascular plants.

2.4.2. Elicitor signal transduction

Results obtained from our pharmacological studies using DPI support the premise that the oxidative burst in *T. testudinum* may involve the activity of a flavoprotein-containing NADPH oxidase complex that is analogous to what is involved in ROS production in terrestrial plants (Liu et al., 2009; Marino et al., 2011). The ability for quinacrine to inhibit the burst provides additional evidence for the possible involvement of a flavonoid component of a membrane-associated gp91-phox subunit. As NaN_3 and KCN could partially inhibit the production of H_2O_2, this prompted speculation as to exactly where ROS were being produced. Both azide and cyanide can block heme-based peroxidases, yet NADPH oxidases, which fall under the flavoprotein family, are typically insensitive to these inhibitors (Davies et al., 2006). It should be noted that DPI has been reported to serve as an effective inhibitor of heme-based peroxidases, suggesting that it may not be very selective when distinguishing between NADPH oxidases and peroxidases (Dwyer et al., 1996; Bolwell et al., 1998; Frahry and Schopfer, 1998). However, it is possible that both enzymatic sources are contributing towards the ROS pool or functioning sequentially as NADPH oxidases could be activated by ROS originating from cell-wall bound peroxidases (Martinez et al., 1998; Grant et al., 2000; Davies et al., 2006; Daudi et al., 2012).

Research identifying the signaling pathway components in aquatic vascular plants is most certainly lagging behind the ever-expanding state of knowledge found in the terrestrial plant literature. To add to the complexity, the activation of different signaling systems may vary based upon the origin of the sensor and on the nature of the stress event. In both the NADPH oxidase and apoplastic peroxidase ROS-generating models, an elicitor molecule is detected by a receptor located on the plasma membrane. In turn, a signal transduction cascade is initiated that activates downstream molecular responses. Data available from terrestrial plants support the involvement of G proteins, ion channels (especially Ca^{2+}), protein kinases, protein phosphatases, phospholipases A and C, and cyclic AMP. This pathway ultimately gives rise to the activation of either an NADPH oxidase complex or a pH-dependent cell wall peroxidase (Wojtaszek, 1997). Our data show that a variety of identifiable signal transduction steps (homologous to what has been reported in terrestrial plants) are involved in ROS production in *T. testudinum* when elicited with LPS.

The phosphorylating activities of kinases are considered to be an integral component of numerous metabolic regulatory mechanisms. Staurosporine was reported to block protein phosphorylation events involved in induction of defense responses in cryptogein-elicited tobacco cells (Viard et al. 1994) and in fungal-elicited spruce cells (Schwacke and Hager, 1992). In our study, staurosporine proved to be an effective inhibitor in *T. testudinum*, suggesting that serine and threonine kinases are involved in an upstream stage of signal transduction. The involvement of protein kinase C (PKC) in respiratory burst signaling cascades, by use of the activator phorbol 12-myristate 13-acetate (PMA), has been demonstrated in fish phagocytes (Castro et al., 2004) and in chlorophytic algae (Ross et al., 2006). In both cases, PMA caused a dose dependent activation of the oxidative burst. Conversely, in *T. testudinum*, there was a moderate inhibition of

ROS produced at the 20 min time point. PMA has been previously shown to have an inhibitory effect on Na^+/H^+ exchange in carcinoma cells (Whiteley et al., 1984) as well as on the phosphorylation-dependent inhibition of anaplastic thyroid cancer cell line proliferation (Afrasiabi et al., 2008). Chlorpromazine-HCl, a selective inhibitor of phospholipase A_2, caused a strong inhibition of the oxidative burst in *T. testudinum*. The production of ROS, involving the activation of phospholipases, has also been demonstrated in soybean (Legendre et al., 1993; Chandra et al., 1996) and in brown algae (Kupper et al., 2001).

Bacterial toxins, such as pertussis toxin, have proven to be useful probes for studying the roles of heterotrimeric G proteins in signal transduction pathways (Moya and Jacobs, 2006). The participation of G proteins was analyzed using cholera toxin in French beans (Bolwell et al., 1991) and was also shown to be involved in the signal transduction cascade associated with the fungal-elicited synthesis of alkaloids in the bloodroot flower *Sanguinaria canadensis* (Mahady et al., 1998). Pertussis toxin proved to inhibit ROS production in *T. testudinum* indicating that G-proteins are involved in this elicitor-triggered oxidative burst.

The regulation of ion flow, mainly Ca^{2+} and H^+ influx and K^+ and Cl^- efflux, is one of the most rapid responses of plant cells to elicitation (Noh and Spalding, 1998; Dennison and Spalding, 2000). The multifunctional roles of ion fluxes in plant systems prompted our investigation into the utilization of ion channels as part of the signal transduction process in *T. testudinum*. The use of the K^+ ionophore valinomycin in conjunction with LPS caused a very large enhancement of ROS production. A similar observation was reported by Kupper et al. (2001) using oligoguluronate elicitation in the brown algae *L. digitata*. Conversely, Bolwell et al. (1995) noted that the pretreatment of valinomycin in French bean cells triggered ion leakage

which subsequently caused a negative impact on the efficiency of pH-dependent cell wall peroxidase(s).

Calcium flux or local fluctuations of internal calcium concentrations are among one of the earliest signaling events associated with the oxidative burst in terrestrial plants (Chandra and Low, 1997; Grant et al., 2000). In addition, positive feedback loops have been identified where the binding of Ca^{2+} promotes ROS formation via NADPH oxidases. This subsequently causes calcium channels to become activated, resulting in a self-amplifying signal (Ogasawara et al., 2008; Takeda et al., 2008). The involvement of elevated cytosolic Ca^{2+} levels also appears to be integrated into the signaling cascade of *T. testudinum*. The combined effects of EGTA chelation, $CaCl_2$ supplementation, and the use of methoxyverapamil and ionophore A23187 all strongly implicate the role of intracellular Ca^{2+} in ROS formation. Similar effects have been noted in French bean (Bindschedler et al., 2001), *Arabidopsis* (Davies et al., 2006), spruce (Schwacke and Hager, 1992) in addition to brown and green algae (Kupper et al., 2001; Ross et al., 2006). This suggests that the ROS-Ca^{2+} relationship represents a more widely used signal codependence that spans the majority of photoautotrophic taxa.

2.4.3. Molecular characterization of an NADPH oxidase from *T. testudinum*

Plant Rbohs are a multigenic family of enzymes with a C-terminal region containing cytosolic FAD- and NADPH-binding domains, responsible for oxidase activity, in addition to six conserved transmembrane helices and an N-terminal regulatory domain (Sagi and Fluhr, 2006). Rboh homologs are unique among ROS-producing enzymes in that they integrate different signal transduction pathways such as calcium, protein phosphorylation and lipid signaling with ROS production (Suzuki et al., 2011). The partial cloning of a gene containing a NAD^+ binding pocket, in conjunction with the signal transduction and pharmacological data presented above,

suggest that seagrass may possess at least one functional respiratory burst oxidase enzyme gene and this would represent the first time this class of enzyme has been identified in a seagrass species.

In conclusion, the present results demonstrate that components of the outer membranes of Gram-negative bacteria can be considered as exogenous elicitors in seagrass and future work should be aimed at identifying other PAMPS in their roles in seagrass defense.

3.

The pseudo-presence of programmed cell death during the interaction between the stramenopile pathogen *Labyrinthula* spp. and the seagrass *Thalassia testudinum*

Abstract

Thalassia testudinum produces reactive oxygen species (ROS) locally in response to microbial exposure. Currently it is undetermined whether or not the increased amount of ROS after infection is simply an antimicrobial agent or a signaling molecule that will initiate programmed cell death (PCD) and lead to the hypersensitive response (HR), a process not yet characterized in marine angiosperms. ROS accumulation was found to increase around the lesion throughout the duration of the infection. However, the only PCD characteristic observed following infection and the increase of ROS was a slight increase in caspase-like protease activity around the lesion. Immunohistochemistry revealed inconsistent activity of proteases, and showed that some of the cells in the lesion-containing may be undergoing PCD, but not in large aggregates or at any localized sites. Detection of nuclear condensation by TUNEL and Hoechst staining were also inconclusive and showed diffuse genetic material throughout the cytoplasm. In conclusion, it appears as though lesions stemming from *Labyrinthula* spp. infection are likely to be a direct result of pathogen-based damage as opposed to host PCD.

3.1. Introduction

Seagrasses are vital to coastal ecosystems and economies. They provide sediment stability and prevent coastal erosion by reducing wave energy and the root and rhizome network help to stabilize the benthos (Dawes et al., 2004). Seagrass meadows also provide nursery habitat for a variety of commercially important fish and invertebrates. Since the 1980's, however, there have been drastic declines in the amount of seagrass habitat due largely in part to population expansion and coastal development (Waycott et al., 2009). Another lesser known cause of the rapid decline in seagrasses is the periodic outbreaks of wasting disease caused by the pathogenic protist slime mold *Labyrinthula* spp. Wasting disease was one of the major factors that lead to the decline of *Zostrera marina* in the Chesapeake during the 1940's (Orth et al., 2006) and has also been associated with declines associated with *Thalassia testudinum* populations in Florida Bay during the 1980's (Fourqurean and Robblee, 1999). While the amount of research on the interaction between *Labyrinthula* spp. and *T. testudinum* has increased in recent years (Trevathan et al., 2011; Bockelmann et al., 2012), there is still little known with respect to the innate immune response of *T. testudinum* upon pathogen attack.

Plant innate immune response to pathogenic invasion includes unique signaling cascades that ultimately give rise to the production of reactive oxygen species, calcium ion fluxes and the involvement of Rboh F genes (Levine et al., 1994, 1996; Lam ,2004) that are involved in localized programmed cell death (PCD), which is an orderly process of biochemical reactions and cellular hallmarks initiated intracellularly (Van Breusegem and Dat, 2006). Indicators of PCD include induction of caspase activity, DNA laddering, nuclear blebbing, loss of ATP, and the cellular membrane becoming compromised allowing for leakage of cellular contents. Cell death is an integral part of development and immunity. During mammalian development PCD is

responsible for removal of the webbing between digits, palate closure, eye and ear development, and the development of male and female reproductive systems (Mirkes, 2002).

Programmed cell death has only been recently documented in plants and was first noticed and charactorized as the mechanism that causes the death of xylem, vasculature that at maturity is dead (Groover, 1997). The hallmarks of plant PCD are similar to that of metazoan PCD, however mechanistically they are slightly different. In plants the proteases involved are caspase like, an actual caspase has not yet been found in plant systems. Several caspase like proteases have been found in terrestrial plants (Groover and Jones, 1999; Lam et al., 2001; Watanabe and Lam, 2004). Metacaspases and vacuolar processing enzymes are responsible for the initiation of PCD. The location of these enzymes is also different. Typically caspases reside in an inactive form in the cytosol. Plant PCD proteases can be in the cytosol but are also contained in plastids and within the vacuole (Sanmartín et al., 2005). DNA laddering is similar in both plants and animals as well. Nucleases cause fragmentation of DNA in an orderly manner. Laddering associated with PCD in plants has been observed in both whole tissue and cell culture (Reape and McCabe, 2010; Mochizuki-Kawai et al., 2013). Membrane permeability is considered to be the the committed step in PCD. Once the cellular and mitochondrial membranes become permeable the cell has reached a point of no return and PCD is initiated (Govrin and Levine, 2000; Jones, 2000; Reape and McCabe, 2010).

PCD in tissue surrounding the initial infection site, which limits pathogen spread, is termed the hypersensitive response (HR) (figure 3.1). Progression of pathogens into living tissue is halted by the HR depriving the pathogen of nutrients and effectively starving the pathogen. The HR is an incompatible plant-pathogen interaction because it halts pathogen progress and confines it to a localized area (Hammond-Kosack and Jones, 1996). This strategy is highly

effective against biotrophic pathogens, those requiring living tissue for sustenance (Gilchrist, 1998) and to extent hemibiotrophic pathogens, those that exhibit both trophic strategies during their lifecycle. More recently it has being demonstrated that necrotrophic pathogens are able to take advantage of the hypersensitive response and induce PCD in the host to provide an ongoing line of continuous nourishment as the lesion expand (Govrin and Le,vine 2000; van Kam, 2006).

The HR has been demonstrated in a wide variety of terrestrial plants but once again knowledge as to whether or not marine angiosperms are capable of undergoing the HR is lacking. While it is clear that seagrass tissues undergo cell death when infected by pathogens (Muehlstein, 1992), it is not evident if this cell death is actually caused by pathogen damage or if the host is undergoing programmed cell death in an attempt to block off the advancement of the progressing pathogen. Unfortunately, due to the lack of knowledge of the seagrass-associated Labyrinthulid life history, it is not clear if this pathogen has a biotrophic, necrotrophic or hemibiotrophic lifestyle. Results from the previous chapter, evidence of calcium fluxes, the detection of an oxidative burst, and the involvement of RbohF gene strongly suggest that the *Thalassia-Labyrinthula* interaction could be a compatible interaction resulting in the induction of the HR. The goal of this study was to determine if *T. testudinum* is undergoing PCD during the early stages of infection (72h) when infected with *Labyrinthula* spp. Characteristic signs of PCD, which include ROS accumulation, activation of caspase-like plant proteases, nuclear condensation and DNA laddering was evaluated.

Figure 3.8 The hypersensitive response in tobacco when induced with the tobacco mosaic virus (TMV) (Lam et al. 2001).

3.2. Methodology

3.2.1. Seagrass Collection and Maintenance

Thalassia testudinum shoots were collected from Pepper Fish Key in Horseshoe Beach, Florida (29°29'N, 83°23'W) and cleaned of epiphytes. Short shoots included the blades, sheath and a 2-inch section of rhizome. Following collection, all specimens were allowed to acclimate for no less than 1 week under greenhouse conditions at the University of North Florida, Jacksonville, FL prior to use in any experiment. Plants were maintained in aquaria at a salinity of 30 under shade cloth allowing a maximal penetration of 15% photosynthetic active radiation (< 300 μmol $m^{-2}s^{-1}$). Diel temperature values ranged between 25-27 °C.

3.2.2. Labyrinthula Culture

A known virulent strain of *Labyrinthula* spp., previously isolated from *T. testudinum*, was used for all *Labyrinthula* elicitation experiments. *Labyrinthula* spp. cultures were maintained in serum-seawater agar (SSA) as previously described in Trevathan et al. (2011).

3.2.3. Infection Experiments

To determine if PCD was initiated during the early stages of *Labyrinthula* infection, selected biochemical responses of T. *testudinum* were evaluated over a 72 h time period. Tissue samples were collected at the following time points: 1) 0 h (prior to infection), 2) 24 h post-infection, 3) 48 h post-infection and 4) 72 h post-infection. Five individual short shoots were used per treatment (n = 5), In addition, uninfected controls (n = 5) were run to test for the effect

of time. For consistency, the second rank blade (i.e., second youngest) was always infected using the methods reported by Steele et al. (2005). Sterilized vectors were placed on week-old *Labyrinthula* spp. agar cultures in areas of equal growth for 1 week prior to infection. Individual *T. testudinum* short shoots (1 short shoot being equivalent to one experimental unit) were placed in 3.8 L polyethylene terephthalate microcosms (Rubbermaid, Winchester, VA, USA) containing 0.22 µm filtered seawater (salinity = 30) All samples were randomly spaced apart at 28 °C under 170 µmol $m^{-2}s^{-1}$ of full spectrum lighting using Power-GLO T5 HO bulbs (Hagen, West Yorkshire, UK) on a 12:12 h L: D photoperiod. Sterilized control vectors and infected vectors were secured onto the middle of the second rank blade with a clamp made from ¼ inch flexible PVC tubing (Aquatic Eco-Systems, Inc., Apopka, FL, USA). During the 72 h ensuing infection period, temperature and salinity were monitored on a daily basis. Deionized water was added as needed to account for evaporation.

3.2.4. Detection of Reactive Oxygen Species in Plant Tissue

Qualitative

To assess the time course of ROS accumulation in *T. testudinum* from *Labyrinthula* infection, the ROS specific probe 2', 7'-dichlorofluorescein-diacetate (H_2DCF-DA; Invitrogen, Carlsbad, CA, USA) was employed. In the reaction a nonfluorescing nonpolar compound, H_2DCF-DA, reacts with cellular esterases cleaving the diacetate group to yield the polar compound DCFH; oxidation of DCFH by ROS yields the fluorescent product DCF.

Post treatment, 2 cm leaf clippings from each replicate were incubated for 15 min in 10 mL of microcosm water containing H_2DCF-DA (5µM final concentration, made from 10 mM stocks in

dimethyl sulfoxide). Prior to microscopic examination, samples were rinsed in filtered seawater to remove any unbound probe. Fluorescent imaging (ex 488 nm, em 525 nm) was taken using a Leica MZ10 F microscope (Leica Microsystems, Inc., Bannockburn, IL, USA) in conjunction with a Canon PowerShot S5-IS (Canon U.S.A. Inc., Melville, NY, USA).

Quantitative

To quantify the levels of ROS generated at the end of each treatment, a peroxide assay was utilized (PeroXOquant^{TM}, Thermo Scientific) according to the methods of Trevathan et al. (2011).

3.2.5. SYTOX Stain

After infections, lesions from *T. testudinum* were excised and stained using SYTOX Green Nucleic Acid Stain (Invitrogen), a quantifiable dead cell indicator. The stain penetrates compromised plasma membranes and cannot penetrate intact or live membranes. Nucleic acids react with the stain and fluoresce green. This fluorescence is indicative of cell death. Cell death was assayed by staining leaves in 2 mM SYTOX for 1 hour in a falcon tube then placed on a microscope slide to be visualized. Fluorescent imaging (ex 488 nm, em 525 nm) was taken using a Leica MZ10 F microscope (Leica Microsystems) in conjunction with a Canon PowerShot S5-IS. Image J (version 1.46) software was used to measure the area of lesion size at each time point.

3.2.6. Caspase Activity

Caspase Assay

Following infection with *Labyrinthula* spp., samples of *T. testudinum* were assayed at specific time points for caspase activity. At the end of each time point the 2 cm section containing the lesion was excised and gently towel dried then flash frozen with liquid $N_{2.}$ Sections were homogenized using a FastPrep 24® bead homogenizer (MP Biomedicals, Irvine, CA, USA) in reaction buffer (400 µL of 100 mM PIPES, pH 7.4, 20 mM EDTA, 1% CHAPS and 10 µL of 1 M dithiothreitol) for 30 seconds at 6 ms^{-1} then again for 1 minute at the same velocity. Samples were centrifuged for 5 minutes at 10,000 x g. The supernatant was collected and centrifuged again for an additional 5 minutes. The supernatant was collected and protein concentration was quantified using a Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Pittsburgh, PA, USA). The Enzchek1 Caspase-3 Assay Kit #2 (Life Technologies, Grand Island, NY, USA) 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 (Sanmartin et al., 2005; Lamkanfi et al., 2007). 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 mL of 1x reaction buffer and 10 mL Z-DEVD-R110 substrate (final substrate concentration, 25 µM). Samples were incubated at room temperature for 25 min and subsequently assayed for caspase catalyzed fluorescent cleavage product Rhodamine-110 using a Biotek, (Winooski, VT, USA) fluorescent platereader (Ex/Em: 496/520 nm; N = 5). The reversible aldehyde caspase inhibitor Ac-DEVD-CHO (Life Technologies) was used as a negative control according to the manufacturer's methods. Background fluorescence was subtracted for no-enzyme controls.

Positive controls using Recombinant Human Active Caspase-3 (Life Technologies) were also run.

Caspase Immunohistochemistry (IHC)

The 2cm section of tissue containing lesions was removed and prepared for immunohistochemistry according to the methods of Sauer et al. (2006). Briefly, samples were fixed in 4% paraformaldehyde overnight at -4 °C. Samples were then rinsed for 1 minute in phosphate buffered saline (PBS, 10mM, pH 7.4). Following the initial rinse, samples were subsequently rinsed an additional 4 times for 4 minutes each with PBS then incubated in 50% ethanol for 2 hours at -4°C. Samples were left overnight in 70% ethanol at -4°C. The next day samples were rinsed 4 times for 4 minutes each with PBS and incubated in 95% ethanol for 2 hours at -4°C. Samples were stored in 100% ethanol at -4°C until chlorophyll removal. Chlorophyll was removed with three washes in 100% methanol for 10 minutes at 37 °C. Samples were made permeable by incubating in 2% Driselase® (Sigma, St. Louis, MO, USA) for 1 hour at 37 °C followed by 3 five minute PBS washes. This was followed by additional 1 hour incubation in 3% IGEPAL® CA-630 (Sigma-Aldrich). A 1:200 dilution of the primary antibody, Anti-Caspase-3 Rabbit (CALBIOCHEM, EMD Millipore, Billerica, MA, USA) was used and a 1:1000 dilution of the secondary antibody, DyLight®488 Anti-Rabbit IgG (Vector Laboratories, Burlingame, CA, USA), was used at a concentration of 20 µg mL^{-1}. Fluorescent imaging was taken using an Olympus BX60 fluorescent microscope (ex 493 nm, em 518 nm; Olympus Corp., Tokyo, Japan).

Caspase Western Analysis

At the end of each time point segments with lesions were excised and gently towel dried then flash frozen with liquid N_2. Sections were homogenized using a FastPrep 24® (MP Biomedicals) bead homogenizer in PBS (.01 M pH 7.4) for 30 seconds at 6 ms^{-1} then again for 1 minute at the same velocity. Samples were centrifuged for 5 minutes at 10,000 x g. The supernatant was collected and centrifuged again for an additional 5 minutes. The supernatant was collected for western blot analysis. Protein concentration was quantified in order to normalize the amount of protein loaded into the wells using a BCA assay (Thermo Scientific) according to the manufacturer's instructions. The amount of protein extract used for each time point was 7474.8 µg/mL of protein. Two Novex Bis-Tris 4-12% gels (NuPage®, Invitrogen Carlsbad, CA, USA) were run using an XCell II Blot Module (Invitrogen) electrophoresis system following manufacturer's instructions. One gel was stained with Coomassie to visualize proteins the other was transferred onto a pre-cut polyvinylidene fluoride membrane (NEN Life Science Products, Boston, MA, USA) using an XCell II™ Blot Module (Invitrogen) for Caspase immunoblotting. The primary antibody used was Anti-Caspase-3 Rabbit at 1:1000 dilution (CALBIOCHEM). The secondary antibody used was DyLight 488 Anti-Rabbit at a 1:5000 dilution and a BCIP/NBT Alkaline Phosphatase Substrate Kit IV (Vector Laboratories) was used to visualize caspase.

3.2.7. TUNEL Assay

The TUNEL assay was used to detect fragmented DNA resulting from nuclease activity in response to infection. Nucleases fragment DNA into 200 bp length fragments and expose the 3' hydroxyl group on the end of the DNA fragment. The breaks in the DNA are labeled by terminal deoxynucleotide dUTP nick end labeling (TUNEL); this method causes the fragmented

DNA to fluoresce. The 2 cm leaf area exhibiting lesions was excised and placed in 5mL of 1% paraformaldehyde in PBS and incubated at -4 °C overnight. Samples were then rinsed for 1 minute in phosphate buffered saline (PBS). Following initial rinse, samples were rinsed an additional 4 times for 4 minutes each with PBS then incubated in 70% ethanol overnight at -4°C. Samples were made permeable by incubating in 2% Driselase® (Sigma) for 1 hour at 37 °C followed by 3 five minute PBS washes and another 1 hour incubation in 3% IGEPAL® CA-630 (Sigma-Aldrich). Detection of apoptosis was performed by using the APO-BrdU TUNEL Assay Kit (Invitrogen) according to the manufacturer's instructions. Infected samples were assayed with positive and negative controls from the kit; uninfected seagrass was used as a negative control. Fluorescent imaging (ex 495 nm, em 519 nm) was taken using an Olympus IX 81 inverted epifluorescent microsope (Olympus) in conjunction with a DP21 digital camera (Olympus).

3.2.8. Stain for Nuclear condensation

A 6 mm cork borer was used to remove tissue from uninfected controls and samples that had been infected for 72 hours. Seagrass tissue was incubated for 10 minutes in 5 mL of 3 µg/mL of Hoechst stain (Invitrogen). Following the incubation, samples were washed 3 times in 5 mL filtered seawater to remove any unbound probe. Individual plant sections were placed on 35 mm glass bottom microwell dishes (MatTek Corp, Ashland, MA, USA) in a drop of filtered seawater. Fluorescent imaging was taken using an Olympus IX 81 inverted epifluorescent microsope (Olympus) in conjunction with a DP21 digital camera (Olympus).

3.2.9. Nuclease Activity

At the end of each time point segments with lesions were excised and gently towel dried then flash frozen with liquid N_2. A 6 mm cork borer was used to remove tissue from *T. testudinum* treatments. Sections were then placed in 1.5 mL Eppindorf tubes with 500 µL of TRI Reagent and homogenized using a FastPrep 24® bead homogenizer for 30 seconds at 6 ms^{-1} then again for 1 minute at the same velocity. Samples were centrifuged for 5 minutes at 10,000 x g. The supernatant was collected and centrifuged again. An additional 500 µL of TRI reagent was added and DNA was isolated and quantified following the manufactures instructions. DNA content was normalized and 50 µg/mL of each sample was loaded into a 0.7% agarose gel for electrophoresis. An additional sample with the same DNA concentration from the zero time point was incubated with RQ1 RNase-Free DNase (Fisher Scientific, Pittsburgh, PA, USA) to serve as a positive control.

3.2.10. Statistical Analysis

All statistical tests were performed at a 95% confidence level. Analysis of the effect of time and infection on H_2O_2 accumulation was performed using a two-way ANOVA. Data was natural log transformed. One-way ANOVA's were used to analyze the effect of infection on lesion area and the amount of Caspase activity at the lesion post-infection. A Tukey's post hoc test was performed to determine significant groupings. All statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

3.3. Results

3.3.1. Detection of Reactive Oxygen Species in Plant Tissue

Upon infection, *T. testudinum* was capable of generating ROS (fig 3.2). ROS were present in the vasculature of sections, even prior to lesion formation (fig 3.2 B). At 48 hours lesions were evident and ROS accumulation was localized to these regions. By 72 hours, lesions were prominent and ROS formation could be clearly observed around the leading edge of necrotic areas (fig 3.2 C). *Labyrinthula* spp. infection significantly increased H_2O_2 production in plant tissue (Fig. 3.3; $F = 10.195$, $p = 0.004$). By the 72 h mark infected plants were producing 0.163 µmol of H_2O_2 g^{-1} fresh weight. Duration of treatment (time) had no effect on H_2O_2 production ($F = 2.279$, $p = 0.156$) and there was no significant interaction (infection*time) between the two factors ($F = 6.003$, $p = 0.60$).

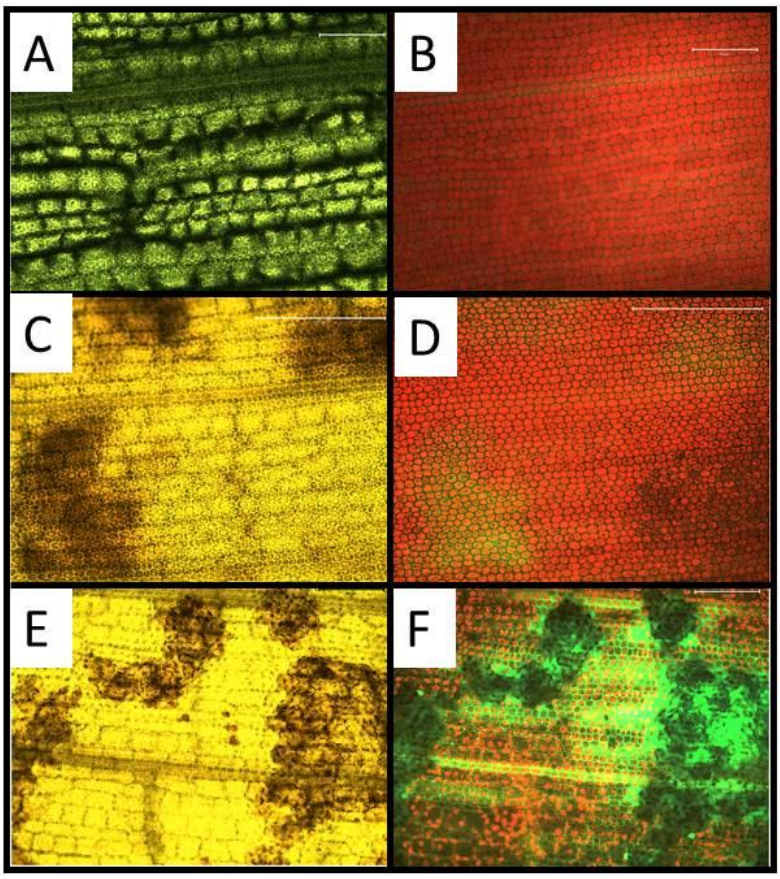


Figure 3.2 Detection of reactive oxygen species following infection over 72 hours with the probe H_2DCF-DA using compound fluorescence microscopy. Left side of panel is brightfield while right side is using fluorescence microscopy. (A-B) 24 hours post infection there is no lesion formation yet ROS is present in the vasculature. (C-D) 48 hours post infection lesions begin to form and ROS accumulation is faint. (E-F) 72 hours post infection lesions continue to form and ROS accumulation occurs at the leading edge of the lesions.

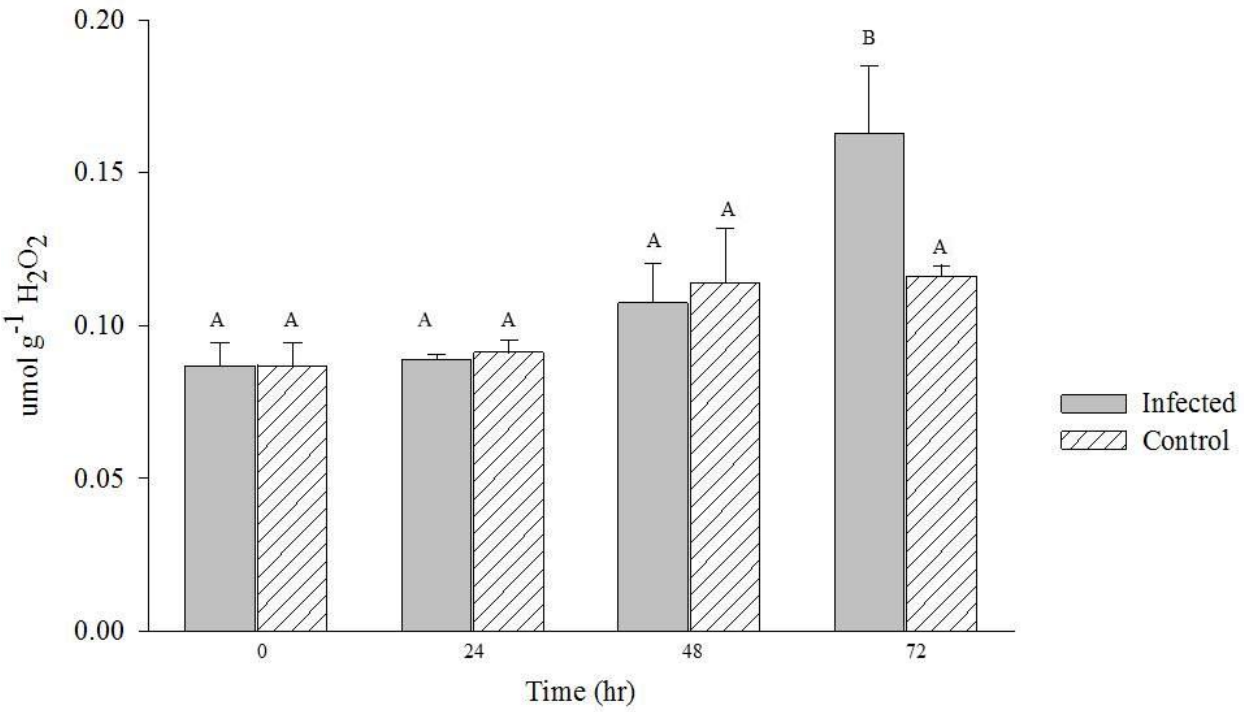


Figure 3.3 Concentration of H_2O_2 in seagrass tissue at the site of infection over 72 hours. Columns represent the means of 3 replicates $\pm$ 1 SEM. Different letters indicate significantly different groups using a Tukey's post hoc test.

3.3.2. Detection of Dead Tissue

Lesions were incubated in SYTOX immediately following infection and were found to contain a high degree of compromised plasma membranes as was indicated by the bright diffuse fluorescence of nucleic acids within the cell (fig. 3.4). The sizes of the lesions increased as a function of infection time (one-way ANOVA, $F= 7.23$, $p= 0.002$) (fig. 3.4).

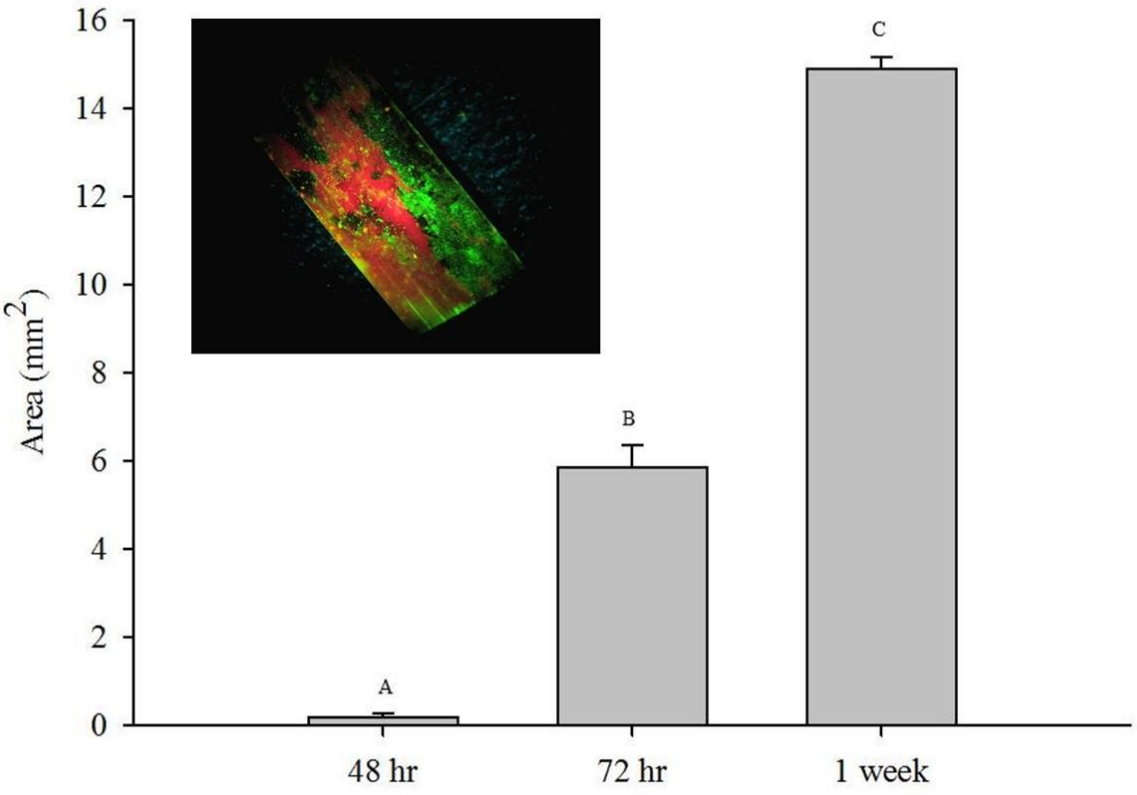


Figure 3.4 Lesion area at the end each infection time point. Points are the mean of 5 replicates ± 1 SEM. Letters designate significantly different groups following a Tukey's post hoc test. Inset is a representative image of SYTOX labeled tissue after five days of infection with *Labyrinthula* spp. (green).

3.3.3. Detection of Caspase activity, an indicator of PCD

Caspases are proteolytic enzymes associated with PCD and are often one of the first indicators of apoptosis. Using a fluorescence assay caspase activity was monitored every 24 hours for 72 hours (fig 3.5). The highest levels of caspase activity were detected at the 72 h time point (6.93 mmol rhodamine mg protein $^{-1}$) and were significantly different than the 0 and 24 hour time points (one-way ANOVA, $F= 7.233$, $p= 0.00277$). Caspase activity was also visualized using IHC. Samples were fixed, permeablized, and stained after each time point (fig 3.6). No staining of caspase was noted at the 0 and 24 hour samples. At 48 and 72 hours caspase presence was enhanced yet was patchy and not always in the region adjacent to lesion tissue. Western blot analysis was inconclusive as immunoreactive bands could not be detected in any samples.

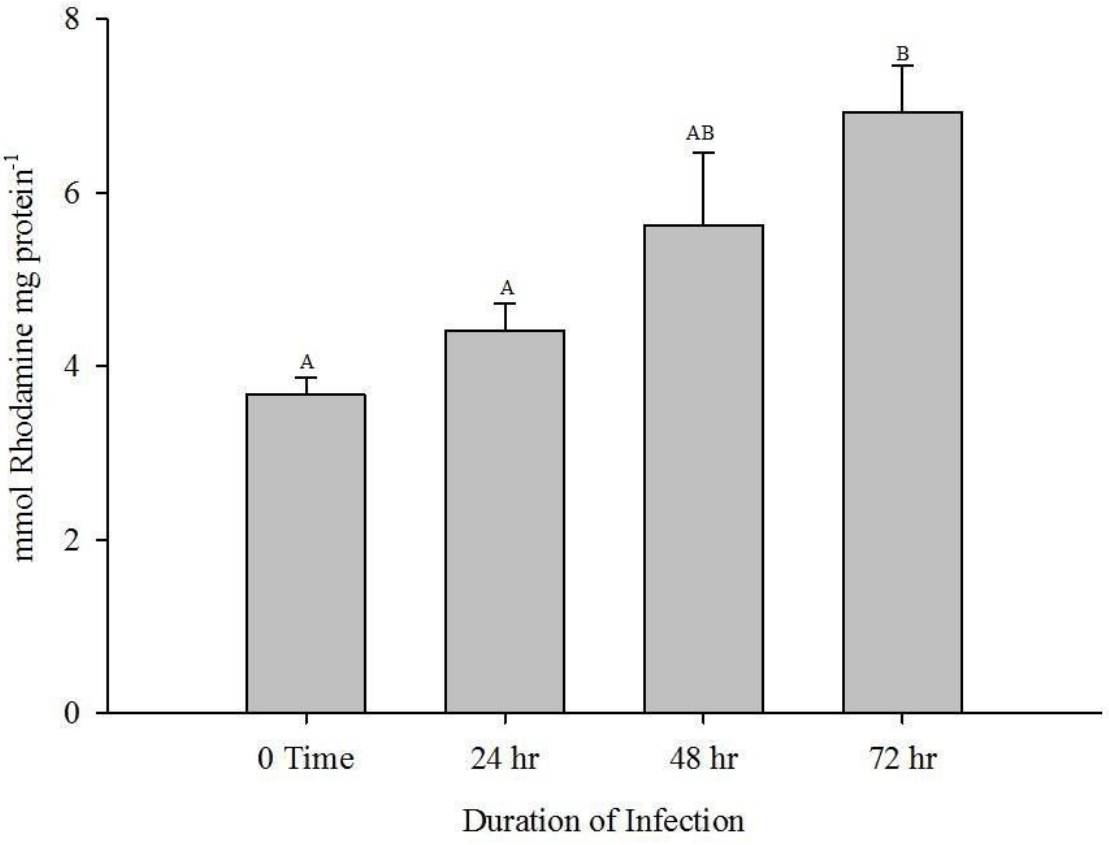


Figure 3.5 Caspase activity over 24 hours as mmol Rhodamine mg protein^{-1}. Columns represent the mean of 5 treatments $\pm$ 1 SEM. Letters indicate significant difference using a Tukey's post hoc test.

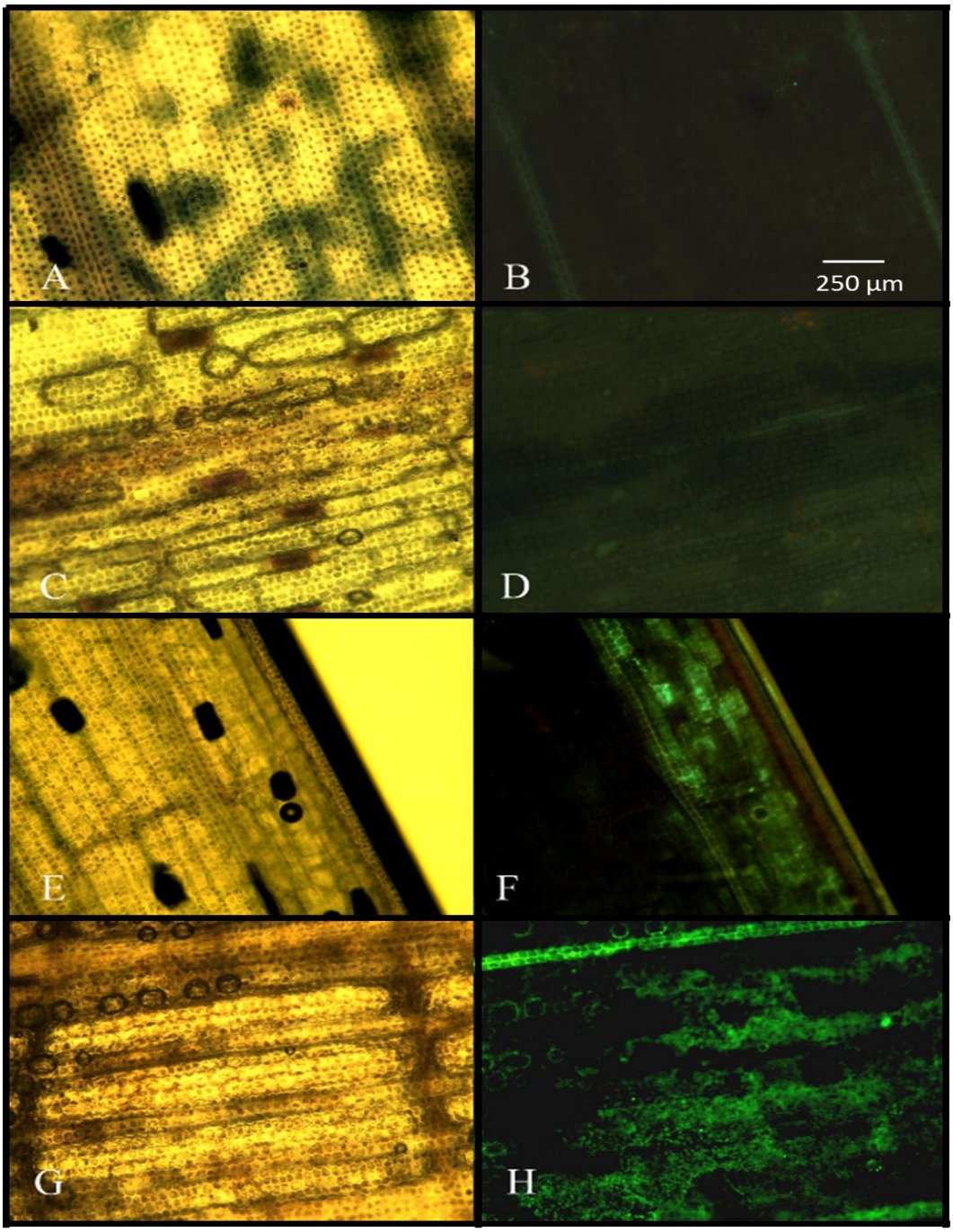


Figure 3.6 Localization of caspase using Anti-caspase-3 Rabbit primary antibody over 72 hours. Left side of panel is brightfield while right side is using fluorescence microscopy. (A-B) Healthy *T. testudinum* tissue displays no caspase activity. (C-D) 24 hours after infection there is no lesion formation or caspase activity. (E-F) 48 hours after infection lesion formation begins and caspase presence is inconsistent and sparse. (G-H) 72 hours after infection lesion formation continues and caspase presence increases but remains patchy and not localized to a specific area.

3.3.4. TUNEL Assay, Hoechst Stain, and Nuclease Activity

Tissue at every time point only showed superficial staining on top of the cells and yielded no conclusive findings associated with the TUNEL assay. Furthermore, no nuclear condensation was detected using the Hoechst stain (Fig. 3.7). Lesion containing area after 72 hours showed a lack of chloroplasts and genetic material was diffuse throughout the entire lesion area whereas chloroplasts in the controls are clearly visible and nuclear material is diffuse but confined to the area within the cell wall. There was no DNA laddering observed in the infected tissue at any time point, all extracted DNA exhibited similar characteristics after gel electrophoresis (data not shown).

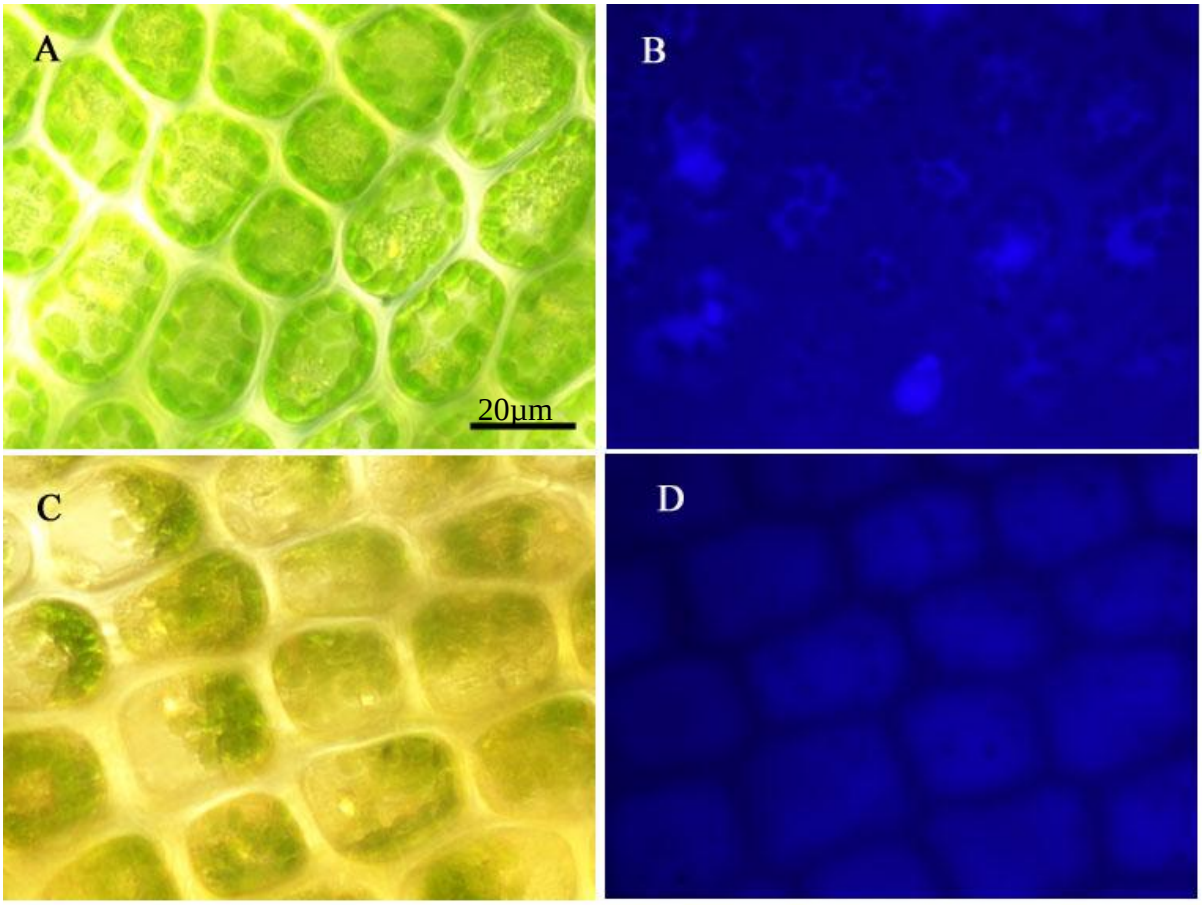


Figure 3.7 Hoechst staining of healthy tissue (A-B) showing orderly chloroplast arrangement and diffuse staining of genetic material within the cell wall. Tissue after 72 hours of infection (C-D), there is no organization of chloroplasts and chlorophyll abundance is drastically reduced. Genetic material is diffuse throughout the lesion area. The left side of panel is brightfield while right side is using fluorescence microscopy.

3.4. Discussion

3.4.1. ROS accumulation and Caspase-Like Proteases

ROS and calcium are widely accepted participants in the induction of the HR. In several terrestrial plants H_2O_2 accumulation and an influx of calcium has been shown to induce the HR (Levine et al., 1994, 1996; Delledonne, 2001; Gechev and Hille, 2005).One of the major enzymes involved in the generation of the ROS responsible for signaling the onset of the HR is an NADPH oxidase, specifically the F form of the gene (RbohF) (Lam, 2004). *T. testudinum* is capable of ROS generation via the oxidative burst and has genes with sequence homology to Rboh F. This would seem to indicate that *T. testudinum* is undergoing the HR upon infection.

After 48 hours of infection characteristic signs of wasting disease are present, concurrent with findings from *Z. marina* (Muehlestein et al., 1989). ROS accumulated around the lesion containing tissue showing the most accumulation after 72 hours of infection. Induction of the HR can take between 12 and 24 hours after pathogen infection in whole leaf tissues (Vanacker et al., 2000). Our results are concurrent with these findings, provided *Labyrinthula* spp. takes 48 hours to infect *T. testudinum* tissue and ROS accumulation was first noticed around the lesion at 48 hours.

Caspase activity in *T. testudinum* increased over the time course of infection following similar patterns to ROS accumulation. Both caspase and ROS were present in the highest amounts after 72 hours of infection. In plants PCD is initiated by ROS accumulation which causes the release of cytochrome C from the mitochondria in turn activating caspase-like proteases (Chichkova et al., 2004). This time course of ROS accumulation followed by protease activity is in line with our results. Immunohistology also revealed caspase presence, although it

was patchy and inconsistent. This does not rule out the possibility of an HR. Necrosis and an HR induced apoptotic region can occur in conjunction with one another (Morel and Dangl, 1997).

Although there was evidence of caspase activity in *T. testudinum* the time course and amount of caspase-like protease activity at the lesion does not coincide with other terrestrial plants (del Pozo and Lam, 1998; Rojo et al., 2004). Both studies reported significant increases in caspase-like protease activity after 24 hours of infection. In our study similar results were reached after 3 days of infection.

Western analysis was also conducted to further characterize the activity of caspase like proteases involved in the HR. Western analysis was unsuccessful. No true caspases have been identified in plants, only proteases with similar activity and tertiary structure (Formicheva et al., 2012). Because protein extracted from lesion tissue was denatured prior to analysis any similarity to Caspase-3 that was not directly related to the amino acid sequence was lost. This would account for the negative results of western analysis.

3.4.2. TUNEL, Hoechst Stain, and DNA Laddering

Aside from the presence of caspases other characteristic indicators of PCD include nuclear condensation as well as DNA fragmentation and laddering. In order to visualize DNA fragmentation the TUNEL assay was employed. There was no clear evidence of fragmented DNA when visualized under the microscope. Hoechst stain was used for nuclear condensation; only diffuse nuclear material was visualized. DNA extracted from infected *T. testudinum* underwent gel electrophoresis and again there was no indication of DNA fragmentation. This also does not rule out the possibility of an HR. Studies have shown induction of the HR from

ROS, calcium influx and caspase-like protease activity in the absence of both nuclear condensation and laddering (Mittler and Lam, 1996; Levine, 1996; Greenberg and Yao, 2004).

3.4.3. Membrane Permeability

Membrane permeability is a late step in the HR and is typically the committed step of PCD (Balk and Leaver, 2001). Staining with SYTOX stain, a stain that can only penetrate dead cells with compromised membranes; revealed large amounts of dead tissue at the lesion. SYTOX stain causes genetic material to fluoresce green. Lesion tissue fluoresced bright green indicating diffuse genetic material in the cytoplasm of the cell. Nuclear condensation and DNA laddering should precede membrane permeability (Govrin and Levine, 2000) and cells involved in the HR should fluoresce later than caspase-like protease activity. The diffuse staining of genetic material throughout the duration of the infection is consistent with the TUNEL and Hoechst stain findings from this study.

3.5. Conclusion

The signaling mechanisms needed to induce PCD and the HR has been demonstrated in *T. testudinum*. *T. testudinum* can generate an oxidative burst upon elicitation, RbohF genes that code for NADPH oxidases are present and ROS does accumulate around infected tissue. Caspase-like proteases are also present following infection; however, no other PCD hallmarks have been demonstrated following *Labyrinthula* spp. infection. Induction of the HR from caspases and ROS alone has been demonstrated in terrestrial plants (Mittler and Lam, 1996;

Levine, 1996; Greenberg and Yao, 2004), but the inconstancy of caspase activity following infection may be an indication that while some cells are undergoing PCD several other neighboring cells are being necrotized at the same time.

4.

General Conclusions and Outlook

The goal of this thesis was to examine the biochemical recognition and response of the seagrass *Thalassia testudinum* to the pathogenic slime mold *Labyrinthula* spp. Chapter 2 sought to determine if *T. testudinum* was capable of generating an oxidative burst when presented with crude extracts of *Labyrinthula* spp. or with lipopolysaccharide (LPS), a commercially available elicitor commonly used to model the oxidative burst in terrestrial plants. There was no oxidative burst generated when *T. testudinum* was presented with crude *Labyrinthula* spp. extracts. However, twenty minutes after addition of 100 µg/mL LPS a characteristic oxidative burst was observed. In conjunction with the oxidative burst an increase in respiration and an alkalization of extracellular media pH was noted. The oxidative burst in *T. testudinum* was dissected using widely used pharmacological activators and inhibitors (Davies et al., 2001; Küpper et al., 2001; Ross, 2006). The enzymatic pathway used to activate the ROS involved in the oxidative burst associated with *T. testudinum* seems to be highly conserved across plant genera. The same mechanisms have been demonstrated in algae, terrestrial plants and now marine angiosperms (Küpper et al., 2001; Doke, 1983; Apostol et al., 1988; Legendre et al., 2003; Desaki, 2006). The oxidative burst has also been shown to be elicited by extracts of plant cell wall, a type of self-induced burst upon detection of damaged self-tissue (Johnson et al., 2003; Legendre et al., 2003). The involvement of this mechanism for activating an oxidative burst in *T. testudinum* was not explored. ROS accumulation was detected in response to *Labyrinthula* spp. as early as 24 hours

after infection so perhaps components of *T. testudinum* cell wall induce this ROS accumulation upon infection.

Chapter 3 investigated whether or not the hypersensitive response, an area of localized self-induced cell death, was responsible for lesion formation at the site of infection. Lesion tissue consisted of dead cells (as opposed to depigmented live cells) as confirmed by SYTOX staining. The accumulation of ROS, a known signaling molecule of PCD in plants, was also visualized around the lesion, (Jones, 2000). This ROS accumulation increased over the time course of the experiment. The only other indicator of the HR was caspase activity, which followed the same trend as ROS accumulation. Caspase activity was greatest at 72 hours post-infection. Other studies that have examined caspase-like protease activity have employed two caspase-like proteases; one that cleaved DEVD amino acid sequences, similar to the one used in our study, and another that cleaved YVAD amino acid sequences (del Pozo and Lam, 1998; Rojo et al., 2004). Both studies showed increased activity using the protease that cleaved YVAD amino acid sequences than the protease that cleaved DEVD amino acid sequences. It would be of interest to determine whether or not there was a similar trend in *T. testudinum*. Immunohistochemistry, in an attempt to visualize caspase at the lesion site, was patchy showing some nearby cells potentially undergoing PCD while the vast majority of cells did not fluoresce and appeared to be necrotic.

No other indicators of PCD were observed. This may indicate that the interaction between *T. testudinum* and *Labyrinthula* spp. is a compatible interaction; in that *T. testudinum* does not have the capability to fend off pathogen attack via the HR or PCD and is being necrotized by the pathogen. The presence of ROS at the lesion site and partial caspase induction

indicate that seagrasses are capable of initiating an immune response; however, not a response capable of repelling or confining *Labyrinthula* spp.

In conclusion this research demonstrates *Thalassia testudinum* is capable of generating an oxidative burst upon elicitation. This study marks the first time the oxidative burst has been detected in marine angiosperms, and that the oxidative burst is conserved across Plantae. However, when *Labyrinthula* spp. becomes pathogenic and infects healthy tissue the oxidative burst is not triggered and PCD is not induced. Early indicators of possible PCD activation are present in small unevenly distributed areas of the lesion following ROS accumulation indicating some cells are attempting to undergo PCD but are not fully capable of initiating the process. This research also provides further insight into the complex interactions between *T. testudinum* and *Labyrinthula* spp. and is the first time the biochemical interaction between the two organisms has been investigated.

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Vita

Kyle Loucks

k.loucks@unf.edu

EDUCATION:

Working towards an M.S., Biology — Present
University of North Florida, Jacksonville, FL

B.S., Biology — 2008
University of West Florida, Pensacola, FL
GPA 3.50

PROFESSIONAL HISTORY:

Graduate Teaching Assistant — 2011-Present
University of North Florida, Jacksonville, FL

QA Chemist/Microbiologist — 2008- 2010
Williams Foods, Lenexa, Kansas

Laboratory Aid, General Botany, Anatomy and Physiology I and II, General Biology — 2006- 2008
University of West Florida, Pensacola, Florida

TEACHING EXPERIENCE

General Biology II Lab, University of North Florida — 2012

Principles of Biology Labs, University of North Florida — 2011

CONFERENCES:

42nd Annual Benthic Ecology Meeting — 2013
Savannah, GA
Seagrass Defenses: The Oxidative Burst and Hypersensitive response in *Thalassia testudinum* in relation with *Labyrinthula* spp., Oral Presentation

2013 UF Marine Biology Symposium — 2013
St. Augustine, FL
Plant pathogen interactions, the relationship between *Labyrinthula* and the immune response of *Thalassia testudinum*, Oral Presentation

34th Annual Southeastern Phycological Colloquy 2012
University of North Florida Jacksonville, FL
Plant-Pathogen Interactions Associated with Wasting
Disease in the Tropical Seagrass *Thalassia testudinum*,
Oral Presentation

Annual Meeting of the American Society of Plant Biology, 2012
Southern Section
Myrtle Beach, SC
Seagrass Defenses: Elicitation of an oxidative burst in the tropical
marine angiosperm *Thalassia testudinum*, Oral Presentation

2012 UF Marine Biology Symposium
St. Augustine, FL
Localization and Dynamics of Reactive Oxygen Species Production 2012
in Diseased Seagrasses, Oral Presentation

33rd Annual Southeastern Phycological Colloquy 2011
Florida International University Miami, FL
Localization and Dynamics of Reactive Oxygen Species Production
in Diseased Seagrasses, Poster presentation

21st Biennial Conference of the Coastal and Estuarine 2011
Research Federation
Daytona Beach, FL
Localization and Dynamics of Reactive Oxygen Species Production
in Diseased Seagrasses, Poster presentation

RESEARCH EXPEDITIONS:

Marine ecology collection and Monitoring 2011
RV Bellows seagrass health assessment in the Florida Keys
and how it relates to Wasting disease

GRANTS, SCHOLARSHIPS AND AWARDS:
Grants

University of North Florida Coastal Biology Travel Grant, $500 (Awarded 2011, renewed 2012)