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Physiology and Biochemistry of the Tropical Seagrass Thalassia testudinum in Response to Hypersalinity Stress and Labyrinthula sp. Infection

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Physiology and Biochemistry of the Tropical Seagrass *Thalassia testudinum* in Response to Hypersalinity Stress and *Labyrinthula* sp. Infection

by

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General Abstract

Seagrass meadows are essential to coastal ecosystems and have experienced declines in abundance due to a series of environmental stressors including elevated salinity and incidence of disease caused by the pathogen *Labyrinthula* sp. This thesis evaluated the dynamics between *T. testudinum* and *Labyrinthula* concerning the impacts of short term elevated salinity stress on the early stages of infection in *Thalassia testudinum* (Chapter 2) as well as the presence of anti-labyrinthulid secondary metabolites produced by the tropical seagrass (Chapter 3). The results showed that short term exposure to hypersalinity alters some cellular processes but does not necessarily lead to an immediate increase in wasting disease susceptibility. Specifically, the occurrence of disease was significantly lower in the hypersalinity treatments possibly due to a direct osmotic shock to *Labyrinthula* or indirectly due to the increase in *in vivo* H_2O_2 concentrations that may have inhibited *Labyrinthula* growth. In addition, it was shown that 4 phenolic acids commonly found in turtlegrass leaf tissue were able to inhibit *Labyrinthula* growth in culture. Using a bioassay-guided fractionation technique, several purified fractions of *T. testudinum* leaf tissue showed anti-labyrinthulid activity, however the detailed characterization of the unknown compounds was inconclusive. The results presented in this thesis highlight the halotolerant characteristics of the seagrass *T. testudinum* as well as suggest that *T. testudinum* has the capability of defending itself against *Labyrinthula* infection using secondary metabolites.

1

Introduction

1.1. Seagrasses

Seagrasses are marine vascular, flowering plants that compromise only 0.001% of all angiosperms. Their polyphyletic origins were first recorded in the mid- to late Cretaceous when they diverged first from terrestrial plants and subsequently from either xerophytic plants or freshwater hydrophytes (Kato *et al.* 2003) while dispersal and diversification began during the mid- to late Eocene. The distribution and diversity of seagrasses today are best explained by both the vicariance hypothesis and the center-of-origin concept which use continental drift and dispersal from a central population, respectively, as mechanisms of isolation and speciation (Dawes 1998). These concepts also account for the presence of sister species in the Caribbean and Indo-West Pacific, namely, *Thalassia testudinum* and *T. hemprichii*, *Syringodium filiforme* and *S. isoetifolium*, and *Halodule wrightii* and *H. uninervis*. Since all seagrasses have similar physiological adaptations to cope with the pressures of living in fully submerged marine habitats, classification is based on vegetative and reproductive characteristics which place the existing 60 species into 12 genera and 4 families.

Globally, seagrass meadows represent a fragile yet vital component of many coastal ecosystems. Aside from serving as a refuge and nursery for a variety of organisms, they are a source of organic matter for associated reef habitats and

commercial fisheries. Their above-ground blades reduce turbidity by acting as a sediment catch while their extensive root and rhizome system stabilizes the sediments and thus reduces coastal erosion. Additionally, seagrass meadows have received increased attention as powerful tools in climate change strategies (Laffoley *et al.* 2009) due to their ability to sequester and store carbon for millennia (Mateo *et al.* 1997).

Living in a submerged marine habitat requires seagrasses to overcome several challenges in order to survive and reproduce successfully. As a result, seagrasses have become adapted to acquiring carbon dioxide and nutrients in the water column, water-mediated reproduction, exponential light attenuation with increasing depth as well as maintaining osmotic balance in a saline environment (Dawes 1998). Seagrass leaves are responsible for the uptake of carbon dioxide from the water column. Aside from CO_2 assimilation, *T. testudinum* has also been shown to efficiently utilize HCO_3^- (Durako 1993) via a carbonic anhydrase-mediated process. Sexual reproduction among marine angiosperms is an annual event and is often temperature dependent (February-May and 20-26°C for Florida species; Phillips 1960; McMillan 1982). With limited conditions under which to sexually reproduce, asexual reproduction by vegetative growth of the rhizome is thought to be the main method of seagrass expansion. This type of growth also allows for resources like soluble carbon and proteins to be shared between ramets (individual short shoots) in a genet (an entire rhizoidal plant) which is particularly useful in stressful environments (see Dawes 2004).

Seagrass photosynthesis and production depend on the amount and quality of light reaching the submerged plants. Depending on the species and location of the seagrass, their minimal light requirement can range from 2-37% of surface irradiance (Lee *et al.*

2007) with Florida Bay populations of *T. testudinum* requiring at least 22% surface irradiance (Fourqurean and Zieman 1991). Being able to maintain a positive carbon budget (balance between photosynthesis and respiration) is essential and has a strong influence on seagrass depth distribution and plant morphology (Lee *et al.* 2007; Ralph *et al.* 2007).

Lastly, maintaining an internal hypertonic environment in a marine setting is possible for seagrasses with a few physiological and metabolic adjustments and adaptations. Active osmoregulation via ion pumps occurs with the assistance of numerous mitochondria and an increase in cell membrane surface area of the epidermal cells (Dawes 1998). Salt tolerance is also aided by the vacuolar sequestration of ions (Na^+, K^+, Cl^-; Touchette 2007). Additionally, the soluble amino acid proline is particularly important as both an organic osmolyte as well as a scavenger of reactive oxygen species which are often byproducts of excessive cellular respiration during osmoregulation (Touchette 2007).

The coastal ecosystems in which seagrasses inhabit are dynamic and undergo daily fluctuations in environmental parameters from both natural and anthropogenic causes. While all marine angiosperms have developed physiological and biochemical adjustments, as mentioned above, to cope with living in a fluctuating habitat, each species has its own optimum range in which environmental factors, such as salinity, light, and temperature, need to lie. The ability of a species to tolerate and recover from conditions beyond their upper and lower thresholds is influenced by the rate, either gradual or pulse, and the length, either acute or chronic, of the stressing episode (Ralph 1998; Koch *et al.* 2007a). Simultaneous variation of more than one parameter outside the tolerable range

has been shown to produce an additive stress response in seagrasses (Ralph 1999; Koch & Erskine 2001; Eldridge *et al.* 2004).

1.2. Wasting Disease and *Labyrinthula* sp.

One such circumstance involving multiple concurrent environmental stressors (high salinity, light reduction, sulfide toxicity, high population density and disease) has been thought to account for the 4000 hectare 1987 *Thalassia testudinum* (Banks ex König; turtlegrass) die-off in Florida Bay as well as subsequent smaller, patchy die-offs in Florida Bay up until 1989 (Roblee *et al.* 1991). Other seagrass species have also been subjected to both larger (*Zostera marina* in the North Atlantic; Short *et al.* 1987; Muehlstein *et al.* 1991) and smaller (*Zostera* sp., New Zealand; Armiger 1964) die-offs. Each of these rapidly evolving events led to a drastic decline in ecosystem functionality. For example, the *Thalassia testudinum* die-off brought about a declined shrimp harvest (Fourqurean and Robblee, 1999). The *Zostera marina* die-off led to a reduction in the waterfowl populations, a loss in scallop industry and a change in the benthic landscape which in some cases was unfavorable for recolonization (Addy and Alyward 1944; Thayer *et al.* 1984; Short *et al.* 1987).

In part, these large die-offs, later termed wasting disease outbreaks, was thought to be caused by an opportunistic marine protist, *Labyrinthula* sp. (Figure 1.1). Taxonomically misplaced for years due to both fungal and slime mold characteristics, recent phylogenetic analysis has placed *Labyrinthula* in the Phylum Labyrinthulomycota under the Stramenopila lineage (Tsui 2009). Having lost the photosynthetic ability of their ancestors (Fig. 1.2), *Labyrinthula* are obligatory, marine heterotrophs often acting as

saprobes, mutualists, and commensalists as well as parasites (Tsui 2009). A specialized organelle of labyrinthulomycetes, the bothrosome (or sagenogentosome), secretes the extracellular ectoplasmic network used for locomotion (Fig. 1.3). The ectoplasmic network also secretes digestive enzymes that act to break down and absorb nutrients (Raghukumar 2002). It is believed that for pathogenic strains of *Labyrinthula,* the digestive enzymes are able to penetrate live seagrass blade tissue by degrading the cell walls of their hosts and subsequently destroying the cellular contents including chloroplasts (Raghukumar 2002; Tsui *et al.* 2009). This results in characteristic black lesions, an indicator of wasting disease (Figure 1.4).

In infected blades, *Labyrinthula* cells can occupy viable, green cells on the periphery of the lesion (Young 1938). Once a seagrass is inoculated with the pathogenic protist, infection can spread rapidly, e.g. 0.8 mm/h (Ralph and Short 2002). It has been shown that once the lesion bisects the width of the blade, the health of the leaf is significantly compromised. Photosynthesis is significantly inhibited, vascular transport is lost or damaged affecting solute transport, and oxygen transport to the roots can be diminished leading to hypoxic conditions belowground (Durako and Kuss 1994). All of these effects on seagrass physiology may leave the seagrass more susceptible to additional environmental stresses (Ralph and Short 2002).

Figure 1.1 *Labyrinthula* sp., the causative pathogen of wasting disease (400x; Photo courtesy of Daniel Martin, University of South Alabama).

Figure 1.2 Summary of Labyrinthulomycota characteristics. Clades A and B represent a basal split between extant genera based on analysis of multiple loci (from Tsui 2009).

Graphic redacted. Paper copy available upon request to home institution

Figure 1.3 Internal cell structure of *Labyrinthula* highlighting the bothrosome organelle that is responsible for the production of the ectoplasmic network. Taken from Porter 1988.

Figure 1.4 *Thalassia testudinum* (turtlegrass) blade exhibiting symptoms of wasting disease.

As mentioned above, *Labyrinthula* travel within their self-produced network and must have a substrate on which to grow. As a result, expansion of wasting disease to a new host must occur by blade-to-blade contact with an infected leaf (Muehlstein *et al.*, 1992). *Labyrinthula* has also been reported to travel long distances via planktonic detritus (Raghukumar 2002). Therefore, the ability of a virulent species of *Labyrinthula* to travel locally within and between populations as well as long distances makes this ubiquitous microbe a potential threat to coastal ecosystems (Short *et al.* 2007).

1.3. Seagrass Defenses

Interestingly, while the seagrass-*Labyrinthula* relationship is long-running and omnipresent, large-scale die-offs are not common. Therefore, it is possible that seagrasses have an effective defense system against this pathogen.

In general, once potentially harmful microorganisms make contact with the hosts‟ cell surfaces, plants can elicit one or more of the following inducible defenses: thickening of the cell wall at the damaged sites, hypersensitive response resulting in localized programmed cell death, increased respiration resulting in increased levels of toxic reactive oxygen species and lastly, the increased production of effective antimicrobial compounds, i.e. secondary metabolites (Cowan 1999; Shetty *et al.* 2008).

The diversity of possible secondary metabolites is incredible. Many of these chemical compounds are produced via the shikimic acid and phenylpropanoid (SA/PP) pathways. These enzymatic pathways, typically start with one of 3 aromatic amino acid precursors, phenylalanine, tyrosine or tryptophan. This biosynthetic pathway can potentially create numerous flavonoids and up to 8000 known phenolics (Herrmann

1995). Plant phenolics are compounds with an aromatic ring and one or more hydroxyl substituents (i.e. phenol). Included in this group are phenols, flavonoids, lignin, tannins, and phenolic acids which are all biosynthesized from precursor phenylalanine (Fig 1.5). The diversity of phenolics begets diverse functions such as scavenging reactive oxygen species, protein synthesis, UV-absorption, and defense mechanisms (reviewed in Stalikas 2007).

In land plants, it has been shown that under ambient conditions, individuals can direct up to 20% of their fixed carbon into the shikimic acid pathway (Haslam 1993). While under attack, resources may be redirected into the SA/PP pathways for the production of antimicrobial secondary metabolites. While these defenses are biochemically expensive, they are essential defense mechanisms to these sessile organisms. Furthermore, while chemical defenses have been shown to be upregulated during microbial attacks in land plants (Arnold & Targett 2002), chronic stress conditions have been shown to decrease the pathway"s efficiency (Weaver & Herrmann 1997), possibly leaving a plant more susceptible to disease. Along these same lines, it has been hypothesized that the recent seagrass wasting disease episodes were attributed to poor environmental conditions which suppress or weaken the hosts" defenses (Muehlstein 1989; Robblee *et al.* 1991).

Biochemical defenses are particularly vital to marine plants, including seagrasses, as they are constantly exposed to potentially harmful microbes. In contrast to their terrestrial counterparts, chemically-mediated defenses of marine plant-pathogen systems are poorly understood (Baskin 2006). However, there has been evidence that sessile marine plants can produce anti-microbial secondary metabolites in response to attacks

(Engel *et al.* 2002). Furthermore, during the past several years there has been growing evidence that seagrasses have the ability to chemically defend themselves against common microorganisms, invading pathogens or wounding (Jensen *et al.* 1998; Arnold & Targett 2002; Arnold *et al.* 2008; Ross *et al.* 2008).

The body of work concerning composition and functions of the secondary metabolites in seagrasses is growing. In a survey of 12 seagrass genera including *Thalassia testudinum*, six phenolic acids were found in more than half of the seagrasses examined (Zapata and McMillan 1979). Four of these phenolic acids were also found to be the product of "pseudo-induction" in *Thalassia testudinum* following wasting disease elicitation (Steele *et al.* 2005; Fig. 1.6). In this study, the lesions bisected the width of the seagrass blade which blocked vascular carbohydrate transport. The 4 phenolic acids were likely accumulating above the damage site in response to altered resource allocation rather than accumulating as a signaled induced defense response to *Labyrinthula* invasion (Steele *et al.* 2005).

There is still much unknown about the specific defense mechanisms of *Thalassia testudinum* against pathogenic *Labyrinthula* sp. Since wasting disease outbreaks have the potential to significantly damage *T. testudinum* meadows and compromise their function in coastal ecosystems, it is crucial to fully understand the dynamics of the *T. testudinum*-*Labyrinthula* relationship. This thesis will contribute to this knowledge base by exploring both physiological and biochemical aspects of *T. testudinum* health and disease.

Figure 1.5 The biosynthesis pathway of plant phenolics originating with phenylalanine in the shikimic acid/phenylpropanoid pathways (from Taiz and Zeiger 2006).

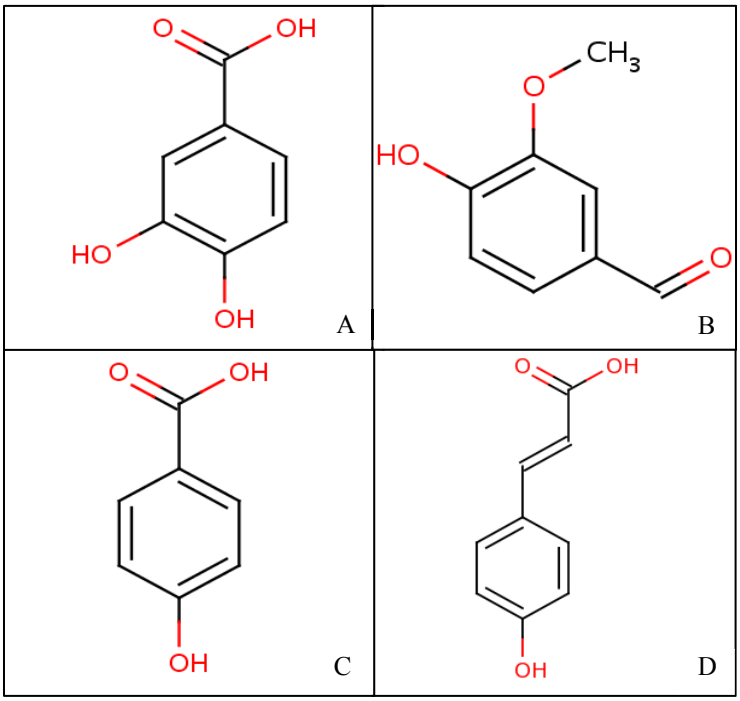

Figure 1.6 Four phenolic acids upregulated in *Thalassia testudinum* leaves during *Labyrinthula* sp. infection (Steele *et al.* 2005). (A) 3,4-dihydroxybenzoic acid (protocatechuic acid), (B) vanillin, (C) *p*-hydroxybenzoic acid, (D) *p*-coumaric acid.

1.4. Objectives

The objectives of the research presented herein were two-fold. Chapter 2 was aimed at providing a better understanding of the interaction between hypersalinity stress and infection dynamics. *Thalassia testudinum* was subjected to hypersalinity exposure, one of the stressors involved in the 1987 wasting disease outbreak. While exposed to this sub-optimal environment, *T. testudinum* was infected with a pathogenic strain of *Labyrinthula.* The seagrass‟ physiological and metabolic responses were monitored during the early stages of infection. In Chapter 3, *T. testudinum*‟s ability to produce effective anti-*Labyrinthula* secondary metabolites will be assessed. While the 4 previously mentioned phenolic acids (3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and vanillin) have been shown to be upregulated during *Labyrinthula* infection, it was not known if these compounds had direct anti-labyrinthulid activities. Therefore, the first part of this chapter was directed towards the determination if these phenolics have anti-labyrinthulid activity and then identify the specific inhibitory concentrations of the phenolic acids both alone and in combination. In the second half of the chapter, a bioassay-guided fractionation technique was used to isolate and subsequently characterize any anti-labyrinthulid compound(s) that have not been previously characterized in the leaf tissue *T. testudinum*.

2

Effects of elevated salinity on the health of the subtropical seagrass *Thalassia testudinum* and its susceptibility to wasting disease

Abstract

Seagrass meadows are essential to coastal ecosystems and have experienced declines in abundance due to a series of environmental stressors including elevated salinity and incidence of disease. This study evaluated the impacts of short term salinity stress on the early stages of infection in *Thalassia testudinum* Banks ex König by assessing changes in cellular physiology and metabolism. Seagrass short shoots were exposed to ambient (30) and elevated (45) salinities for 7 days and subsequently infected for one week by the causative pathogen of wasting disease, *Labyrinthula* sp. The occurrence of wasting disease was significantly lower in the hypersalinity treatments. Additionally, while exposure to elevated salinity caused a reduction in chlorophyll *a* and *b* content, photosynthetic activity was not affected by salinity or infection. In contrast, plant respiratory demand was significantly enhanced as a function of infection. Elevated salinity caused *T. testudinum* to significantly increase its *in vivo* H_2O_2 concentrations to levels that exceeded those which inhibited *Labyrinthula* growth in an *in vitro* assay. The results suggest that while short term exposure to hypersalinity alters some cellular

processes this does not necessarily lead to an immediate increase in wasting disease susceptibility.

2.1. Introduction

Globally, seagrass meadows represent a fundamental, yet delicate, component of many coastal marine ecosystems that, aside from serving as a refuge for numerous organisms, fulfill other important roles such as sediment stabilization and biogeochemical cycling. Unfortunately, over the past eighty years these ecologically and economically important habitats have been declining in area at an increasing rate partially due to anthropological reduction in habitat and water quality (Waycott *et al.* 2009). These coastal habitats can be subjected to extreme fluctuations in light levels and salinities, elevated temperatures (Vergeer *et al.* 1995; Blakesley *et al.* 2002), and hypoxic conditions (Koch *et al.* 2007b), all which contribute to an overall decrease in seagrass viability. While seagrasses are, to a certain degree, able to cope with environmental variability, unfavorable conditions that occur simultaneously have been noted to cause an additive or synergistic decline in seagrass health (Ralph 1999; Koch *et al.* 2007b; Koch *et al.* 2007c).

Such unfavorable environmental conditions have been thought to be precursory to large-scale die-offs of eelgrass (*Zostera marina* L.) and turtlegrass (*Thalassia testudinum* Banks ex König) habitats during which virulent forms of *Labyrinthula* spp. (Division Stramenopila) infect seagrass blades (Muehlstein 1989; Robblee *et al.* 1991). The resulting infection, termed wasting disease, is characterized by black lesions at sites of

cell degradation (Raghukumar 2002; Tsui *et al.* 2009). *Labyrinthula* spp. move within a self-produced ectoplasmic network, degrade host cell wall tissue with extracellular enzymes, and are believed to travel from host to host via direct contact (Muehlstein 1992). Since most *Labyrinthula* spp. isolated from the field do not create lesions, such incidences of disease are hypothesized to be attributed to poor environmental conditions which suppress or weaken the hosts" defenses (Muehlstein 1989).

Previous research has supported the idea that multiple co-occurring environmental stressors likely work in concert to diminish the health of *Thalassia testudinum* (Robblee *et al.* 1991; Blakesley *et al.* 2002; Koch *et al.* 2007c). This in turn would render the host more susceptible towards opportunistic pathogen infection. Salinity fluctuations likely represent one of the leading drivers behind disease transmission. Elevated salinities up to 70 have been reported in areas of Florida Bay (Florida, USA) which are subject to episodes of drought, high evaporation, and/or low circulation (Robblee *et al.* 1991; Kelble *et al.* 2007c). While the optimum salinity range for *T. testudinum* lies between 30-45 (Kahn and Durako 2006), hypersalinity thresholds can be 45 and 65 under pulsed (sudden change) and gradual increases, respectively (Koch *et al.* 2007a). Laboratory-based studies have demonstrated that salinity affects the virulence and growth of *Labyrinthula* spp. in both marine and terrestrial systems (Young 1943; Muehlstein *et al.* 1988; Martin *et al.* 2009; McKone and Tanner 2009). Furthermore, a series of field-based studies have provided strong evidence for the positive correlation between elevated salinity and increase in symptoms of seagrass wasting disease (Burdick *et al.* 1993; Blakesley *et al.* 2002).

While the importance of pathogens in terrestrial ecosystems has long been documented (Grenfell and Dobson 1995; Anderson *et al.* 2004), the role of diseases in coastal settings has not been thoroughly explored (Baskin *et al.* 2006). Studies on seagrass wasting disease are lagging behind in terms of our understanding of plant pathogenesis as well as the intricate relationship between environmental stress and disease transmission. Despite the reports that pathogenic strains of *Labyrinthula* spp. have been identified as primary etiological agents of wasting disease in seagrass meadows (Short *et al.* 1987; Muehlstein 1989; Muehlstein *et al.* 1991), there is still much to be elucidated regarding this host-pathogen interaction.

The goal of this current study was to further understand the impacts of hypersalinity on the physiological responses of *T. testudinum* during the early stages of *Labyrinthula* infection. Specimens of *T. testudinum* were exposed to ambient (30) or elevated (45) salinities for a one week duration under laboratory conditions prior to being infected. One week after the onset of infection, physiological and metabolic responses were measured including lesion area, maximum and effective quantum yield, rapid light curves, total chlorophyll content, dark-adapted respiration, and detection of reactive oxygen species. It was hypothesized that plants that were exposed to elevated salinity levels would exhibit a decrease in health and thus, be more likely to become infected by pathogenic *Labyrinthula* sp.

2.2. Materials and Methods

2.2.1. Seagrass Collection & *Labyrinthula* Culture

Thalassia testudinum Banks ex König short shoots were collected from the Indian River Lagoon, Ft. Pierce, Florida, USA (27°47‘‘N, 80°31‘W and 27°58‘N, 80°31‘W), cleaned of epiphytes, and maintained at the University of North Florida, Jacksonville, Florida, USA in aquaria tanks at the in situ salinity of 30 and light intensity of 115 µmol m^{-2} s^{-1} under a 12:12 h L:D photoperiod. Short shoots included the blades, sheath and short segments of rhizome in either side. A known virulent strain of *Labyrinthula* sp., kindly provided by the laboratory of Dr. Anne Boettcher (University of South Alabama, USA), was utilized in all experiments. *Labyrinthula* sp. cultures were maintained in serum-seawater agar (SSA) media as previously described by Martin *et al.* (2009) with slight modification. Briefly, the SSA recipe contained 500 mL of 0.22 µm filtered seawater (salinity of 25) using Instant Ocean Sea Salt which was combined with 6 g agar, 0.5 g glucose, 0.05 g nutritional yeast, 0.05 g peptone, 1.5 mg germanium dioxide, 12.5 mL streptomycin/penicillin (stock: 1.25 g streptomycin + 1.25 g penicillin per 100 mL de-ionized H_2O), and 5 mL horse serum. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2. Experimental Design

To evaluate the effects of hypersalinity exposure and *Labyrinthula* sp. infection on *T. testudinum* health, five individual short shoots were used per treatment (n=5). Treatment groups were as follows: 1) uninfected seagrass at a salinity of 30 (control), 2) infected seagrass at 30, 3) uninfected seagrass at 45 and 4) infected seagrass at 45. 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* sp. 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 acclimated in 3.8 L polyethylene terephthalate microcosms (Rubbermaid, Winchester, VA, USA) containing 0.22 µm filtered seawater of the desired salinity. Plants in the hypersalinity treatments had the salinity raised in a pulsed fashion, i.e., placed directly in seawater of a salinity of 45, and were allowed to acclimate for one week. All samples were randomly spaced apart at 28°C under 170 µmol m^{-2} s^{-1} of light using a 12:12 h L:D photoperiod. Following one week of acclimation, 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 one-week ensuing infection period, temperature and salinity were monitored on a daily basis. De-ionized water was added as needed to account for evaporation.

2.2.3. Lesion Measurements

Post treatment, lesions from the infected samples were measured using a Kodak© Gel 1500 Imaging System (Rochester, NY, USA). Photographs were captured using a GL 1500 digital camera. Kodak© Molecular Imaging Software v.5.0.1.27 was used to take accurate measurements of lesion areas and were reported in square millimeters (mm^2).

2.2.4. Pulse Amplitude Modulated Fluorometry

Chlorophyll a fluorescence measurements using the Diving Pulse Amplitude Modulated (PAM) fluorometer (Heinz-Walz GmbH ©,Effeltrich, Germany) were accomplished in a randomized fashion for maximum quantum yields (MQY) as well as effective quantum yields (EQY) and rapid light curves (RLC) to avoid results influenced by light histories. PAM settings were as follows: measuring intensity=5, gain=3, saturation intensity=2 and damp=2. A dark leaf clip (DIVING-LC) was attached at the measurement sites to hold the fiber optic cable in place (Durako and Kunzelman 2002). Each measurement was taken within 5 s of attaching the leaf clip to minimize dark acclimation (i.e., quasi-darkness yield; Durako and Kunzelman 2002) with the distance of 5 mm from the fiber optic cable to the adaxial leaf surface.

Maximum quantum yield measurements were taken before the onset of light. At that point, photosynthetic structures were fully dark-adapted, i.e., the PSII reaction centers were completely oxidized. MQYs were calculated with MQY= $(F_v)/F_m$ where $F_v = (F_m-F_o)$. F_m represents the dark adapted maximum fluorescence and F_o represents the dark-adapted initial fluorescence. Two hours after the diurnal light regime began, measurements for EQYs and RLCs were taken. The initial measurement taken for each rapid light curve was equivalent to the effective quantum yield for that sample. EQYs were calculated with EQY= $(\Delta F/ F'_m)$ where $(F'_m-F)/ F'_m$. F'_m is the light-adapted maximum fluorescence, and F is the minimum fluorescence of light-adapted leaves. Using the fluorescence measurements from MQY and EQY, non-photosynthetic quenching (NPQ) was calculated with $(F_m - F'_m)/ F'_m$. For RLCs, eight increasing light intensities (i.e. photon flux densities or PFDs) were given at 10 second intervals

beginning at the lowest actinic light intensity. The respective average PFDs (μmol m^{-2} s^{-1}) were measured in triplicate using an Apogee Quantum Light Meter® (Apogee Instruments, Inc., Logan, UT, USA): 23.3, 69, 142.3, 230, 346.7, 481.7, 716.7, and 984.3 μmol m^{-2} s^{-1}. To create the light curve, relative electron transport rates (rETR) were plotted against the PFDs where rETR= Yield * PFD * AF * 0.5. PFD is the photon flux density or the photosynthetically active radiation (PAR; nm) that is being emitted. The plants were light-adapted for this measurement, so the Yield parameter represents the EQY. As actual leaf absorbance was not measured in this experiment, the absorbance factor (AF) was set to one, and so the electron transport rate was relative (rETR). The 0.5 in the equation is to take into account the light accepted by photosystem II (PSII) only.

Using SigmaPlot©, the methods and equations reported by Ralph and Gademann (2005) were used to quantitatively compare RLCs. The curve was fitted with a double exponential decay function using a Marquardt-Levenberg regression algorithm. For samples that did not exhibit photoinhibition, a rectangular hyperbola was used. Relative electron transport rates and saturation irradiances (E_k) were also calculated (Ralph and Gademann 2005).

2.2.5. Dark-Adapted Respiration

To evaluate the impact of salinity and *Labyrinthula* infection on dark-adapted respiration, 2 cm sections of each sample of *T. testudinum*, including lesion area, were analyzed for their oxygen uptake post-treatment. 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. In the reaction chamber, the leaf sections were submerged in 10 mL of O_2 -saturated filtered seawater (0.45 μm). Oxygen uptake was measured over the course of 20 min for each experiment. Respiration was calculated as nanomoles of oxygen consumed per minute per gram of tissue. Following respiration measurements, samples were immediately flash frozen in liquid nitrogen and stored at -20°C for pigment analysis.

2.2.6. Pigment Analysis

Previously frozen samples were ground with a mortar and pestle in the dark in a small amount of 95% acetone until no large fragments remained. The total volume of crude extract (10 mL) from each individual sample was wrapped in aluminum foil and refrigerated for 4 hours to allow for additional chlorophyll extraction with minimal degradation. Chlorophyll *a* and *b* content was subsequently evaluated using the spectrophotometric technique and equations described by Jeffrey and Humphrey (1975) using absorbance readings at 647, 664, and 750 nm.

2.2.7. Detection of Reactive Oxygen Species in Plant Tissue

To assess whether elevated salinity compromised plant health and induced oxidative stress in *T. testudinum*, reactive oxygen species (ROS) accumulation was qualitatively monitored as previously described in Lauer *et al.* (2011) using the ROS specific 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. After the two-week incubation period, 2 cm leaf clippings, obtained from each replicate, were incubated for 15 min in 10 mL of microcosm water containing H_2DCF-DA (5 μM final concentration). Prior to microscopic examination, samples were rinsed in fresh 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).

2.2.8. Quantification of Hydrogen Peroxide in Plant Tissue

Following the 2-week incubation at ambient (30) and elevated (45) salinities, hydrogen peroxide levels in *T. testudinum* blade tissue were accurately quantified using the aqueous compatible PeroXOquant ™ Quantitative Peroxide Assay Kit (ThermoScientific, Rockford, IL, USA). Protocols were followed as per the manufacturer‟s specifications with slight modification. Immediately after the end of the incubation, blade sections were excised from the middle of the second rank blade, and wet weights at approximately 0.1 g were measured. Samples were homogenized in 1 mL de-ionized water using a Fast Prep 24 bead homogenizer (MP Biomedicals, Irvine, CA, USA). Following centrifugation at 13,000 x g (10 min), supernatants were combined with working reagent for a 1:10 ratio (modified from Cheeseman 2006). Samples were read at 595 nm against H_2O_2 standards ranging from 0-1000 μM.

2.2.9. *In Vitro Labyrinthula* Growth Inhibition Assays

To evaluate the potential for ecologically relevant concentrations of H_2O_2 (from *T. testudinum*) to inhibit *Labyrinthula* growth, an *in vitro* liquid culture assay was developed based upon a protocol previously reported by Martin *et al.* (2009). Varying concentrations of H_2O_2 (0-100 μM; stock solution 30 % H_2O_2, Sigma-Aldrich St. Louis, MO, USA) were diluted with liquid media (SSA media without agar). Each replicate sample (final volume of 2 mL) was transferred to a single well of a 12-well microplate (Costar ®, Corning Inc., Corning, NY, USA). A standard area of SSA agar-based *Labyrinthula* sp. culture was cut using a 6 mm diameter cork borer and placed upside down in each well to start the assay. Following 24 hours of growth, the liquid media was gently removed and replaced with 1 mL of 0.125% Crystal Violet histological stain (Fisher Scientific, Fair Lawn, NJ, USA). After 1 minute, the stain was removed, rinsed with de-ionized water then dried at 60°C for ten minutes. Using a dissecting scope, the well was inverted and the growth edge was traced. The area of *Labyrinthula* sp. colony growth was quantified using a Kodak© Gel 1500 Imaging System.

2.2.10. Statistical Analysis

All statistical tests were performed at a 95 % confidence level. Lesion size and *in planta* H_2O_2 content were statistically analyzed using a two-tailed independent t-test with salinity as the independent variable. Analysis of respiration, MQY, EQY, NPQ, alpha and beta slopes, $rETR_{max}$, E_k, and chlorophyll concentrations were performed using a two-way ANOVA with salinity and infection as the independent variables. MQY and EQY were compared within each treatment using independent t-tests. *Labyrinthula* sp. colony growth inhibition was statistically analyzed using a one-way ANOVA with H_2O_2

concentration as the independent variable and was followed by a Tukey‟s post-hoc analysis.

2.3. Results

Specimens of *Thalassia testudinum* were infected with pathogenic *Labyrinthula* sp. under ambient (30) and elevated (45) salinities for a 1 week period. One sample from the 45 hypersalinity uninfected treatment defoliated. Another replicate from the same treatment had browning of the second rank blade from senescence including areas under the control vector. The former replicate was omitted from all analyses while the latter was only omitted from the fluorescence measurements. In general, lesions were noted to form near the edges of the vector and ranged in appearance from small dots to continuous larger necrotic areas up to 15.5 mm^2. Average lesion size for the infected samples was significantly smaller in the 45 treatments when compared to the 30 controls ($t= 3.019$, $p= 0.03$; Fig. 2.1). Using the Wasting Index (WI) interpolation method of Burdick ***et al.*** (2003), ambient salinities had approximately 5 % lesion cover while elevated salinities had roughly 2-3 % cover.

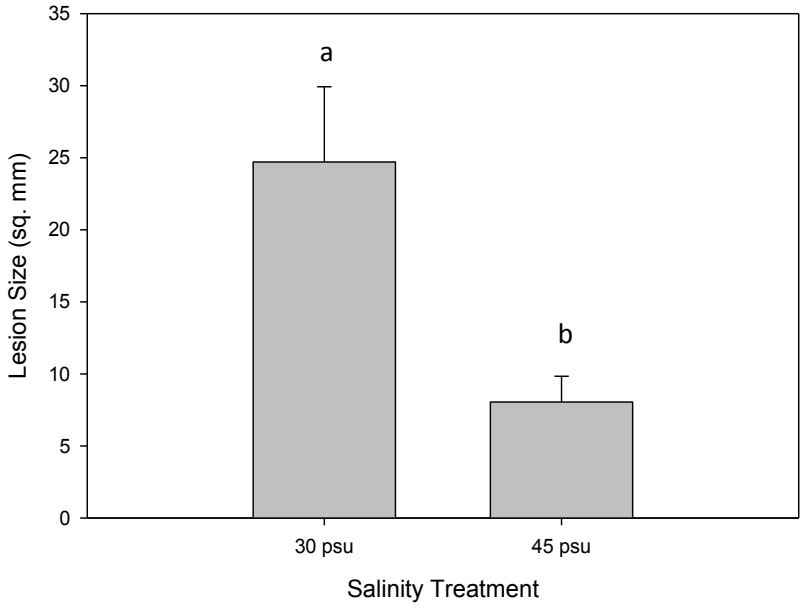

Figure 2.1 Lesion area of *Thalassia testudinum* blades infected with *Labyrinthula* sp. following a 1-week incubation period under ambient (30) and elevated (45) salinities. Letters indicate significant differences ($p \leq 0.05$). Values represent mean ± SE (n=5).

There were no significant differences at a p-value of 0.05 among treatments with respect to both dark- and light-adapted photosynthetic efficiency measurements (MQY and EQY; Table 2.1). Similarly, the differences in rapid light curve parameters among treatments (alpha slope, α; relative electron transport rate maximum, $rETR_{max}$; saturation irradiance, E_k; and downregulation irradiance/ beta slope, β; Table 2.1 and Fig. 2.2) were insignificant. However, both uninfected and infected treatments under a salinity of 30 had significantly lower yields for EQY than MQY (t= 3.462, p= 0.02 and t= 3.262, p= 0.011, respectively; Fig. 2.3) while those of the infected treatment under a salinity of 45 were marginally significant (t= 2.030, p= 0.077; Fig. 2.3). Across all treatments, EQY means were 4.8 to 8.8 % lower than MQY means. Values of NPQ were not significantly different among treatments (Fig. 2.4), yet there was a marginally significant effect for salinity (F= 3.915; p=0.068) with hypersalinity NPQ values being 26-68 % higher than controls maintained at 30.

Post exposure, samples of *T. testudinum* were monitored to determine if infection or elevated salinity had an impact on respiratory demand. There was a significant effect of infection as well as a salinity by infection interaction (2-way ANOVA; F= 10.323, p= 0.006 and F= 5.047, p= 0.040, respectively). *Labyrinthula* sp. infection caused an increase in respiration rates for both salinity treatments. Furthermore, the infected plants under a salinity of 30 exhibited a significantly high degree of respiration than those at 45 (Fig. 2.5). Following exposure to a salinity of 45, samples of *T. testudinum* demonstrated a significant reduction in total chlorophyll (chlorophyll *a* + *b*) content regardless of infection status (F= 14.699, p= 0.002; Fig. 2.6).

Table 2.1 Chlorophyll *a* fluorescence measurements of *Thalassia testudinum* under salinity (30 & 45) and *Labyrinthula* sp. infection treatments.

Treatment	MQY	EQY	NPQ	α	$rETR_{max}$	E_k	β
30 U	0.801 (0.00574)a	0.733 (0.0187)a	0.594 (0.242)a	0.470 (0.0149)a	28.0 (2.69)a	60.1 (6.95)a	0.0121 (0.00356)a
30 I	0.796 (0.00736)a	0.758 (0.00888)a	0.687 (0.211)a	0.451 (0.0262)a	26.4 (3.30)a	58.6 (6.46)a	0.0135 (0.00227)a
45 U	0.781 (0.00767)a	0.712 (0.0340)a	1.87 (0.961)a	0.446 (0.0267)a	25.5 (6.02)a	57.0 (12.5)a	0.00694 (0.000722)a
45 I	0.783 (0.0165)a	0.722 (0.0254)a	0.932 (0.249)a	0.423 (0.0348)a	28.5 (2.76)a	68.4 (7.29)a	0.00982 (0.00200)a

U= uninfected, I= infected, MQY= Maximum Quantum Yield, EQY= Effective Quantum Yield, NPQ= Non-Photosynthetic Quenching, α= Alpha slope, $rETR_{max}$= relative electron transport rate maximum, E_k= saturation irradiance, β= downregulation irradiance/Beta slope. Two-way ANOVAs were performed with salinity and *Labyrinthula* sp. infection as the independent factors. Letters indicate significant differences ($p \leq 0.05$). Values represent mean ± SE (n=3-5).

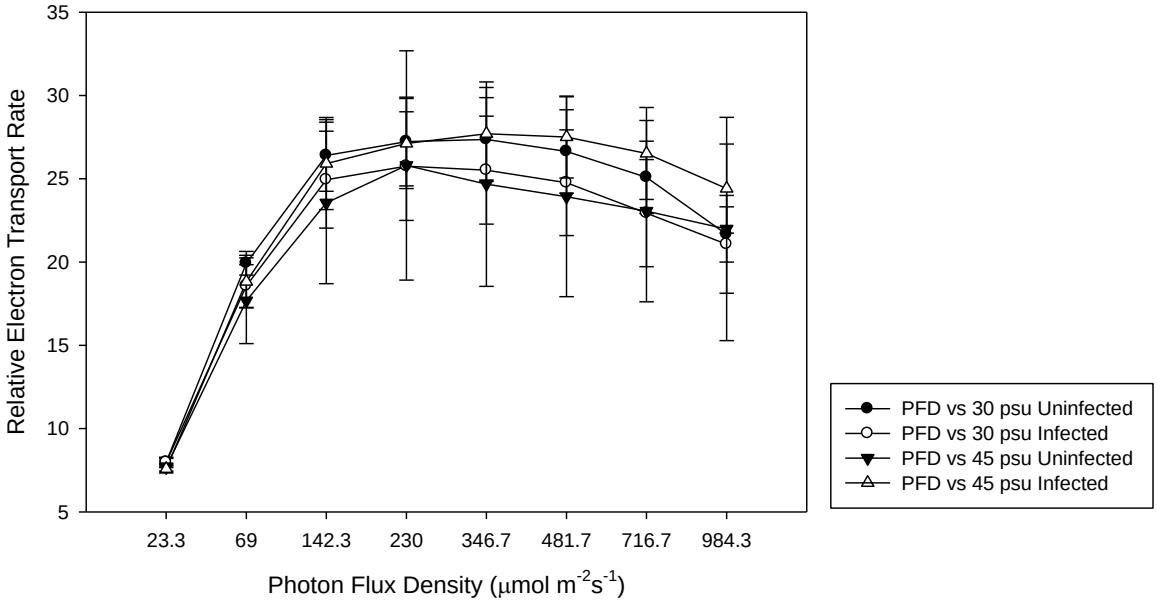

Figure 2.2 Rapid light curves of *Thalassia testudinum* blades under elevated salinity and *Labyrinthula* sp. infection. Values represent mean ± SE (n=3-5).

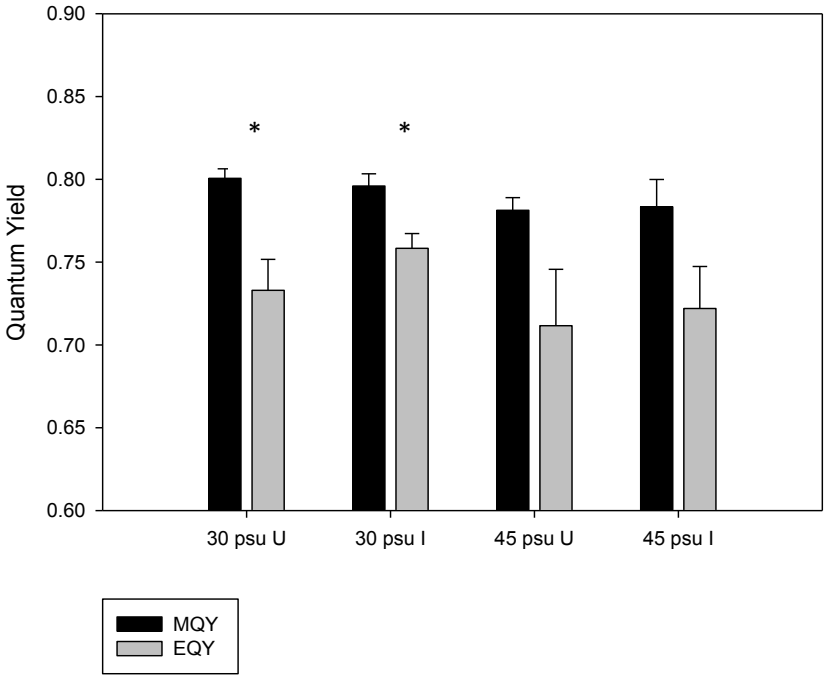

Figure 2.3 Comparison between *Thalassia testudinum* maximum and effective quantum yields taken before the onset of light and taken two hours after the diurnal photoperiod began, respectively. *T. testudinum* samples were subjected to elevated salinity and *Labyrinthula* sp. infection. U= uninfected, I= infected. Letters indicate significant differences ($p \leq 0.05$). Values represent mean ± SE (n=3-5).

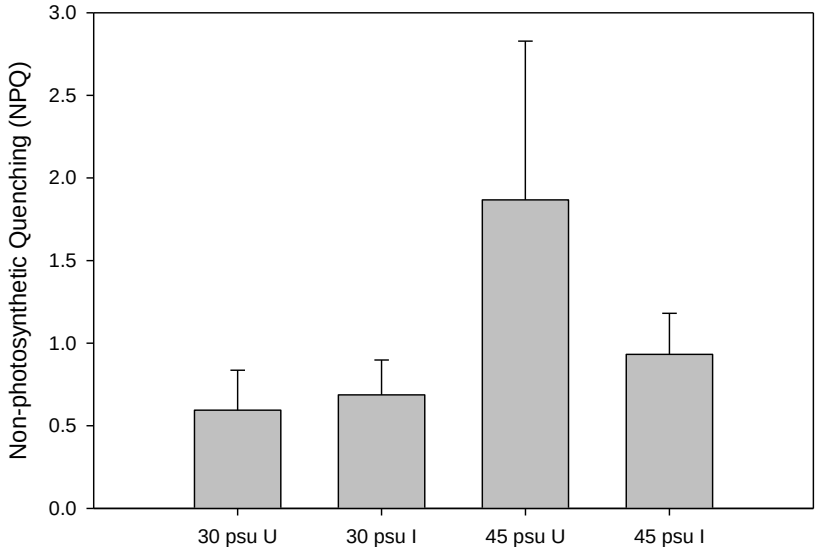

Figure 2.4 Heat dissipation (i.e., non-photosynthetic quenching, NPQ) of photosystem II of *Thalassia testudinum* under ambient (30) and elevated (45) salinity and *Labyrinthula* sp. infection. U= uninfected, I= infected. The salinity treatment has a marginally significant effect (2-way ANOVA, p= 0.068). Values represent mean ± SE (n=3-5).

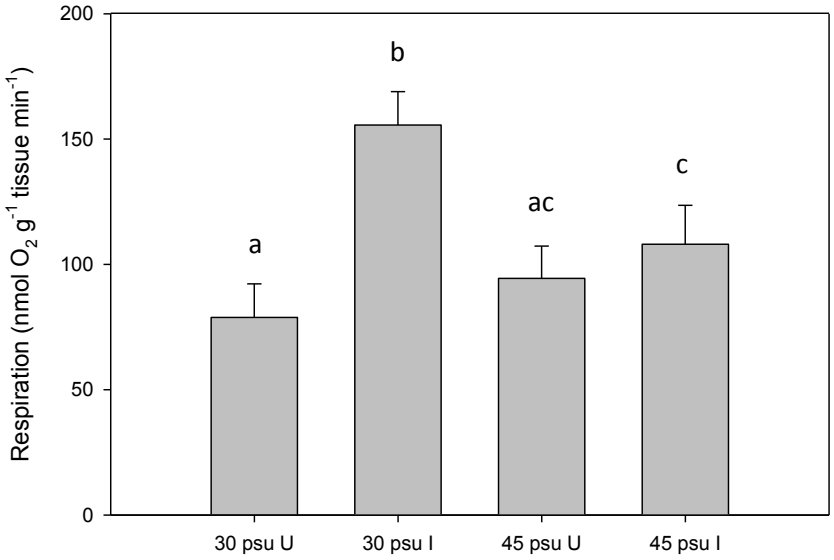

Figure 2.5 Dark-adapted respiration rates of *Thalassia testudinum* under ambient (30) and elevated (45) salinity and *Labyrinthula* sp. infection. U= uninfected, I= infected. Letters indicate significant differences ($p \leq 0.05$). Values represent mean ± SE (n=4-5).

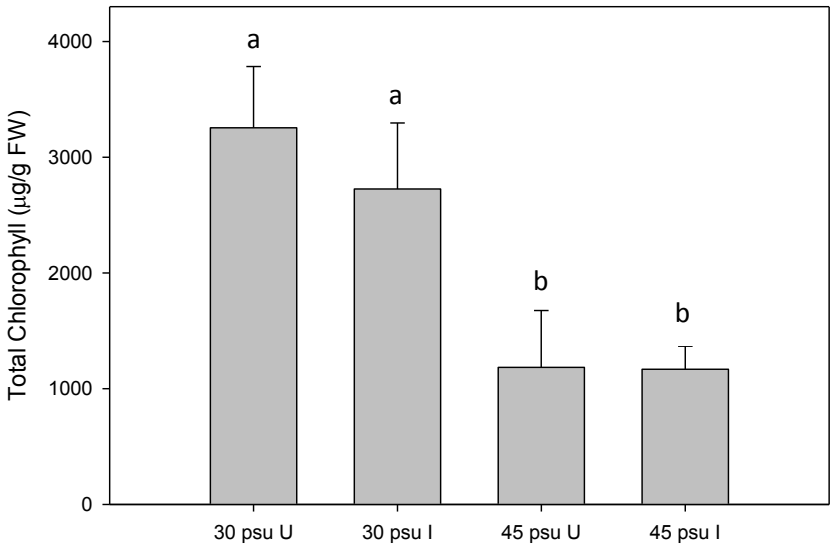

Figure 2.6 Total chlorophyll (*a* + *b*) content of *Thalassia testudinum* blades under ambient (30) and elevated (45) salinity and *Labyrinthula* sp. infection. U= uninfected, I= infected. Letters indicate significant differences ($p \leq 0.05$). Values represent mean ± SE (n=4-5).

Reactive oxygen species (ROS) production was upregulated in *T. testudinum* samples following osmotic stress. Specimens that were exposed to hypersalinity treatment displayed a pronounced release of ROS that was localized to the apoplastic regions between cells (Fig. 2.7). Specimens that were maintained at a salinity of 30 displayed much lower ROS production. Furthermore, quantitative measurements showed that the amount of hydrogen peroxide in the second rank blades of *T. testudinum* was significantly lower at salinity of 30 (0.231 ± 0.0354 μmol H_2O_2/g FW) compared to levels at 45 (0.604 ± 0.117 μmol H_2O_2/g FW) values ($t= -3.058$, $p= 0.016$; Fig. 2.8). These values correspond to 290 μM and 755 μM H_2O_2 on a tissue water basis in the 30 and 45 salinity treatments, respectively.

Labyrinthula sp. Growth in the *in vitro* assay was negatively impacted upon the addition of hydrogen peroxide ($F= 21.153$, $p< 0.001$; Fig. 2.9). *Labyrinthula* sp. colony size showed a 19 % reduction in area when exposed to 60 μM H_2O_2 when compared to controls. When samples were incubated in 80 μM and 100 μM H_2O_2 there was a significant reduction in colony size (52 % and 55 %, respectively) when compared to control values ($p< 0.001$).

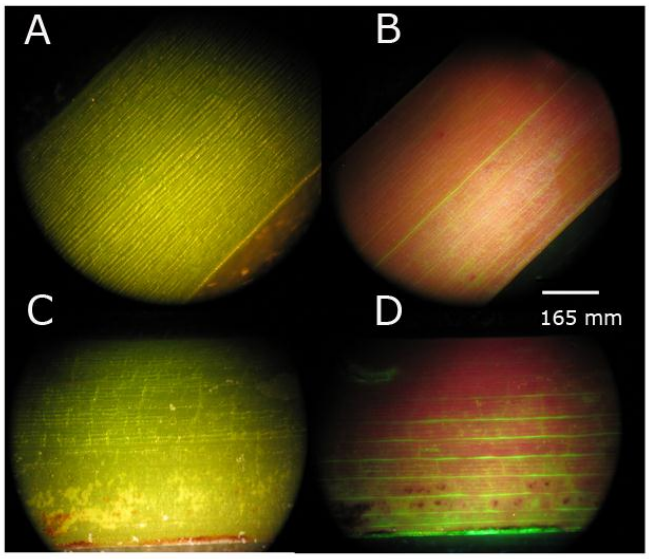

Figure 2.7 Hypersalinity stress induces ROS production in *Thalassia testudinum* blade tissue. Following salinity treatments, plants were stained with the fluorescent probe H_2DCF-DA. This probe detects the localized accumulation of ROS using fluorescent microscopy (Panels B & D, salinities of 30 & 45, respectively; ex 488 nm, em 525 nm). The presence of ROS is indicated by green, while chlorophyll is revealed in red. Light microscopy shown in Panels A & C for salinities of 30 and 45, respectively, are for visual reference.

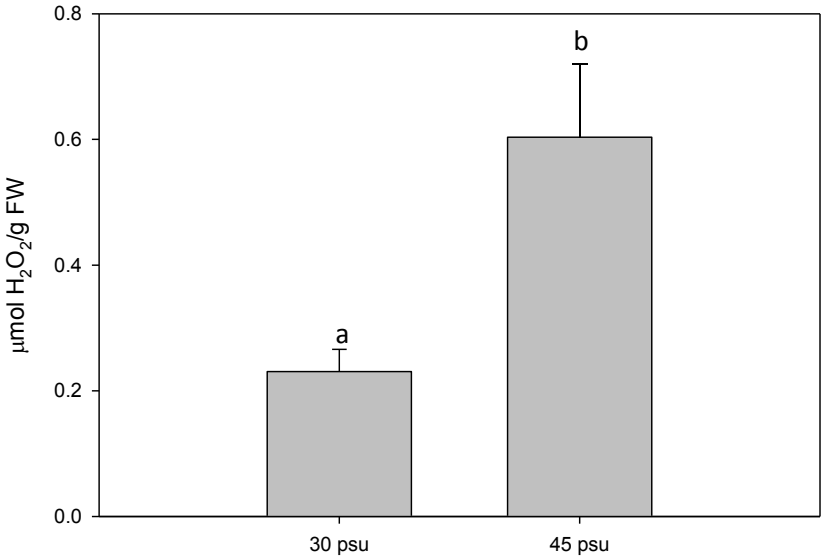

Figure 2.8 *In planta* hydrogen peroxide concentrations in *Thalassia testudinum* blades following a 2-week incubation period at salinities of 30 and 45. Letters indicate significant differences (p= 0.03). Values represent mean ± SE (n=5).

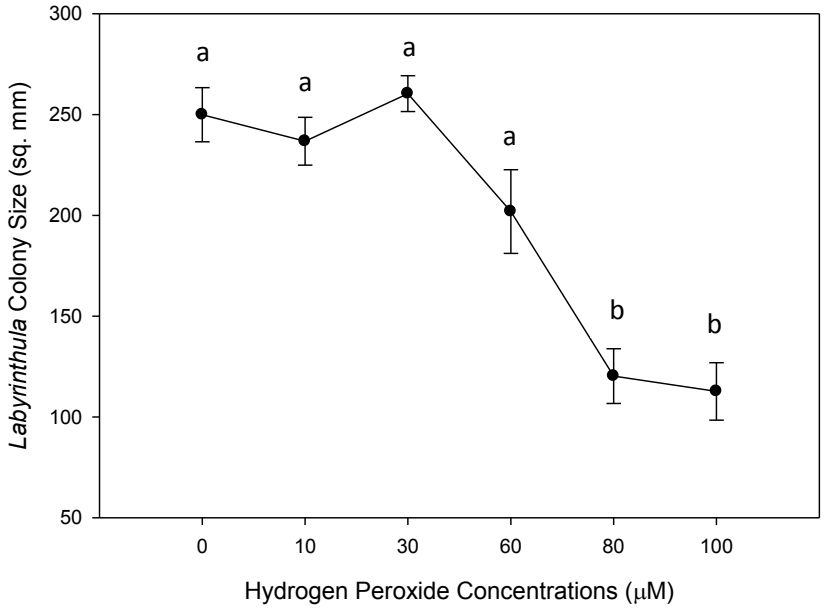

Figure 2.9 Effect of exogenous hydrogen peroxide (0- 100 μM) on *Labyrinthula* sp. colony growth. Letters indicate significant differences ($p \leq 0.004$). Values represent mean ± SE (n=6).

2.4. Discussion

2.4.1. Effect of Salinity on Wasting Disease

When compared to the wealth of knowledge concerning plant pathogenesis in terrestrial ecosystems, the roles of diseases in aquatic settings remain poorly understood (Baskin 2006). In this study, we evaluated the relationship between exposure to elevated salinity and susceptibility to infection using the *T. testudinum – Labyrinthula* host pathogen model system.

The extent of necrotic lesion coverage on *T. testudinum* blades was significantly lessened when plants were exposed to a hypersalinity environment suggesting that salinity clearly influences *Labyrinthula* sp. infection (Fig. 2.1). Previous reports focusing on infected specimens of *Zostera marina* demonstrated that a positive correlation existed between lesion size and exposure to elevated salinities (McKone and Tanner 2009). Unfortunately, available data on the relationship between infection responses and elevated salinity in *T. testudinum* is limiting. A single report (Blakesley *et al.* 2002) has provided documentation showing that *T. testudinum* beds in Florida Bay that were exposed to elevated salinity (>15) had higher incidence of wasting disease. Using a liquid culture assay, Martin *et al.* (2009) demonstrated that colony surface area of *Labyrinthula* "T" (isolated from turtlegrass) declined over 90% when grown at a salinity of 50 when compared to samples maintained at 30. While cell replication continued, the spreading of the ectoplasmic network was significantly diminished Martin *et al.* (2009). The negative impact of elevated salinity of *Labyrinthula* sp. may influence the pathogen‟s ability to adhere and to infect host blade tissue.

2.4.2. PAM Fluorometry

Several previous studies have explored the relationship between *Labyrinthula* infection and the photosynthetic activity of host seagrasses. Ralph and Short (2002) reported that compared to healthy uninfected tissue *Zostera marina* displayed drastically reduced photosynthetic activity at the lesion site as well as up to 5 cm away from infection in healthy green tissue. Durako and Kuss (1994) utilized photosynthetic versus irradiance responses and noted significant impairment of *Thalassia testudinum*‟s photosynthetic capacity as lesion growth increased. Once lesions span the width of the blade, a decrease in photosynthesis as well as a reduction of oxygen transport and carbohydrate transport would likely occur (Durako and Kuss 1994; Ralph and Short 2002). While these studies have contributed to the overall knowledge of seagrass wasting disease in terms of plant health and pathogen identification, they have all involved field samples that have previously been infected for an unknown amount of time.

We observed that during the 1-week infection period of *T. testudinum* neither *Labyrinthula* presence nor changes in salinity significantly inhibited the seagrass‟ photosynthetic response. Durako and Kuss (1994) found that in natural populations of *T. testudinum* there was also little variation in alpha (α) values when comparing uninfected samples and samples with 25 % lesion cover. However, their study noted a significant reduction of the photosynthetic maximum at 25 % coverage suggesting infection caused a reduction in photosynthetic capacity (Durako and Kuss 1994). RLC values and oxygen-based P-I curves are cannot be interpreted synonymously, however, the data from the two methods are correlative (Ralph and Burchett 1995; Beer *et al.* 1998; Ralph and Gademann 2005). Therefore, the differences between the photosynthetic capacities from

of Durako and Kuss (1994) and this study may be due to the differences in lesion cover (25% and 2-5%, respectively).

Salinity stress has been shown to affect photosynthesis by interrupting the electron transport chain as well as altering the chlorophyll content and chloroplast structure (reviewed in Touchette 2007). Clearly salinity levels as well as duration of exposure will influence plant physiological responses. After a 2-week incubation period at a salinity of 45, there was no detectable reduction in the photosynthetic activity of *T. testudinum* (Table 2.1 and Fig. 2.2). These observations are in agreement with previous reports that have demonstrated that *T. testudinum* shows no significant decrease in photosynthetic efficiency (MQY and α) or capacity ($rETR_{max}$) when exposed to pulsed increases in salinity up to 45 (Kahn and Durako 2006; Koch *et al.* 2007a). While PSII can be a target of hypersalinity stress (Xia *et al.* 2004), *T. testudinum* has the ability to make adjustments to maintain levels of photosynthesis comparable to ambient salinities.

Light-adapted EQY measurements of *T. testudinum* have been reported to fluctuate diurnally as a function of open reaction centers available to receive light energy (Durako and Kunzelman 2002; Qiu *et al.* 2003; Belshe *et al.* 2007). This variation may indicate PSII downregulation (β) and non-photosynthetic quenching processes (Belshe *et al.* 2007). In the present study, lack of variation in MQY among treatments indicates that PSII reaction centers were undamaged (Maxwell and Johnson 2000), while after the onset of light EQY values declined (Fig. 2.3). Quantum yield values in every treatment exhibited a decline with the onset of light as may be expected, though NPQ values had a marginally significant increase due to elevated salinities ($p= 0.068$; Fig. 2.4). Non-photosynthetic quenching (NPQ) is correlated with the xanthophyll cycle, an inherent

photoprotective mechanism that naturally dissipates excess heat (Ralph *et al.* 2002; Qiu *et al.* 2003). While the NPQ and xanthophyll cycle have often been associated with stress caused by high light, they have also been seen to be upregulated under hypersaline conditions in green algae (Masojídek *et al.* 2000) and coastal plants (Kao and Tsai 1999; Qiu *et al.* 2003). For example, Naumann *et al.* (2007) reported that after 8 days under hypersalinity stress salt-tolerant *Phragmites australis* did not show any change in MQY but had a reduction in EQY with a concomitant increase in NPQ. Similar chlorophyll *a* results indicating a constant MQY with variable EQY and NPQ could be useful in indicating sublethal stress before permanent photodamage occurs (Naumann *et al.* 2007).

2.4.3. Dark-Adapted Respiration

In aquatic flora, physiological changes that occur under salt stress may include increases in respiratory demand that are often necessary to meet the metabolic challenges of osmotic imbalance (Touchette 2007). On the cytological level, *T. testudinum* possesses a well-developed mitochondrial-plasmalemma transport system which enables this halotolerant genus to maintain suitable ion levels despite exposure to elevated levels of salinity when compared to other aquatic families such as Zosteracae or Ruppiaceae (Jagels 1973; Jagels 1983). In the present study, exposure of *T. testudinum* to a salinity of 45 did not cause any significant increase in respiratory demand. Similar observations have been made in *T. testudinum* seedlings that were grown under a salinity range of 10-50 over a 30 day period (Kahn and Durako 2006). These data suggest that *T. testudinum* may have additional mechanisms to cope with elevated salinity other than ATP-mediated processes, such as active ion transport (Touchette 2007).

In contrast to the results obtained from elevated salinity, the response to *Labyrinthula* infection showed a significant increase in cellular respiration at a salinity of 30 when compared to non-infected controls (Fig. 2.5). This work is in agreement with data from Durako and Kuss (1994) who demonstrated that in natural populations of infected *T. testudinum* there was a positive correlation existed between lesion cover and plant respiratory oxygen demand. Enhanced oxygen consumption is a classic response of infected plants to a wide range of pathogens (Millerd and Scott 1962). Numerous studies have provided evidence for enhanced respiratory activity or an increase in the expression of genes encoding enzymes associated with glycolosis or the tricarboxylic acid cycle (Major *et al.* 2010). Mitochondrial activity is typically upregulated in order to support the resource demands (e.g. shikimic acid/phenylpropenoid pathway) associated with pathogen resistance (Miller and Scott 1962; Bolton 2009; Major *et al.* 2010).

2.4.4. Pigment Analysis

Following the incubation period, *T. testudinum* samples that were exposed to a salinity of 45 (regardless of infection status) showed the strongest visible signs of chlorosis and loss of pigmentation (Fig. 2.6). Total chlorophyll (chlorophyll $a + b$) content (Fig. 2.6) was significantly reduced under hypersalinity ($p= 0.002$) which is a common response to osmotic stress in plants (Parida and Das 2005; Silva *et al.* 2010). In seagrasses, decreased PSII function has been correlated with decreased chlorophyll content (McMillan and Moseley 1967 reported in Koch *et al.* 2007c). However, high chlorophyll pigment concentrations do not always coincide with high PSII function (Ralph 1998; Silva *et al.* 2010; Wan *et al.* 2010), especially in salt tolerant species (Pak *et*

al. 2009). A study using the watermoss *Salvinia natans* revealed that under high salinities MQY did not change even though a significant reduction in total chlorophyll (*a*+ *b*) content occurred (Jampeetong and Brix 2009). Quantum yield measurements, thus, reflect the status of the photosystem reaction center and are not always tightly linked to the amount of working chlorophyll present (Ralph and Gademann 2005). It has been suggested that one strategy to increase light absorption efficiency under stressed conditions would be for seagrasses to forego costly production of pigments and use internal light scattering (Cummings and Zimmerman 2003).

2.4.5. Reactive Oxygen Species

When plants are exposed to periods of enhanced salinity, the disruption of ion homeostasis may trigger the malfunction of any cellular electron transport processes, in particular, those located within the chloroplast and mitochondria. In turn, excess production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) may ensue. While oxidative stress may serve as an indicator of compromised health, the buildup of ROS may serve beneficial roles in a plant such as inhibiting the growth of pathogenic microorganisms (Kuźniak and Urbanek 2000). Plants respond to microbial challenge with a rapid transient accumulation of ROS, termed oxidative burst. However, if a plant is already undergoing oxidative stress and is subsequently infected, the plant may inadvertently already have sufficient levels of ROS to inhibit infection.

In this study, a greater accumulation of ROS was observed in *T. testudinum* samples that were exposed to a salinity of 45 when compared to plants maintained at 30

(Fig. 2.7 B & D). This accumulation was localized to the apoplastic regions between the cells which is a response consistent with peroxidase-dependent apoplastic oxidative bursts also seen in *Arabidopsis thaliana* when infected by the pathogen *Fusarium oxysporum* (Bindschedler *et al.* 2006). In addition to being produced in the apoplast, H_2O_2 can induce programmed cell death if a sustained burst follows the initial, rapid burst (Apel and Hirt 2004).

Although the exact roles that H_2O_2 play in the seagrass‟ defenses against *Labyrinthula* are still unclear, in this study it was found that *T. testudinum*, while living in a hypersaline environment, can produce H_2O_2 in quantities that greatly exceed that which can inhibit *Labyrinthula* colony growth *in vitro* (750 µM vs.80 µM, respectively; Fig. 2.9). This suggests that *T. testudinum* could in fact inadvertently defend itself against *Labyrinthula* sp. invasion while under periods of oxidative stress.

2.5. Conclusion

It was initially hypothesized that elevated salinity would lower the resistance of *T. testudinum*, which in turn would render the plant more susceptible to pathogenic infection. However, in terms of susceptibility to infection, the results indicate that while *T. testudinum* is experiencing oxidative stress and pigment degradation under elevated salinity, *Labyrinthula* infection is not enhanced. This may be due to the direct effect of osmotic stress on the pathogen as well as the indirect effect of reactive oxygen species derived from the host tissue. *T. testudinum* responded to infection with enhanced respiratory demand which is indicative of a classic plant defense response. However, the

upregulation of defense molecules or proteins within *T. testudinum* has yet to be determined. Changes in chlorophyll content and ROS levels are responses that may be most useful in the early detection of osmotic stress in *T. testudinum*. Additionally, observing changes in EQY and NPQ after exposure to an elevated salinity environment with no simultaneous adjustments in MQY may indicate sublethal stress before permanent damage can be done to photosystem II.

In conclusion, initial exposure to elevated salinity stress does not necessarily imply that *T. testudinum* is more prone to infection. This study emphasized the halotolerant characteristics of *T. testudinum* as well as useful indicators of early sublethal stress. This work also highlighted novel dynamics of the effects of hypersalinity and ROS on *Labyrinthula* sp. growth and infection.

3

Anti-labyrinthulid compounds produced by the tropical seagrass *Thalassia testudinum*

Abstract

Historically, seagrasses have been subjected to incidences of wasting disease caused by the marine protist, *Labyrinthula* sp. While seagrasses are rich in phenolics that have been observed to accumulate in response to *Labyrinthula* infection, the exact anti-labyrinthulid properties of these secondary metabolites produced by the tropical seagrass *Thalassia testudinum* are unknown. In this study, *in vitro* bioassays were used to test the anti-labyrinthulid properties of 4 phenolics acids (previously shown to be produced in *T. testudinum* leaves during infection) as well as unknown compounds extracted and partially purified from *T. testudinum* leaf tissue. The 4 phenolic acids, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and vanillin, were shown to inhibit *Labyrinthula* growth at concentrations much lower than what was found in *T. testudinum* tissue. Additionally, it was found that several combinations of phenolic acids had a synergistic response against *Labyrinthula* colony growth. The bioassay-guided fractionation technique was useful in isolating several anti-labyrinthulid fractions. Preliminary characterization of these compounds using ^{1}H-NMR spectroscopy and

HPLC-MS was inconclusive thus highlighting the need for further characterization. The results suggest that *T. testudinum* has the capability of defending itself against *Labyrinthula* infection using secondary metabolites, yet, further research is required to identify the location and biosynthetic processes involved in the production of these *in situ*.

3.1. Introduction

Phenolic secondary metabolites derived from the shikimic acid and phenylpropanoid (SA/PP) pathways provide plants with numerous selective advantages including protection from ultraviolet radiation, the attraction of potential pollinators, cell wall strengthening, defense signaling and direct antimicrobial and antiherbivory defenses (Lattanzio *et al.* 2006; Stalikas 2007). Several reports have documented the presence of phenolic-based compounds in seagrass tissues, and it has been speculated that these compounds serve similar roles to that which are found their terrestrial relatives.

In a broad survey of 25 seagrass species, simple phenolic acids were commonly found in both the above- and belowground tissues (Zapata and McMillan 1979). Similarly, sulphated phenolic acids and/or flavones were also frequently found in 43 seagrass species with their presence often conserved taxonomically at the family or genus level (McMillan *et al.* 1980).

In addition to these surveys on phenolic content, research has begun to shed light on the antimicrobial functions of phenolic acids in seagrasses, particularly against the pathogen associated with wasting disease, *Labyrinthula*. It has been shown that there is a

significant positive correlation between phenolic acid concentration and *L. zosterae* infection in the temperate eelgrass *Z. marina* (Vergeer et al 1995; McKone and Tanner 2009). A similar correlation was found when turtlegrass *Thalassia testudinum* was infected by pathogenic *Labyrinthula* sp. (Steele *et al.* 2005). However, it was suggested that the upregulation of 4 phenolic acids, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and vanillin, was a result of pseudo-induction. The phenolic acids were not accumulating at the infection site, rather they were upregulated only at sites above the lesion. It was speculated that the necrotic tissue was serving as a physical barrier allowing the buildup of carbohydrates (precursors of phenolics via the shikimic acid pathway; Steele *et al.* 2005).

While these positive correlations between *Labyrinthula* abundance and phenolic content suggest a bioactive role, there have been no extensive studies that have directly tested whether secondary metabolites from *T. testudinum* have true anti-labyrinthulid properties. One single report has tested this assumption using *Z. marina* (eelgrass) leaf tissue as a source of phenolics. Using an agar-based *in vitro* assay, it was found that caffeic acid had variable inhibitory activity against *Labyrinthula zosterae* colony growth (Vergeer and Develi 1997).

Work by Jensen *et al.* (1998) demonstrated that *Thalassia testudinum* is able to produce a compound that effectively inhibits the growth and spore attachment of *Schizochytrium aggregatum*, a marine saprobe related to *Labyrinthula*. This thraustrochytrid was inhibited by the novel flavone glycoside, luteolin7-β-D-glucopyranosyl-2”-sulphate (Thalassiolin A), at concentrations 15x lower then what was found in *T. testudinum* leaf tissue.

While it seems plausible that seagrasses, in particular *T. testudinum*, may have the secondary metabolite arsenal that could assist in the prevention or reduction of *Labyrinthula* infection, it is still unclear if *T. testudinum* can specifically produce compounds that inhibit the growth of *Labyrinthula.* With the increasing frequency of marine diseases and the devastating effects wasting disease has on seagrass populations and ecosystem functions (Robblee and DiDomenico 1991; Fourqurean and Robblee 1999; Harvell *et al.* 1999), it is critical to fully understand the factors that prevent or reduce wasting disease occurrences.

The objective of this chapter was to further understand this relationship between the secondary metabolites of *Thalassia testudinum* and their effects on *Labyrinthula* colony propagation *in vitro*. While the 4 previously mentioned phenolic acids (3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and vanillin) have been shown to be upregulated during *Labyrinthula* infection, it is not known if these compounds have direct anti-labyrinthulid activities. Therefore, the first part of this chapter was to determine if these phenolics have anti-labyrinthulid activity and then identify the specific inhibitory concentrations associated with these compounds, both alone and in combination. It was hypothesized that these phenolic acids have multiple functions, and that in addition to accumulation in response to blocked vascular transport, they are able to inhibit *Labyrinthula* colony growth. In the second part of this chapter, a bioassay-guided fractionation approach was used to isolate compounds (some of which may be previously uncharacterized) that inhibit *Labyrinthula* colony growth. By using this broad exploratory approach, it was hypothesized that compounds that actively inhibit *Labyrinthula* proliferation will be found in *T. testudinum* leaf tissue.

3.2. Materials and Methods

3.2.1. *Labyrinthula* Cultures & Seagrass Collection

A known virulent strain of *Labyrinthula* sp., kindly provided by the laboratory of Dr. Anne Boettcher (University of South Alabama), was utilized in all bioassay experiments. *Labyrinthula* sp. cultures were maintained in serum seawater agar (SSA) media as previously described by Martin *et al.* (2009) with slight modification. Briefly, the SSA recipe contained 500 mL of 0.22 μm filtered seawater (salinity of 25) using Instant Ocean® sea salt which was combined with 6 g agar, 0.5 g glucose, 0.05 g nutritional yeast, 0.05 g peptone, 1.5 mg germanium dioxide, 12.5 mL streptomycin/penicillin (stock: 1.25 g streptomycin/1.25 g penicillin per 100 mL de-ionized H_2O), and 5 mL horse serum. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Samples of the seagrass *Thalassia testudinum* (Banks ex. König) were collected from a shallow water habitat (~ 1m) on June 2010 from Long Key, Florida, USA (24°49'N, 80°31'W). Blades were cleaned of epiphytes, patted dry and immediately frozen at -80°C until processing.

3.2.2. Liquid Culture Assay

To test for anti-*Labyrinthula* properties of the phenolic acids and the unknown compounds extracted from *T. testudinum* blades, a liquid culture assay was utilized (modified from the methods of Martin *et al.* 2009). Selected concentrations of the compound of interest were made by dilution via heated liquid media (SSA media without agar). Each replicate sample (final volume of 2 mL) was transferred to a single well of a

12-well microplate (Costar©, Corning Inc., Corning, NY, USA). A standard area of SSA agar-based *Labyrinthula* sp. culture was cut using a 6 mm diameter cork borer and placed upside down in each well to start the assay. Following 24 h of growth, the liquid media was gently removed and replaced with 1 mL of 0.1% Crystal Violet histological stain (Fisher Scientific, Fair Lawn, NJ, USA). After 1 minute, the stain was removed, rinsed with deionized water and dried at 60°C for ten minutes. Using a dissecting scope, the well was inverted and the growth edge was traced. The area of *Labyrinthula* sp. colony growth was quantified using a Kodak© Gel 1500 Imaging System.

3.2.3. Phenolic Acid Inhibition of *Labyrinthula* sp.

The four phenolic acids that have been previously reported to be upregulated in *T. testudinum* during *Labyrinthula* infection (Steele *et al.* 2005) were assayed. Inclusive were 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and vanillin. To assay for anti-labyrinthulid bioactivity, 50 mg of each phenolic acid (Fisher Scientific, Fair Lawn, NJ, USA) was solubilized in 1 mL acetone (100 mg for vanillin) to create stock solutions. Beginning with a final concentration of 0.05 mg/ml for each phenolic acid, each treatment (replicates of 6) was sequentially diluted by adding the stock solution to hot media, keeping the acetone volume the same in each treatment. *Labyrinthula* sp. samples were added to the liquid media and monitored using the assay method described above. Preliminary tests showed that the acetone controls (acetone + media) did not show any inhibitory activity against *Labyrinthula* sp. colony growth compared to negative controls (only media).

Percent inhibition exhibited by the *Labyrinthula* colonies in response to the phenolic acid dose was calculated and graphed against the log of the phenolic acid concentration (mM). A sigmoidal dose-response curve (logistic 4-parameter) was fitted and the half maximal inhibitory concentration (IC_{50}) was calculated for each phenolic acid using GraphPad Prism version 4 (San Diego, California, USA).

The interactive effects of two phenolic acids on *Labyrinthula* growth was tested in the following combinations using a 1:1 molar ratio of each compound‟s IC_{50} (Lane and Kubanek 2006): 3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid and *p*-coumaric acid; *p*-hydroxybenzoic acid and *p*-coumaric acid; and *p*-coumaric acid and vanillin. The dose-response curve derived from each combination was compared to the theoretic additive curve developed from the two individual dose-response curves (Tallarida *et al.* 1997). Additionally, an interactive index was used to compare the IC_{50} values of the combination and theoretical additive curves (Observed IC_{50}/Theoretical Additive IC_{50}). An index of less than one suggests synergism while an index equal to one indicates an additive response (Tallarida 2002).

To visualize the presence of phenolic compounds, live *T. testudinum* blades were infected for 5 days using the pathogenic strain of *Labyrinthula* described above (see infection methods, Chapter 2). Infected tissue was visualized using an Olympus BX60 fluorescent microscope (ex 350 nm, em $\geq$ 450 nm; Olympus Corp., Tokyo, Japan).

3.2.4. Bioassay-Guided Fractionation

General

Fisher Scientific (Fair Lawn, NJ, USA) HPLC-grade solvents were used in all fractionation and characterization steps. All solvent fractions were dried in vacuo using a Büchi Rotavapor R210 (Büchi Labortechnik AG, Flawil, Switzerland) and a Labconco Freezone 6 lyophilizer (Labconco, Kansas City, MO, USA). Column chromatography was carried out using Varian Bond Elut C18 60 mL columns (Agilent Technologies, Santa Clara, CA, USA) under pressure. HPLC was performed using a Zorbax SB-C18 column (9.4 x 250 mm, 5µm; Agilent Technologies, Santa Clara, CA, USA) in association with a Waters 1525 Binary pump, Waters 2487 Dual Wavelength Absorbance Detector and Breeze Software Version 3.30 SPA (Waters Corp., Milford, MA, USA). Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was performed using a Varian VNMRS (500 MHz; Agilent Technologies, Santa Clara, CA, USA). HPLC-MS was performed using a Synergi-Hydro RP column (Phenomenex, Inc., Torrance, CA, USA) in association with Applied Biosystems (ABI) 3200 QTrap LC/MS/MS (International Equipment Trading Ltd., Vernon Hills, IL, USA).

Extraction and Isolation

Thalassia testudinum biomass (1.17 kg fresh weight) was homogenized in a blender then extracted three times in 60:40 EtOH:H_2O (v/v). The solvent extract was combined, filtered, and dried in vacuo yielding 108 g of crude extract. A total of 17 g of extract was solvent partitioned in 1g aliquots between 3:2 MeOH: H_2O (v/v) and chloroform (400 mL ea.). Fractions were dried, and the chloroform fraction (Fraction C)

was stored at -80°C until further use. The MeOH:H_2O fraction (14.12 g) was dissolved 1 g at a time in 300 mL deionized water (Fraction W) and partitioned with an equal part of *n*-butanol (Fraction B). Both Fraction W and Fraction B were separately applied to a C-18 column and eluted sequentially with 80:20, 60:40, 40:60, 20:80 and 0:100 H_2O:MeOH (v/v; fractions 1-5, respectively).

Compound Characterization

^{1}H NMR spectroscopy and HPLC- mass spectroscopy were used to further characterize metabolites from bioactive HPLC fractions. For ^{1}H NMR spectroscopy, samples were solubilized in 1 mL deuterated chloroform. For HPLC-MS, samples were solubilized in HPLC-grade MeOH to a final concentration of 100 µg/mL. The LC-MS gradient was as follows: 50:50 H_2O:MeOH (0.1% formic acid) to 100% MeOH (0.1% formic acid) in 15 min. 100% MeOH was run for 5 min then returned to 50:50 H_2O:MeOH in 1 min.

3.2.5. Statistical Analyses

The differences among the IC_{50} values of each compound as well as between the original and theoretical curves were statistically analyzed with an F test ($p= 0.05$) using GraphPad Prism Version 4.

3.3. Results

3.3.1. *Labyrinthula* Growth Inhibition by Phenolic Acids

Each phenolic acid was assayed in order to assess its inhibitory activity against *Labyrinthula* colony growth, beginning with a concentration of 0.05 mg/mL

(approximately 0.3 mM) and increasing the dose until 100% growth inhibition occurred. A dose-response curve was created for each phenolic acid, and the concentration that inhibited *Labyrinthula* colony growth by half (IC_{50}) was calculated (Fig. 3.1). Each IC_{50} was significantly different (F test, $p \leq 0.0011$). *P*-coumaric acid (p-coum) was the most potent *Labyrinthula* inhibitor (IC_{50} = 0.6625 mM), followed by *p*-hydroxybenzoic acid (p-hyd; IC_{50}= 0.9622 mM), 3,4-dihydroxybenzoic acid (3,4-dihyd; IC_{50}= 1.048 mM), then vanillin (IC_{50}= 5.613 mM).

Using the phenolic acid concentrations found in infected *T. testudinum* tissue reported by Steele *et al.* (2005), *in planta* levels were converted from % dry weight to a mM basis. These values were then compared with the anti-labyrinthulid IC_{50} values for each of the phenolic acids used in this study (Fig. 3.2). Each phenolic acid was produced in *T. testudinum* leaves in excess of what was required to inhibit *Labyrinthula* colony growth (IC_{50} value). Notably, the IC_{50} values of *p*-hydroxybenzoic and vanillin were 45x and 14x lower than the *in planta* concentrations of 43.44 mM and 78.87 mM, respectively (Fig. 3.2).

Bioassays using a combination of two phenolics were performed to determine if there was an additive or synergistic effect on *Labyrinthula* growth. The combination of *p*-coumaric acid and vanillin did not show any activity against *Labyrinthula* growth. For combinations that exhibited anti-labyrinthulid activity, additive curves and IC_{50} values were calculated using the values from the individual curves. The interaction index quantitatively indicated an additive or synergistic interaction between two compounds. The combination of 3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid had an index value closest to 1 (0.905) while the combination of *p*-coumaric acid and *p*-

hydroxybenzoic acid had the lowest index value (0.7306; Table 1).The IC_{50} values resulting from each combination of phenolic acids (3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and *p*-coumaric acid, and *p*-coumaric acid and *p*-hydroxybenzoic acid) were all significantly different from their respective theoretical additive curves (F test, $p< 0.0001$; Figs. 3.3-3.5, respectively).

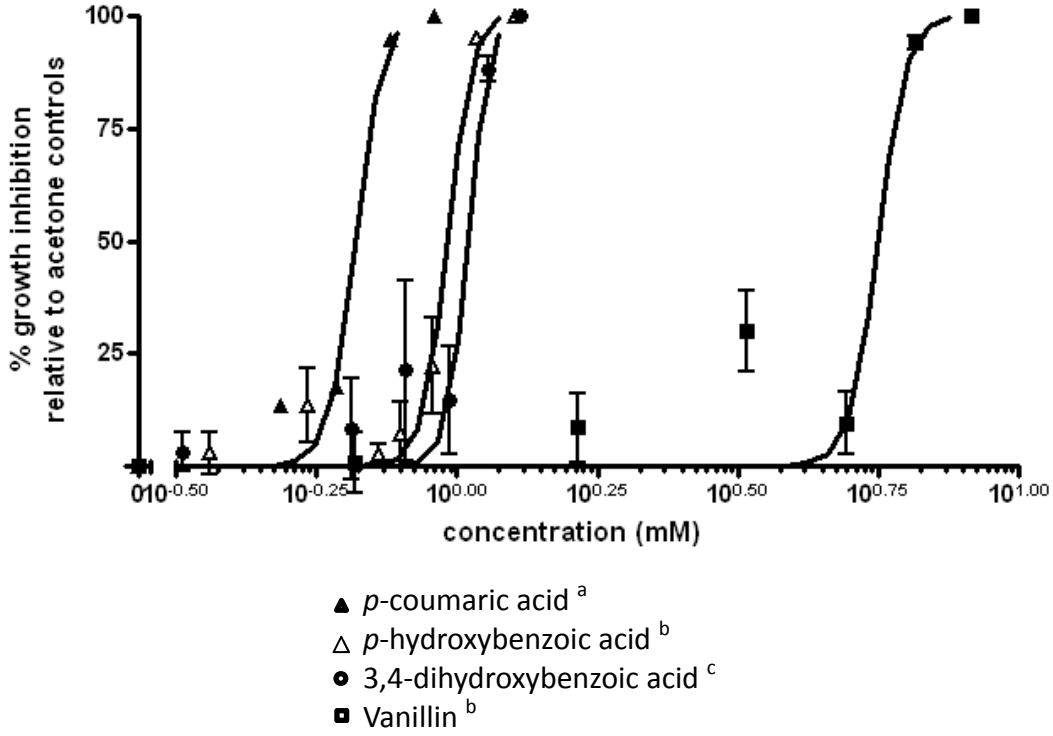

Figure 3.1 Effect of phenolic acid concentrations on *Labyrinthula* colony growth for *p*-coumaric acid, *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and vanillin. Letters indicate significantly different IC_{50} values (F test, $p \leq 0.0011$).

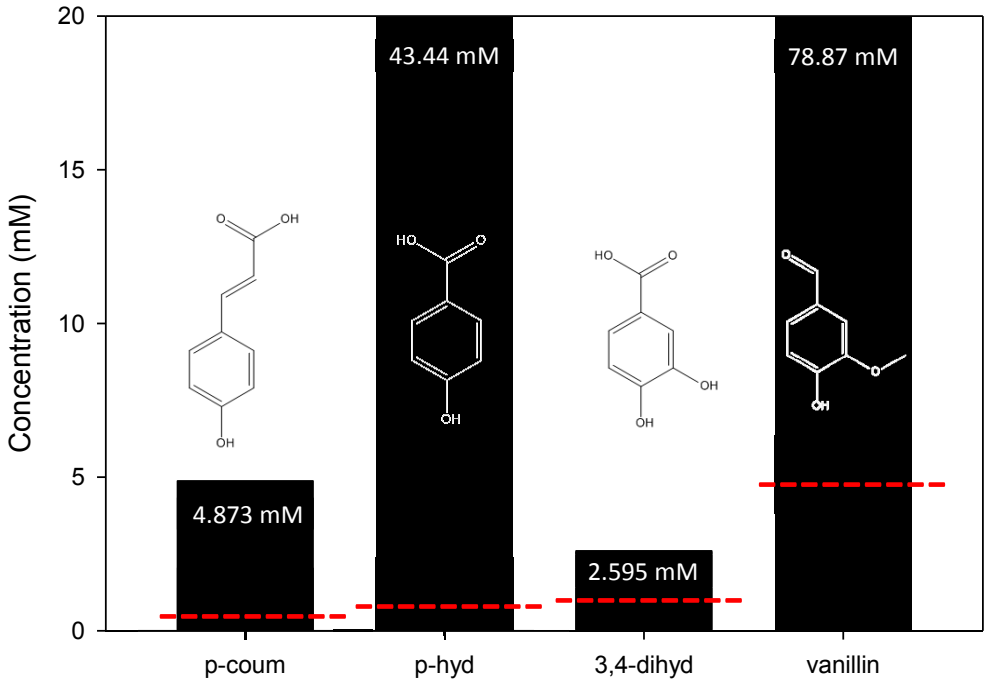

Figure 3.2 Comparison of the phenolic acid IC_{50} values reported in this study (red) with the *in planta* concentrations reported by Steele *et al.* (2005). Dose-response curves were used to calculate the IC_{50} values (Fig. 3.1).

Table 3.1 The interaction index used to quantify the degree of synergism on *Labyrinthula* sp. colony growth in the presence of two of phenolic acids.

	Observed IC_{50}	Theoretical Additive IC_{50}	Interaction Index[a]
3,4 dihyd + *p*-hyd	0.9078	1.003	0.905
3,4 dihyd + *p*-coum	0.5982	0.8118	0.7306
p-coum + *p*-hyd	0.6688	0.7847	0.8523

[a] Index calculated as Observed/Theoretical. Values < 1 suggest a synergistic interaction is occurring.

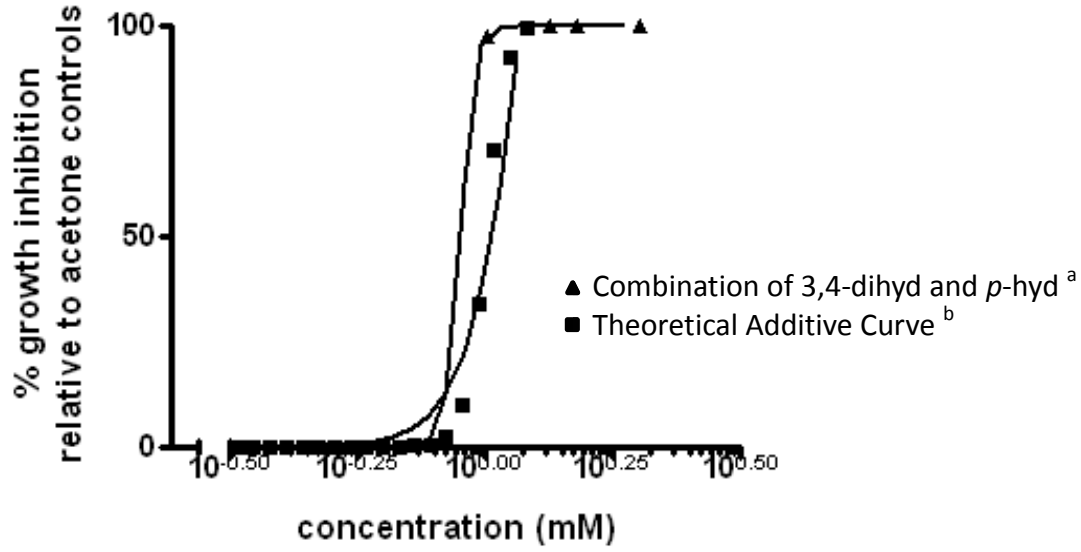

Figure 3.3 Comparison of the combined dose-response curve of 3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid with the theoretical additive curve. Letters indicate significant differences in IC_{50} values (F test, $p<0.0001$).

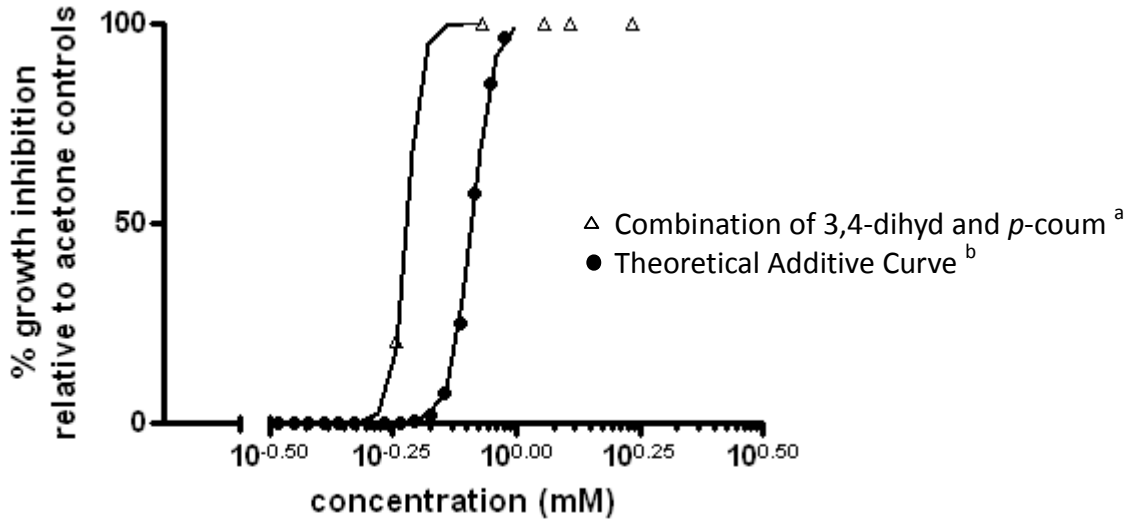

Figure 3.4 Comparison of the combined dose-response curve of 3,4-dihydroxybenzoic acid and *p*-coumaric acid with the theoretical additive curve. Letters indicate significant differences in IC_{50} values (F test, $p<0.0001$).

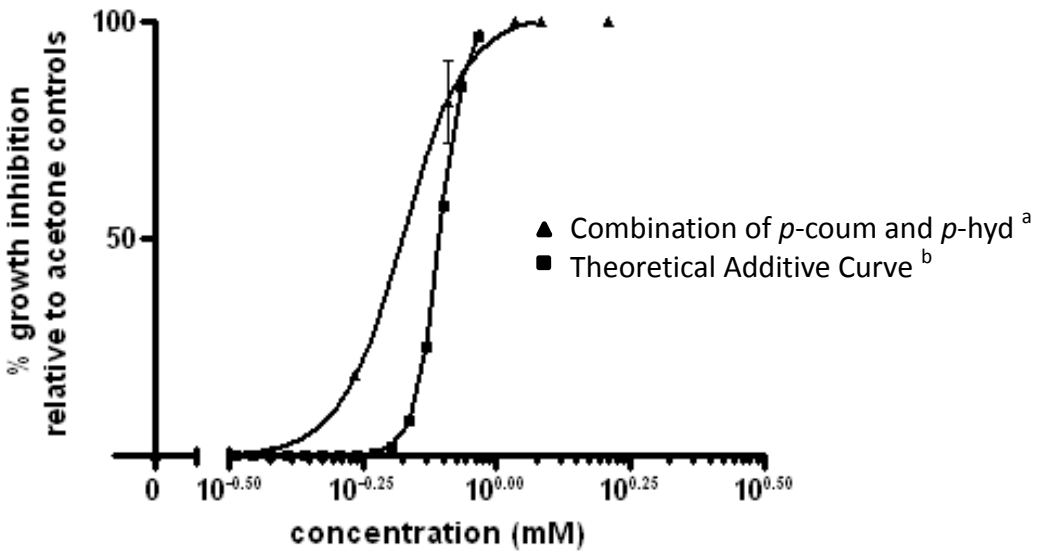

Figure 3.5 Comparison of the combined dose-response curve of *p*-coumaric acid and *p*-hydroxybenzoic acid with the theoretical additive curve. Letters indicate significant differences in IC_{50} values (F test, $p<0.0001$).

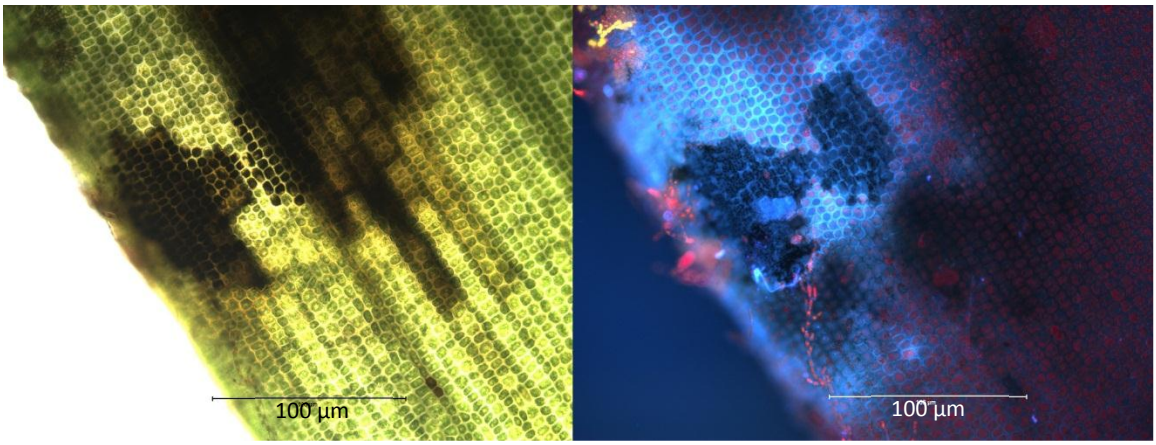

Figure 3.6 Autofluorescence of phenolic compounds in *Thalassia testudinum* leaf tissue 5 days post-infection with *Labyrinthula*. (A) Light microscopy shows pronounced lesions (brown) surrounded by healthy green tissue. (B) Excitation using a DAPI filter set (ex 350, em ≥ 450) shows accumulation of phenolics (blue) on lesion periphery. Red indicates the localization of chloroplasts and other photosynthetic epiphytes.

To visually detect the presence of phenolic compounds during *Labyrinthula* infection, 5 days post-infection *Thalassia testudinum* blades were visualized using fluorescent microscopy (Fig. 3.6). The presence of blue autofluorescence suggested the accumulation of phenolics less than 50 μm from lesion site (Fig. 3.6B). Red fluorescence indicated the localization of chloroplasts and other photosynthetic epiphytes (Fig. 3.6B).

3.3.2. Bioassay-Guided Fractionation

Crude extract was solvent partitioned between 3:2 MeOH:H_2O (v/v) and chloroform (Fraction C). The MeOH:H_2O phase was dried and further partitioned between equal parts water (Fraction W, 10.59 g) and *n*-butanol (Fraction B, 1.973 g). All fractions were dried and tested negative for anti-*Labyrinthula* activity. Fractions W and B were further separated using reverse-phase liquid chromatography resulting in fractions W1-W5 and B1-B5, respectively. Liquid culture assays with these fractions revealed fractions B4, B5 and W2 inhibited *Labyrinthula* colony growth by 100% relative to solvent only controls (Fig. 3.7-3.8).

Fraction W2 was further purified using reverse phase high pressure liquid chromatography (RP-HPLC). Chromatographic fractions were collected in 5 min increments (0-30 min; fractions W2-A through F, respectively; Fig. 3.9) and then assayed for activity. None of these fractions exhibited anti-labyrinthulid activity. Because anti-labyrinthulid activity was not chemically tractable in fraction W2, isolation of compounds from this fraction was not further pursued.

Fractions B4 and B5 were also purified using RP-HPLC. Fractions were collected in 5 min increments (0-35 min; fractions B4-A-G and B5-A-G, respectively) and then

assayed for activity. Fraction B4 showed anti-labyrinthulid activity in fractions B4-B and B4-D at 250 μg/mL and 200 μg/mL, respectively (Fig. 3.10). Bioassays of the B5 fractions revealed activity in B5-D at 133 μg/mL and B5-E at 88.9 μg/mL (Fig. 3.11).

Fractions B5-D and B5-E showed the greatest bioactivity and so were further characterized using ^{1}H NMR and RP-HPLC coupled with mass-spectroscopy. The signal to noise ratio in the ^{1}H NMR was too poor for adequate analysis due to the insufficient quantity of sample. LC-MS data were also inconclusive, possibly owing to the complexity of the molecular fragmentation patterns.

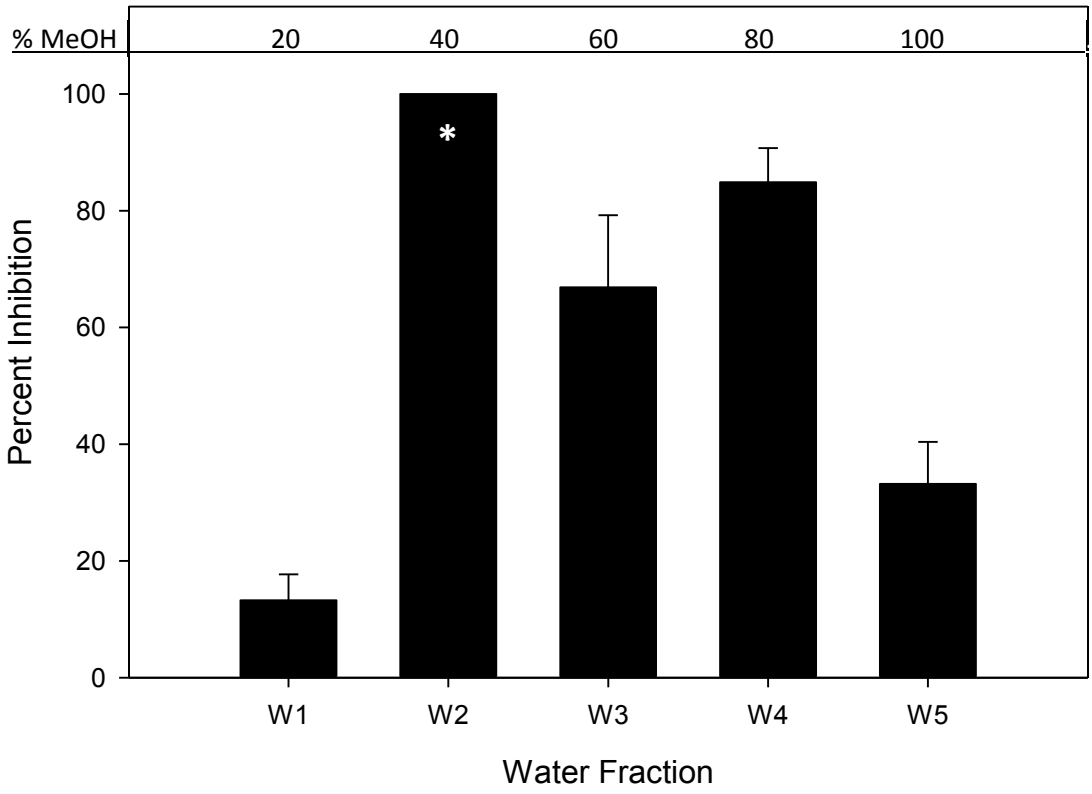

Figure 3.7 Percent inhibition of *Labyrinthula* colony growth by *Thalassia testudinum* chromatographic fractions originating from the water fraction (Fraction W) of the solvent partition. Fractions W1- W5 correspond to a solvent gradient solvent gradient of 20% to 100% MeOH in H_2O. The asterisk indicates the fraction that exhibited 100% growth inhibition and was subsequently subjected to RP-HPLC.

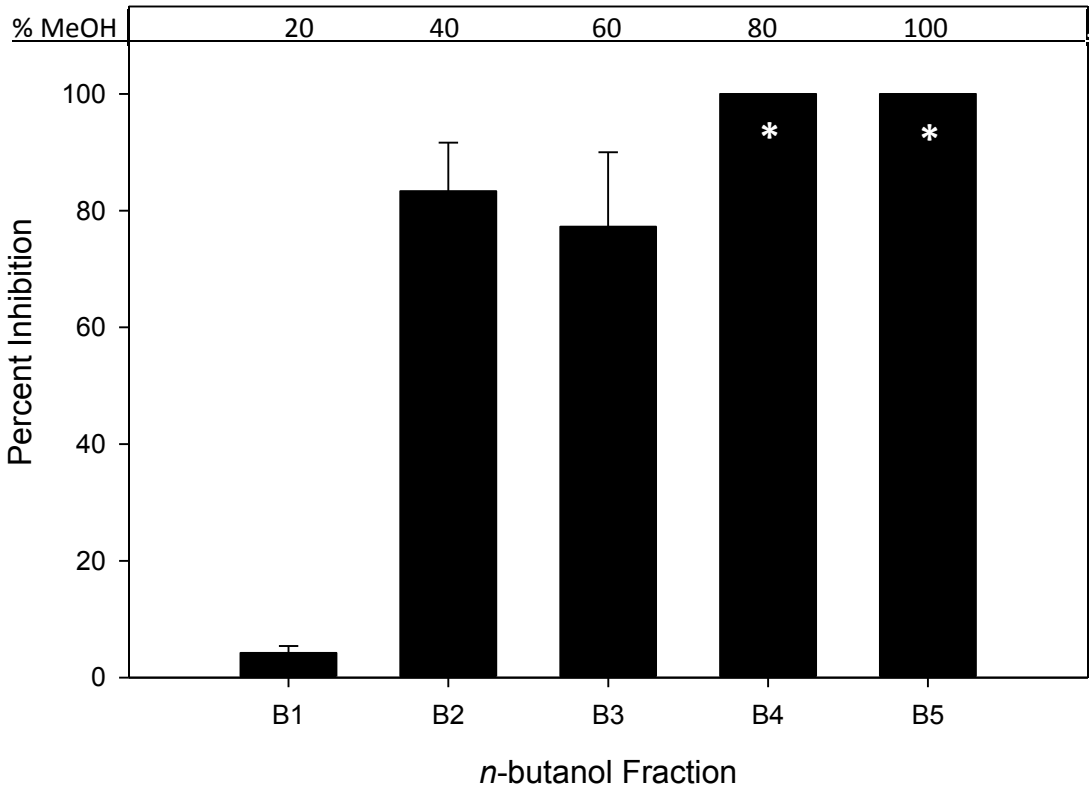

Figure 3.8 Percent inhibition of *Labyrinthula* colony growth by *Thalassia testudinum* chromatographic fractions originating from the n-butanol fraction (Fraction B) of the solvent partition. Fractions B1- B5 correspond to a solvent gradient of 20% to 100% MeOH in H_2O. Asterisks indicate the fractions that exhibited 100% growth inhibition and were subsequently subjected to RP-HPLC.

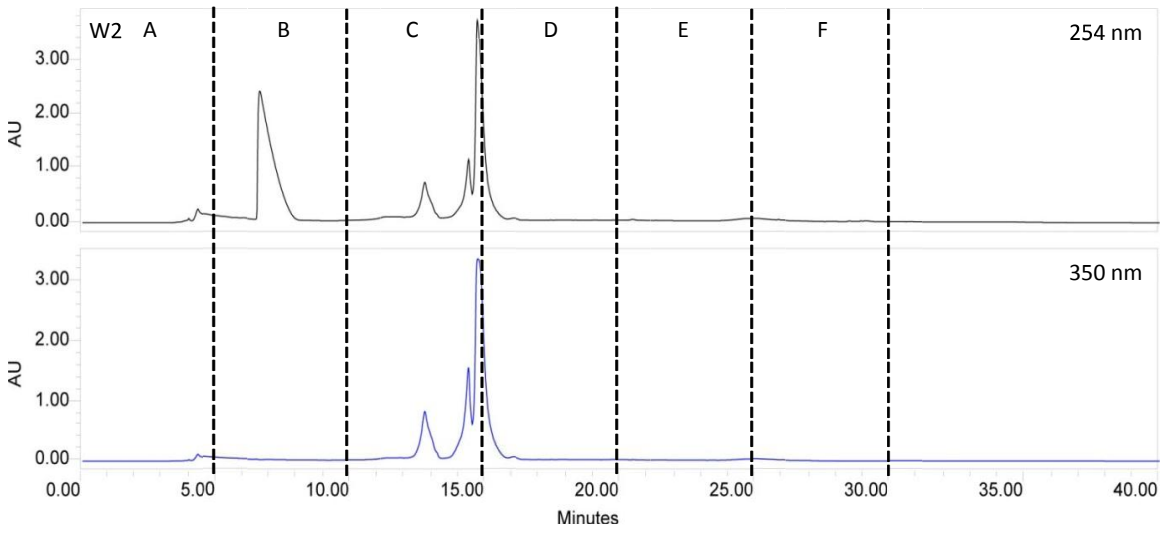

Figure 3.9 Representative RP-HPLC chromatogram of fraction W2, with monitoring at 254 and 350 nm. Letters represent the fractions collected in 5 min intervals. Subsequent bioassays showed no anti-labyrinthulid activity.

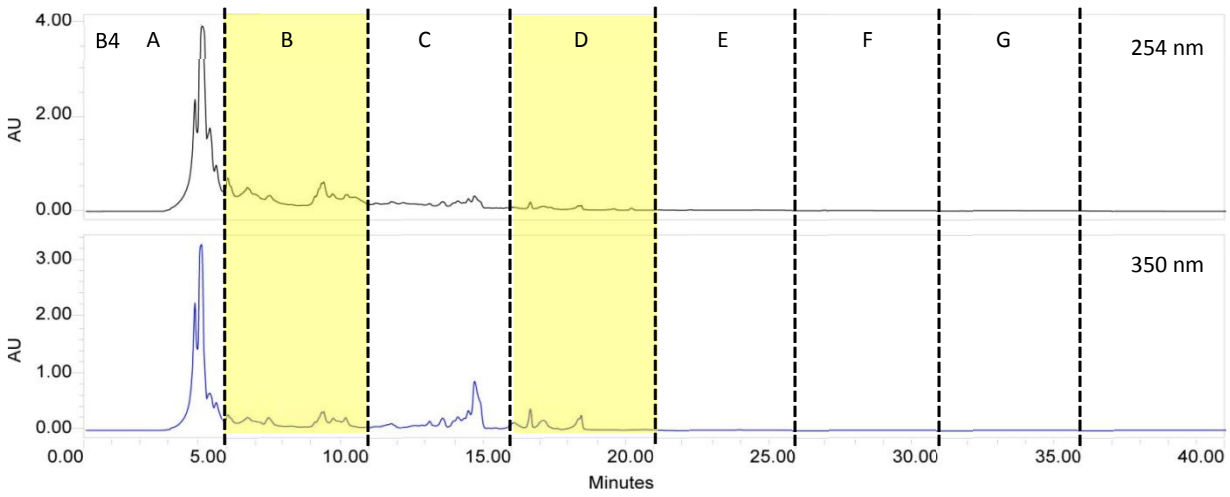

Figure 3.10 Representative RP-HPLC chromatogram of fraction B4, with monitoring at 254 and 350 nm. Letters represent the fractions collected in 5 min intervals. Highlights indicate fractions exhibiting anti-labyrinthulid activity.

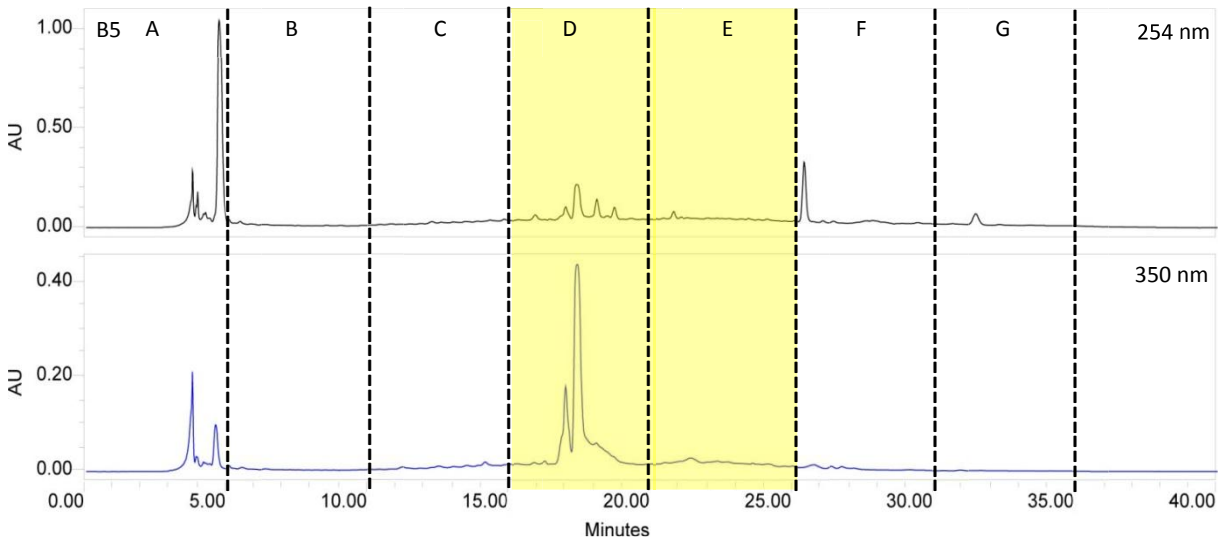

Figure 3.11 Representative RP-HPLC chromatogram of fraction B5, with monitoring at 254 and 350 nm. Letters represent the fractions collected in 5 min intervals. Highlights indicate fractions exhibiting anti-labyrinthulid activity.

3.4. Discussion

3.4.1. Phenolic Acid Inhibition of *Labyrinthula* sp.

Phenolic compounds provide plants with an array of benefits including protection against microorganisms. Seagrasses are rich in phenolics, including phenolic acids and their conjugates (Zapata and McMillan 1979; McMillan *et al.* 1980). Four phenolic acids were previously identified as being pseudo-induced in *T. testudinum* leaf tissue while under attack by pathogenic *Labyrinthula* (Steele *et al.* 2005). In the current study, it was demonstrated that these metabolites, alone (Fig. 3.1) and in combination (Table 3.1), exhibited direct anti-*Labyrinthula* properties. *P*-coumaric acid, a hydroxycinnamic acid, was the most potent antibiotic with the lowest IC_{50} (0.6625 mM). The other phenolic acids, all hydroxybenzoic acid derivatives, had higher IC_{50} values. Vanillin, a reduced form of vanillic acid, had the highest IC_{50} (5.613 mM).

In combination, *p*-coumaric acid and vanillin had no effect on *Labyrinthula* growth which suggests a negative, antagonistic interaction between these two compounds. However, a positive, synergistic interaction was observed with the other combinations of phenolic acids (3,4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid and *p*-coumaric acid; and, *p*-hydroxybenzoic acid and *p*-coumaric acid). Each IC_{50} value calculated from the combination dose-response curve was significantly different than the theoretical additive IC_{50} values (Figs. 3.3-3.5). Each interaction index was less than 1 suggesting that *Labyrinthula* growth was affected synergistically in the presence of these phenolic acid combinations (Table 3.1). In general, secondary metabolites often have resource and metabolic costs associated with production (Bolton 2009). When the ecological benefits outweigh these costs, these

metabolites are said to maximize the plant‟s fitness (Optimal Defense Theory; Rhoades 1979). Synergistic interactions of secondary metabolites are thought to increase a plant‟s fitness since a greater toxic effect is reached with lower concentrations of metabolites resulting in lower associated resource costs (Nelson and Kursar 1999). Phenolic acids, particularly *p*-coumaric acid, *p*-hydroxybenzoic acid and vanillic acid, have been shown to produce synergistic inhibitory responses on invading fungi in conifers (Cates 1999).

Interestingly, when the IC_{50} values of the individual phenolic acid inhibition trials in this study were compared to the *in planta* levels reported by Steele *et al*. (2005), the IC_{50} values were 2.5x – 45x lower than the levels reported during *Labyrinthula* infection (Fig 3.2). This suggests that although there are ample phenolic acids available to inhibit *Labyrinthula,* this may not be their primary role. Despite what would be considered high levels of phenolics, the plants are still capable of being infected. The following are possible explanations of the disconnection between the phenolic content with respect to the *in vitro* and *in plant* results:

- When testing secondary metabolites for antimicrobial activity, bioassays may not always predict their ecological functions since the full range of host and pathogen responses and tactics are not being utilized (Engel *et al*. 2002). In this study, the whole colony of *Labyrinthula* was fully exposed to the phenolic acids while growing on a 2-D surface. This is unlike the 3-D environment of a cell in which a few cells may be exposed to host metabolites.
- When invading seagrass tissue, *Labyrinthula* uses enzymes to degrade live epidermal cells. While not much is known about these enzymes, it is possible that the molecules utilized during infection of live tissue may not be expressed *in*

vitro and may cause *Labyrinthula* to response differently to the phenolic acids produced *in planta*.

- Free phenolic acids were used in the anti-*Labyrinthula* bioassays. While free phenolics are often more toxic to pathogens, they may be naturally sequestered in plant organelles or vacuoles or present in inactive forms (bound to cells walls or stored as glycosides) until invasion occurs (van Sumere *et al.* 1975; Lattanzio *et al.* 2006). This helps to prevent self-damage while also being readily available for activation by enzymes in response to pathogen challenge (Lattanzio *et al.* 2006).
- Figure 3.6 shows accumulation of phenolics less than 100 µm from the lesion site while the phenolic acids extracted by Steele *et al.* (2005) were taken 2 cm from the lesion. Low molecular weight phenols, including phenolic acids, have been shown to accumulate at the site of infections as a rapid, initial lines of defense against microorganisms (Nicholson and Hammerschmidt 1992). Coinciding with defense strategies in land plants, the phenolic acids in *T. testudinum* may be serve as the first line of defense to slow *Labyrinthula* growth while secondary responses are being synthesized (Nicholson and Hammerschmidt 1992).

The relatively low levels of phenolic acids needed to inhibit *Labyrinthula* growth in culture-based bioassays and the accumulation of phenolics at the lesion sites on infected blades suggest that *T. testudinum* possibly employs these secondary metabolites as initial defenses to wasting disease. To fully understand the defense mechanisms, it is necessary to identify the inducible defenses that are often the end-products of signal-transduction pathways. Such defenses include an oxidative burst of reactive oxygen

species (see Chapter 2), synthesis of specific anti-microbial compounds (i.e. phytoalexins) and pathogenesis-related (PR) proteins, as wells as protective cross-linking of phenolic compounds as physical barriers to further infections (Dixon and Paiva 1996; Heath 2000; Lattanzio *et al.* 2006).

3.4.2. Bioassay-Guided Fractionation

A bioassay-guided fractionation technique was used to survey potential anti-labyrinthulid secondary metabolites found in *Thalassia testudinum* blade tissue. Thus far, activity was followed to two fractions of the n-butanol partition (fractions B4 and B5; Fig. 3.8). This coincides with the activity found in the *n*-butanol fractions reported by Regalado *et al.* (2009) from which a flavone glycoside (Thalassiolin B) and phenolic acid (*p*-hydroxybenzoic acid) were subsequently isolated.

LC-MS analysis of fraction B5-D indicated a compound of high molecular weight ($[M^+]$ m/z = 986.6). These compounds were initially detected at 254 nm which is the wavelength used to detect phenolics acids (Steele *et al*. 2005; Stalikas 2007). It is possible that B5-D contains polymers of phenolic acids or perhaps a new compound(s) that has yet to be fully characterized.

3.4.3. Conclusion

In this chapter, it was concluded that (1) phenolics acids are inhibitory toward *Labyrinthula* growth in culture and (2) by using a bioassay-guided fractionation technique, anti-labyrinthulid compounds could be isolated directly from *T. testudinum* tissue. The pure phenolic acids used in this study where able to inhibit *Labyrinthula*

colony growth at concentrations naturally found *T. testudinum* tissue. In some cases, the combination of two phenolic acids was able to elicit a synergistic inhibitory response on *Labyrinthula* proliferation. Even though the phenolic acids were previously shown to be pseudo-induced in response to *Labyrinthula* infection, the strong inhibitory properties and the accumulation of phenolics adjacent to the lesion sites suggest that these phenolic acids may in fact be a component of an initial line of defense against wasting disease. Additionally, despite the inconclusive characterization of the active compounds, metabolites exhibiting anti-labyrinthulid properties have been partially isolated directly from *T. testudinum* tissue.

In conclusion, these initial results on the biochemical-mediated defenses of *T. testudinum* to *Labyrinthula* infection suggest that turtlegrass produces anti-labyrinthulid compounds. Future work should investigate the timing and location of pre-formed and induced compounds associated with a defense response in *T. testudinum*.

4

General Conclusions and Outlook

It was the goal of this thesis to explore the physiological and biochemical responses of the tropical seagrass *Thalassia testudinum* to hypersalinity stress and *Labyrinthula* infection. In Chapter 2, after exposure to elevated salinity, *T. testudinum* experienced chlorophyll degradation as well as oxidative stress yet was able to maintain photosynthetic functions. Interestingly, exposure to a hypersaline environment did not increase *T. testudinum*‟s susceptibility to wasting disease during the initial stages of infection. The cause of the decreased susceptibility is unclear since *Labyrinthula* infections may have been affected directly by the hypersaline environment or indirectly by the ROS levels in the host tissue.

While ROS accumulation in response to infection was not measured in this study, it was shown that *Labyrinthula* growth can be significantly inhibited *in vitro* in the presence of ROS (i.e., H_2O_2) at levels well below the concentrations found in *T. testudinum* leaf tissue. Additionally, while ROS are known to have several roles as defense mechanisms such as direct microbial toxicity, cell wall reinforcement and signaling of other defense-related molecules, few studies have explored the relationship between the ROS response of seagrass and microbial invasion (Ross *et al.* 2008; Sureda *et al.* 2008). More research is needed to understand the *in vivo* effects of ROS on *Labyrinthula* during inoculation.

After one week, *T. testudinum* exhibited increased respiration in response to *Labyrinthula* infection. This change in metabolism has been reported in higher plants to support the higher resource demands associated with pathogen resistances. For example, routing raw materials to the shikimic acid and phenylpropanoid pathways for the *de novo* production of secondary metabolites can require intense metabolic demands (Bolton 2009). In Chapter 3, it was shown that 5 days post-infection, phenolic compounds were upregulated in the immediate vicinity of *Labyrinthula* lesions. Phenolics that accumulate at the infection site are often associated with a rapid first line of defense in order to slow or stop further disease progression while other, more effective strategies can be employed (Nicholson and Hammerschmidt 1992). These secondary responses may include the *de novo* production of specific compounds such as phytoalexins and pathogenesis-related proteins (Nicholson and Hammerschmidt 1992; Lattanzio *et al.* 2006). It would be interesting to see if the increased respiration in *T. testudinum* is directly involved in a secondary stage of defense.

Using *in vitro* bioassays, unidentified compounds extracted from *T. testudinum* tissue as well as 4 phenolic acids that are known to be present during wasting disease exhibited anti-labyrinthulid properties (Chapter 3). This suggests that *T. testudinum* does in fact have the secondary metabolite arsenal to defense itself during *Labyrinthula* infection. However, there was a disconnection between the concentrations of 4 phenolics acids that inhibit *Labyrinthula in vitro* and their concentrations during infection of *T. testudinum* tissue (Steele *et al.* 2005). This highlights the importance of using ecologically relevant assays to help detect the effects of secondary metabolites on

microbial behavior (e.g., adhesion, settlement, and surface swarming; Jensen *et al.* 1998; Engel *et al.* 2002).

Few studies have explored the regulation of the SA/PP pathways as well as other defense-related signal-transduction pathways in seagrasses. Two hormones involved in defense signaling in plants, salicylic acid and jasmonic acid, do not seem to be involved in seagrass phenolic production during microbial invasion or wounding (Steele *et al.* 2005; Arnold *et al.* 2008). It may also be important to explore the location and timing of biochemical defenses in seagrasses, particularly at the seagrass leaf surface when microbial invasion is initiated (Lane *et al*. 2009) as well as the genomic level (Lane and Moore 2011).

In conclusion, the results of this thesis suggest that *Thalassia testudinum* does have biochemical and metabolic defense mechanisms to at least manage *Labyrinthula* infection. *T. testudinum* is also able to physiologically adjust to survive under (short term conditions) in a hypersaline environment without increasing its susceptibility to wasting disease. This supports the currently hypothesis that multiple concurrent environmental stressors are involved the significant decline in seagrass health preceding large wasting disease outbreaks.

Literature Cited

Addy CE, Aylward DW. 1944. Status of eelgrass in Massachusetts during 1943. J. Wildl. Manage. 8: 269-275.

Anderson P, Cunningham A, Patel N, Morales F, Epstein P, Daszak P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol. 19: 535-544.

Apel K, Hirt H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55: 373-399.

Armiger LC. 1964. An occurrence of *Labyrinthula* in New Zealand *Zostera*. New Zeal. J. Bot 2: 3-9.

Arnold TM, Targett NM. 2002. Marine Tannins: The importance of a mechanistic framework for predictive ecological roles. J. Chem. Ecol. 28: 1919-1934.

Arnold TM, Tanner CE, Rothen M, Bullington J. 2008. Wound-induced accumulations of condensed tannins in turtlegrass, *Thalassia testudinum*. Aquat. Bot. 89: 27-33.

Baskin Y. 2006. Sea Sickness: The Upsurge in Marine Diseases. BioScience 56: 464-469.

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A. 1998. Measuring photosynthetic rates in seagrass by pulse amplitude modulated (PAM) fluorometry. Mar. Ecol. Prog. Ser. 174: 293-300.

Belshe E, Durako M, Blum J. 2007. Photosynthetic rapid light curves (RLC) of *Thalassia testudinum* exhibit diurnal variation. J. Exp. Mar. Biol. Ecol. 342: 253-268.

Bindschedler L, Dewdney J, Blee K, Stone J, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies D, Ausubel F, Bolwell G. 2006. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. The Plant Journal doi: 10.1111/j.1365.313X.2006.02837.x.

Blakesley B, Berns D, Merello M, Hall M, Hyniova J. 2002. The Dynamics and Distribution of the Slime Mold *Labyrinthula* sp. and its potential impacts on *Thalassia testudinum* populations on Florida, www.tbeptech.org.

Bolton M. 2009. Primary metabolism and plant defense- Fuel for the fire. Mol. Plant Microbe Interact. 22: 487-497.

Burdick D, Short F, Wolf J. 1993. An index to assess and monitor the progression of wasting disease in eelgrass *Zostera marina*. Mar. Ecol. Prog. Ser. 94: 83-90.

Cates RG. 1996. The role of mixtures and variation in the production of terpenoids in conifer-insect-pathogen interactions. Recent Adv. Phytochem. 30: 179-216.

Cheeseman J. 2006. Hydrogen peroxide concentrations in leaves under natural conditions. J. Exp. Bot. 57: 2435-2444.

Cowan MM. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564-582.

Cummings M, Zimmerman R. 2003. Light harvesting and the package effect in the seagrasses *Thalassia testudinum* Banks ex König and *Zostera marina* L.: optical constraints on photoacclimation. Aquat. Bot. 75: 261–274.

Dawes CJ. 1998. Marine Botany. 2nd Ed. New York: John Wiley & Sons.

Dawes CJ, Phillips RC, Morrison G. 2004. Seagrass Communities of the Gulf Coast of Florida: Status and Ecology. Florida Fish and Wildlife Conservation Commission Fish and Wildlife Research Institute and the Tampa Bay Estuary Program. St. Petersburg, FL, pp. iv-74.

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. The Plant Cell 7: 1085-1097.

Durako MJ. 1993. Photosynthesis utilization of $CO_{2(aq)}$ and HCO_3^- in *Thalassia testudinum* (Hydrocharitaceae). Mar. Biol. 115: 373-380.

Durako M, Kuss K. 1994. Effects of *Labyrinthula* infection on photosynthetic capacity of *Thalassia testudinum*. Bull. Mar. Sci. 54 (3): 727-732.

Durako M, Kunzelman J. 2002. Photosynthetic capacity of *Thalassia testudinum* measured in situ by pulse-amplitude modulated (PAM) fluorometry: methodological and scale-based considerations. Aquat. Bot. 73: 173-185.

Eldridge PM, Kaldy JE, Burd AB. 2004. Stress response model for the tropical seagrass *Thalassia testudinum*: The interactions of light, temperature, sedimentation, and geochemistry. Estuaries 27: 923-937.

Engel S, Jensen PR, Fenical W. 2002. Chemical ecology of marine microbial defense. J. Chem. Ecol. 28: 1971-1985.

Fourqurean JW, Zieman JC. 1991. Photosynthesis, respiration and whole plant carbon budget of the seagrass *Thalassia testudinum*. Mar. Ecol. Prog. Ser. 69: 161-170.

Fourqurean JW, Robblee MB. 1999. Florida Bay: A history of recent ecological changes. Estuaries 22: 345–357.

Grenfell B, Dobson A. 1995. Ecology of Infectious Diseases in Natural Populations. Cambridge: Cambridge University Press. p. 521.

Harvell CD, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hoffman EE, Lipp EK, Osterhaus ADME, Overstreet RM, Porter JW, Smith GW, Vasta GR. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science 285: 1505-1510.

Haslam E. 1993. Shikimic Acid: Metabolism and Metabolites. Chichester: John Wiley and Sons.

Heath MC. 2000. Nonhost resistance and nonspecific plant defenses. Current Opinion in Plant Biology 3: 315-319.

Herrmann KM. 1995. The shikimate pathway: Early steps in the biosynthesis of aromatic compounds. The Plant Cell 7: 907-919.

Jagels R. 1973. Studies of a marine grass, *Thalassia testudinum*: I. Ultrastructure of the osmoregulatory leaf cells. Amer. J. Bot. 60: 1003–1009.

Jagels R. 1983. Further evidence for osmoregulation in epidermal leaf cells of seagrasses. Amer. J. Bot. 70: 327-333.

Jampeetong A, Brix H. 2009. Effects of NaCl salinity on growth, morphology, photosynthesis and proline accumulation of *Salvinia natans*. Aquat. Bot. 91: 181–186.

Jeffrey S, Humphrey G. 1975. New spectrophotometric equations for determining chlorophylls *a, b,* c_1, and c_2 in higher plants algae, and natural phytoplankton. Biochem. Physiol. Pflanz. 167: 191-194.

Jensen PR, Jenkins KM, Porter D, Fenical W. 1998. Evidence that a new antibiotic flavones glycoside chemically defends the sea grass *Thalassia testudinum* against zooporic fungi. Appl. Environ. Microbiol. 64: 1490-1496.

Kahn A, Durako M. 2006. *Thalassia testudinum* seedling responses to changes in salinity and nitrogen levels. J. Exp. Mar. Biol. Ecol. 335: 1-12.

Kao W-Y, Tsai H-C. 1999. The photosynthesis and chlorophyll *a* fluorescence in seedlings of *Kandelia candel* (L.) Druce grown under different nitrogen and NaCl controls. Photosynthetica 37: 405-412.

Kato Y, Aioi K, Omori Y, Takahata N, Satta Y. 2003. Phylogenetic analysis of *Zostera* species based on rbcL and matK nucleotide sequences: Implications for the origin and diversification of seagrasses in Japanese waters. Genes & Genetic Systems 78: 329-342.

Kelble C, Johns E, Nuttle W, Lee T, Smith R, Ortner P. 2007. Salinity patterns of Florida Bay. Estuar. Coast. Shelf Sci. 71: 318-334.

Koch MS, Erskine JM. 2001. Sulfide as a phytotoxin to the tropical seagrass *Thalassia testudinum*: interactions with light, salinity and temperature. J. Exp. Mar. Biol. Ecol. 266: 81-95.

Koch M, Schopmeyer S, Kyhn-Hansen C, Madden C, Peters J. 2007a. Tropical seagrass species tolerance to hypersalinity stress. Aquat. Bot. 86: 14-24.

Koch M, Schopmeyer S, Holmer M, Madden C, Kyhn-Hansen C. 2007b. *Thalassia testudinum* response to the interactive stressors hypersalinity, sulfide and hypoxia. Aquat. Bot. 87: 104–110.

Koch M, Schopmeyer S, Neilsen O, Kyhn-Hansen C, Madden C. 2007c. Conceptual model of seagrass die-off in Florida Bay: Links to biogeochemical processes. J. Exp. Mar. Biol. Ecol. 350: 73-88.

Kuźniak E, Urbanek H. 2000. The involvement of hydrogen peroxide in plant responses to stresses. Acta Physiol. Plantarum 22: 195–203.

Laffoley Dd‟A, Grimsditch G. (eds). 2009. The management of natural coastal carbon sinks. IUCN, Gland, Switzerland. 53 pp.

Lane AL, Kubanek J. 2006. Structur-activity relationship of chemical defenses from the freshwater plant *Micranthemum umbrosum*. Phytochemistry 67: 1224-1231.

Lane AL, Moore BS. 2011. A sea of biosynthesis: marine natural products meet the molecular age. Nat. Prod. Rep. 28: 411-428.

Lane AL, Nyadong L, Galhena AS, Shearer TL, Stout EP, Parry RM, Kwasnik M, Wang MD, Hay ME, Fernandez FM, Kubanek J. 2009. PNAS 106: 7314-7319.

Lattanzio V, Lattanzio VMT, Cardinali A. 2006. Role of phenolics in the resistance mechanism of plants against fungal pathogens and insects. Phytochemistry: Advances in Research pp. 23-67.

Lauer N, Yeager M, Kahn A, Dobberfuhl D, Ross C. 2011. The effects of short term salinity exposure on the sublethal stress response of *Vallisneria americana* Michx. (Hydrocharitaceae). Aquat. Bot. 95: 207-213.

Lee KS, Park SR, Kim YK. 2007. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. J. Exp. Mar. Biol. Ecol. 350: 144-175.

Li G, Wan S, Zhou J, Yang Z, Qin P. 2010. Leaf chlorophyll fluorescence, hyperspectral reflectance, pigments content, malondialdehyde and proline accumulation responses of castor bean (*Ricinus communis* L.) seedlings to salt stress levels. Ind. Crops Prod. 31: 13–19.

Major I, Nicole M, Duplessis S, Séguin A. 2010. Photosynthetic and respiratory changes in leaves of poplar elicited by rust infection. Photosyn. Res. 104: 41-48.

Martin D, Boone E, Caldwell M, Major K, Boettcher A. 2009. Liquid culture and growth quantification of the seagrass pathogen, *Labyrinthula* spp. Mycologia. 101: 632-635.

Masojídek J, Torzillo G, Kopecký J, Koblížek M, Nidiaci L, Komendal J, Lukavskál A, Sacchi A. 2000. Changes in chlorophyll fluorescence quenching and pigment composition in the green alga *Chlorococcum* sp. grown under nitrogen deficiency and salinity stress. J. Appl. Phyc. 12: 417–426.

Mateo MA, Romero J, Pérez M, Littler MM, Littler DS. 1997. Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass *Posidonia oceanica*. Estuar. Coast. Shelf Sci. 44: 103-110.

Maxwell K, Johnson G. 2000. Chlorophyll fluorescence- a practical guide. J. Exp. Bot. 51: 659-668.

McKone K, Tanner C. 2009. Role of salinity in the susceptibility of eelgrass *Zostera marina* to the wasting disease pathogen *Labyrinthula zosterae*. Mar. Ecol. Prog. Ser. 377: 123-130.

McMillan C, Zapata O, Escobar L. 1980. Sulphated phenolic compounds in seagrasses. Aquat. Bot. 8: 267-278.

McMillan C. 1982. Reproductive physiology of tropical seagrasses. Aquat. Bot. 14: 245-258.

McMillan C, Moseley F. 1967. Salinity tolerances of five spermatophytes of Redfish Bay, Texas. Ecology 48: 503–506.

Millerd A, Scott K. 1962. Respiration of the diseased plant. Annu. Rev. Plant Physiol. 13: 559-574.

Muehlstein L, Porter D, Short F. 1988. *Labyrinthula* sp., a marine slime mold producing the symptoms of wasting disease in eelgrass, *Zostera marina*. Mar. Biol. 99: 465-472.

Muehlstein L. 1989. Perspectives on the wasting disease of eelgrass, *Zostera marina*. Dis. Aquat. Org. 7: 211-221.

Muehlstein L, Porter D, Short F. 1991. *Labyrinthula zosterae sp. nov.,* the causative agent of wasting disease of eelgrass, *Zostera marina*. Mycologia 83: 180-191.

Muehlstein L. 1992. The host-pathogen interaction in the wasting disease of eelgrass, *Zostera marina*. Can. J. Bot. 70: 2081-2088.

Naumann J, Young D, Anderson J. 2007. Linking leaf chlorophyll fluorescence properties to physiological responses for detection of salt and drought stress in coastal plant species. Physiol. Plant. 131: 422-433.

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9: 81-92.

Nicholson RL, Hammerschmidt R. 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30: 369-389.

Pak V, Nabiour M, Meskarbashee M. 2009. Effect of salt stress on chlorophyll content, fluorescence, Na^+ and K^+ ions content in rape plants (*Brassica napus* L.). Asian J. Agric. Res. 3: 28-37.

Parida A, Das A. 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60: 324–349.

Phillips RC. 1960. The ecology of marine plants of Crystal Bay, Florida. Quartley Journal of the Florida Academy of Science. 23: 328-337.

Porter D. 1988. Labyrinthulomycota. In Handbook of Protoctista, L. Margulis *et al.*, eds. Boston: Jones and Bartlett. p 391.

Qiu N, Lu Q, Lu C. 2003. Photosynthesis, photosystem II efficiency and the xanthophyll cycle in the salt-adapted halophyte *Atriplex centralasiatica*. New Phytol. 159: 479-486.

Raghukumar S. 2002. Ecology of the marine protists, the Labyrinthulomycetes (Thraustochytrids and Labyrinthulids). Eur. J. Protistol. 38: 127-145.

Ralph P. 1998. Photosynthetic response of *Halophila ovalis* (R.Br.) Hook. *f.* to osmotic stress. J. Exp. Mar. Biol. Ecol. 227: 203-220.

Ralph P. 1999. Photosynthetic response of *Halophila ovalis* (R.Br.) Hook. *f.* to combined environmental stress. Aquat. Bot. 65: 83-96.

Ralph P, Burchett M. 1995. Photosynthetic responses of the seagrass *Halophila ovalis* (R. Br.) Hook. *f*. to high irradiance stress, using chlorophyll *a* fluorescence. Aquat. Bot. 51: 55-66.

Ralph P, Short F. 2002. Impact of the wasting disease pathogen, *Labyrinthula zosterae*, on the photobiology of eelgrass *Zostera marina*. Mar. Ecol. Prog. Ser. 226: 265-271.

Ralph P, Gademann R. 2005. Rapid light curves: A powerful tool to assess photosynthetic activity. Aquat. Bot. 82: 222-237.

Ralph P, Polk S, Moore K, Orth R, Smith, Jr. W. 2002. Operation of the xanthophyll cycle in the seagrass *Zostera marina* in response to variable irradiance. J. Exp. Mar. Biol. Ecol. 271: 189-207.

Ralph PJ, Durako MJ, Enríquez S, Collier CJ, Doblin MA. 2007. Impact of light limitation on seagrasses. J. Exp. Mar. Biol. Ecol. 350: 176-193.

Regalado EL, Rodriguez M, Menendez R, Conception AA, Nogueiras C, Laguna A, Rodriguez AA, Williams DE, Lorenzo-Luaces P, Valdes O, Hernandez Y. 2009. Repair of UVB-damaged skin by the antioxidant sulphated flavone glycoside Thalassiolin B isolated from the marine plant *Thalassia testudinum* Banks ex Kong. Mar. Biotechnol. 11: 74-80.

Rhoades DF. 1979. Evolution of plant chemical defense against herbivores in herbivores: Their interaction with secondary plant metabolites. G.A. Rosenthal and D.H. Janzen, eds. New York: Academic Press. p. 13.

Robblee M, Barber T, Carlson, Jr. P, Durako M, Fourqurean J, Muehlstein L, Porter D, Yarbro L, Zieman R, Zieman J. 1991. Mass mortality of the tropical seagrass *Thalassia testudinum* in Florida Bay (USA). Mar. Ecol. Prog. Ser. 71: 297-299.

Robblee MB, DiDomenico WJ. 1991. Seagrass die-off threatens ecology of Florida Bay. Park Science 11:21-22.

Ross C, Puglisi MP, Paul VJ. 2008. Antifungal defenses of seagrasses from the Indian River Lagoon, Florida. Aquat. Bot. 88: 134-141.

Shetty NP, Jørgensen HJL, Jensen JD, Collinge DB, Shetty HS. 2008. Roles of reactive oxygen species in interactions between plants and pathogens. Europ. J. Plant Pathol. 121: 267–280.

Short F, Muehlstein L, Porter D. 1987. Eelgrass wasting disease: Cause and recurrence of a marine epidemic. Biol. Bull. 173: 557-562.

Short F, Carruthers T, Dennison W, Waycott M. 2007. Global seagrass distribution and diversity: A bioregional model. J. Exp. Mar. Biol. Ecol. 350: 3-20.

Silva E, Ribeiro R, Ferreira-Silva S, Viégas R, Silveira J. 2010. Comparative effects of salinity and water stress on photosynthesis, water relations and growth of *Jatropha curcas* plants. J. Arid Environ. 74: 1130-1137.

Stalikas CD. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. J. Sep. Sci. 30: 3268-3295.

Steele L, Caldwell M, Boettcher A, Arnold T. 2005. Seagrass-pathogen interactions: „pseudo-induction" of turtlegrass phenolics near wasting disease lesions. Mar. Ecol. Prog. Ser. 303: 123-131.

Sureda A, Box A, Terrados J, Deudero S, Pons A. 2008. Antioxidant response of the seagrass *Posidonia oceanica* when epiphytized by the invasive macroalgae *Lophocladia lallemandii*. Mar. Environ. Res. 66: 359-363.

Taiz L, Zeiger E. 2006. Plant Physiology. 4th Ed. Sunderland: Sinauer Associates, Inc. p. 324.

Tallarida RJ. 2002. The interaction index: a measure of drug synergism. Pain. 98: 163-168.

Tallarida RJ, Kimmel HL, Holtzman SG. 1997. Theory and statistics of detecting synergism between two active drugs: cocaine and buprenorphine. Psycopharmacology 133: 378-382.

Thayer GW, Kenworthy WJ, Fonesca MS. 1984. The ecology of eelgrass meadows of the Atlantic coast: a community profile. U.S. Fish Wildl. Serv. FWS/OBS-84/02, p. 147.

Touchette B. 2007. Seagrass-salinity interactions: Physiological mechanisms used by submersed marine angiosperms for a life at sea. J. Exp. Mar. Biol. Ecol. 350: 194-215.

Tsui C, Marshall W, Yokoyama R, Honda D, Lippmeier C, Craven K, Peterson P, Berbee M. 2009. Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. Mol. Phylogen. Evol. 5: 129-140.

van Sumere CF, Albrecht J, Dedoner A, De Pooter H, Pé I. 1975. Plant Proteins and Phenolics *in* The Chemistry and Biochemistry of Plant Proteins. New York: Academic Press, Inc. pp. 247-248.

Vergeer LHT, Develi A. Phenolic acids in healthy and infected leaves of *Zostera marina* and their growth-limiting properties towards *Labyrinthula zosterae*. Aqaut. Bot. 58: 65-72.

Vergeer L, Aarts T, de Groot J. 1995. The „wasting disease" and the effect of abiotic factors (light intensity, temperature, salinity) and infection with *Labyrinthula zosterae* on phenolic content of *Zostera marina* shoots. Aquat. Biol. 52: 35-44.

Waycott M, Duarte C, Carruthers T, Orth R, Dennison W, Olyarnik S, Calladine A, Fourqurean J, Heck K, Hughes A, Kendrick G, Kenworthy W, Short F, Williams S. 2009. Accelerating loss of seagrasses across the global threatens coastal ecosystems. Proc. Natl. Acad. Sci. U.S.A. 106: 12377-12381.

Weaver LM, Herrmann KM. 1997. Dynamics of the shikimate pathway in plants. Trends Plant Sci. 2: 346-351.

Xia J, Li Y, Zou D. 2004. Effects of salinity stress on PSII in *Ulva lactuca* as probed by chlorophyll fluorescence measurements. Aquat. Bot. 80: 129–137.

Young EL. 1938. Recent investigation on the eel-grass problem: preliminary report. Bull. Mt. Desert Isl. Biol. Lab. p. 26-28.

Young EL. 1943. Studies on *Labyrinthula*. The etiologic agent of the wasting disease of eelgrass. Am. J. Bot. 30: 586-593.

Zapata O, McMillan C. 1979. Phenolic acids in seagrass. Aquat. Bot. 7: 307-317.

Curriculum Vitae

Stacey M. Trevathan-Tackett

FUTURE INTEREST AND OBJECTIVES

In the near future, I will be completing a Masters of Science in Biology from the Coastal Biology Program at the University of North Florida (December 2011). Afterwards, I plan on entering into a PhD program at the University of Technology, Sydney in Sydney, Australia. The research will involve the effects of degradation on seagrass meadows‟ abilities to store long-term sedimentary carbon and the related effects of CO_2 emissions.

EDUCATION

Present	M.S. Biology, University of North Florida, Jacksonville, FL. Thesis Title: Physiology and Biochemistry of the Tropical Seagrass *Thalassia testudinum* in Response to Hypersalinity Stress and *Labyrinthula* sp. Infection. December 2011.
2007	B.S. Marine Science & Biology, Summa Cum Laude, Coastal Carolina University, Conway, SC. Senior Thesis Project: Food Web Development and Analysis of the Tropical Seagrass Bed Habitats of Discovery Bay, Jamaica.

RELATED PROFESSIONAL EXPERIENCE

2011- Present	Adjunct Lecturer, Department of Biology, University of North Florida. Principles of Biology & General Biology II.
2009- 2011	Graduate Research Assistant, Department of Biology, University of North Florida.
2007- 2009	Executive Secretary, National Headquarters, Wildlife Action, Inc.
	Summer Camp Instructor/Nurse, National Headquarters, Wildlife Action, Inc.
2005-2006	Teaching Workshop Leader & Tutor, Chemistry Departments, Coastal Carolina University, Conway, SC.

RESEARCH GRANTS

University of North Florida

Coastal Biology Flagship Program Travel Grant (2011) $279.00 Funded.
Dodson Grant, Department of Biology (2011) $2,000.00 Funded.
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Graduate Scholars Program Scholarly Projects Grant (2009) $500.00 Funded.

PUBLICATIONS

Trevathan, S.M., Kahn, A. and C. Ross. 2011. Effects of short-term hypersalinity exposure on the susceptibility to wasting disease in the subtropical seagrass *Thalassia testudinum*. Plant Physiology and Biochemistry 49, 1051-1058.

Trevathan-Tackett S.M., Lane, A. and C. Ross (2011) Isolation and characterization of anti-labyrinthulid secondary metabolites using bioassay-guided fractionation methodology. (In Preparation for *Phytochemistry*).

PRESENTATIONS

Trevathan-Tackett, S.M., Kahn, A. and C. Ross. Short term impacts of elevated salinity and infection on the physiology and biochemistry of the seagrass. 21st Biennial Conference of the Coastal and Estuarine Research Federation. Poster Presentation. November 2011.

Trevathan-Tackett, S.M., Lane, A. and C. Ross. Secondary metabolites produced by the seagrass *Thalassia testudinum* exhibit anti-labyrinthulid properties. 33rd Annual Southeastern Phycological Colloquy, Miami, Florida. Oral Presentation. October 2011.

Trevathan, S.M., Kahn, A. and C. Ross. Effects of elevated salinity on the health of the seagrass *Thalassia testudinum* and its susceptibility to wasting disease. 40th Annual Benthic Ecology Meeting, Mobile, Alabama. Oral Presentation. March 2011.

Trevathan, S.M., Kahn, A. and C. Ross. Seagrass Wasting Disease: Physiological Impacts of Environmental Stressors. Southeastern Estuarine Research Society Conference, St. Augustine, Florida. Poster Presentation. November 2010.

Trevathan, S.M., Kahn, A. and C. Ross. Elevated Salinity and Wasting Disease as Stressors on seagrass *Thalassia testudinum*. Scholars Transforming Academic Research Symposium, Jacksonville, Florida. Poster Presentation. April 2010.

Trevathan, S.M., Kahn, A. and C. Ross. Physiological responses of the seagrass *Thalassia testudinum* against the causative agent of wasting disease, *Labyrinthula* sp. 39th Annual Benthic Ecology Meeting, Wilmington, North Carolina. Poster Presentation. March 2010.

RESEARCH EXPEDITIONS- Marine Ecology, Collection and Monitoring

2011 *R/V Bellows*
Assessed the health of 6 seagrass beds in Key West, Dry Tortugas, and Marquesas, Florida, USA and how it was related to incidence of "wasting disease."

2010 NOAA/DEP Key West, FL
Helped with coral tissue collection and seagrass monitoring.

2010 Mote Marine Laboratory, Summerland Key, FL
Collected *Thalassia testudinum* samples and isolated *Labyrinthula* spp. seagrass blades. Performed assays on healthy *Thalassia testudinum* samples to determine *Labyrinthula* pathogenicity.

2010 Keys Marine Laboratory, Long Key, FL
Collected *Thalassia testudinum* biomass for biochemical analysis and surveyed *T. testudinum* for virulent forms of *Labyrinthula* spp.

2009 *R/V Bellows*
Surveyed areas of Key West, Dry Tortugas, Marquesas for "wasting disease" associated with seagrasses.

ACADEMIC & PROFESSIONAL HONORS

Dr. Nancy Foster Scholarship Program, National Oceanic and Atmospheric Administration (2009-2011)

Scholarship Recipient NA09SEC4810016
One of seven scholarships given to graduate students for outstanding scholarship with the goal to encourage independent graduate level research -- particularly by female and minority students -- in oceanography, marine biology and maritime archaeology.

Wildlife Action, Inc.

President Award (2008)
Awarded by the National President of the grassroots conservation organization for those who have gone beyond what is expected in a volunteer, non-profit organization. Four have been given in 31 years.

Coastal Carolina University

President‟s Award (2007)
Awarded to graduates for attaining the highest cumulative grade point average in the graduating class.

SERVICE ACTIVITIES

Wildlife Action, Inc., National Headquarters, Ambassador (2008-2009)

- Attended Chamber of Commerce, county council, various government, and community meetings.
- Presented information about wildlife, conservation, and Wildlife Action at expos, to clubs, and to organizations in the community.

Wildlife Action, Inc., Carolina Forest Chapter, Secretary& Treasurer (2007-2009)

- Fulfilled the duties of the local chapter‟s secretary and treasurer.
- Coordinated activities such as the monthly meeting, canoe trips, and school and Earth Day events;
- Acted as the chapter‟s Director of Land Management of the 480-acre wetland the chapter utilizes and stewards.
- Worked closely with local and state government agencies and departments & headed and conducted meetings with said entities

SKILLS

Working knowledge of the following instruments & methodology:

- Plant physiology (Pulse Amplitude Modulated Fluorometry/ DIVING-PAM; respiration; pigment extraction and analysis).
- Reverse Phase- High Performance Liquid Chromatography
- Rotary Evaporator/ Distillation
- Fluorescence/light microscopy
- Sterile technique
- Histological techniques

ORGANIZATIONS- Membership

Sigma Xi- Scientific Research Society
November 2009- Present
Wildlife Action, Inc. - Non-profit Conservation Organization
Life Member since 2007
Omicron Delta Kappa- Leadership
December 2005- May 2007