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# The Effects of *Labyrinthula sp.* Infection, Salinity, and Light on the Production of Phenolic Compounds in *Thalassia testudinum*

by

Jennifer M. Sneed

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology
College of Arts and Sciences
University of South Florida

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Table of Contents

List of Tables ........................................................................................iv

List of Figures ........................................................................................v

Abstract ........................................................................................vii

Introduction ........................................................................................1
*Labyrinthula* ........................................................................................2
The Induction of Phenolic Defenses ........................................................................................4
Hypotheses ........................................................................................7
Question 1a (Field Collection) ........................................................................................7
Question 1b (Laboratory Perturbation) ........................................................................................8
Question 2 ........................................................................................8

Experimental Design ........................................................................................9
Determining the Possible Correlation of Phenolic Production with *Labyrinthula sp.* Infection in *Thalassia testudinum* ........................................................................................9
Determining the Effect of *Labyrinthula sp.* Infection on the Phenolic Content in *Thalassia testudinum* ........................................................................................9
Determining the Effects of Salinity and Light on the Ability of *T. testudinum* to Produce Phenolics in Response to Infection by *Labyrinthula sp.* ........................................................................................10

Methodology ........................................................................................13
Seagrass Collection ........................................................................................13
Laboratory Seagrass Cultures ........................................................................................14
Preparation of Infected and Healthy Leaf Blade Sections ........................................................................................15
Harvesting of Samples ........................................................................................15
Extraction of Phenolics ........................................................................................16
Extraction for HPLC Analysis ........................................................................................16
Extraction for the Folin-Denis Assay ........................................................................................17
Analysis of Phenolic Acids: HPCL Analysis ........................................................................................17
Analysis of Total Phenolics: Folin-Denis Assay ........................................................................................21
Labyrinthula Cultures ........................................................................................21
Preparation of Agar Plates ........................................................................................21
Inoculation of Plates ........................................................................................22
Statistical Analysis ........................................................................................23

Results ....................................................................................................24
Effects of *Labyrinthula sp*. Infection of Phenolic Content in Field Samples of *Thalassia testudinum*....................................................................24
Total Phenolics....................................................................................24
Individual Phenolic Acids....................................................................25
Effects of *Labyrinthula sp*. Infection on the Phenolic Content of *Thalassia testudinum* Three, Four, and Seven Days Post Infection ..............................28
Total Phenolics....................................................................................28
Individual Phenolic Acids....................................................................29
Effects of *Labyrinthula sp*. Infection and Light Level on the Phenolic Content of *Thalassia testudinum*...................................................................31
Total Phenolics....................................................................................31
Individual Phenolic Acids....................................................................32
Effects of *Labyrinthula sp*. Infection and Salinity on the Phenolic Content of *Thalassia testudinum*....................................................................34
Total Phenolics....................................................................................34
Individual Phenolic Acids....................................................................35
Combination of Time Data with Salinity and Light Data ...................................38
Salinity....................................................................................................38
Light.......................................................................................................42

Discussion ...................................................................................................43
Induction of Phenolic Compounds in *Thalassia testudinum* in Response to *Labyrinthula sp*. Infection ........................................................................44
Field Study.............................................................................................44
Laboratory Perturbation........................................................................45
Effects of Environmental Stress on the Induction of Phenolic Compounds .........47
Light.......................................................................................................47
Salinity....................................................................................................49
Future Research ..........................................................................................50
Conclusions .................................................................................................51

Literature Cited ............................................................................................52

Appendices ...................................................................................................56
Appendix 1: HPLC chromatogram of gentisic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, and ferulic acid standards. ..........................................................................................56
Appendix 2: HPLC chromatogram of *Thalassia testudinum* extract...................57
Appendix 3: Univariate ANOVA: The effects of *Labyrinthula sp.* infection on the total phenolic content in field samples of *Thalassia testudinum*...........................................................................................58
Appendix 4: Multivariate ANOVA: The effects of *Labyrinthula sp.* infection on individual phenolic acid content in field samples of *Thalassia testudinum*.........................................................................59

Appendix 5: Univariate ANOVA: Effects of *Labyrinthula sp.* infection on the total phenolic content of *Thalassia testudinum* three, four, and seven days post infection. ........ 60
Appendix 6: Multivariate ANOVA: Effects of *Labyrinthula sp.* infection on individual phenolic acid content of *Thalassia testudinum* three, four, and seven days post infection. ........ 61
Appendix 7: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the total phenolic content of *Thalassia testudinum* ........ 62
Appendix 8: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the individual phenolic acid content of *Thalassia testudinum* ........ 63
Appendix 9: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the total phenolic content of *Thalassia testudinum* ........ 64
Appendix 10: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the individual phenolic acid content of *Thalassia testudinum* ........ 65
Appendix 11: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the total phenolic content of *Thalassia testudinum* with the addition of previous data ........ 66
Appendix 12: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the individual phenolic acid content of *Thalassia testudinum* with the addition of previous data ........ 67
Appendix 13: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the total phenolic content of *Thalassia testudinum* with the addition of previous data ........ 68
Appendix 14: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the individual phenolic acid content of *Thalassia testudinum* with the addition of previous data ........ 69

List of Tables


Table 1. Equations of standard curves used for HPLC quantification of phenolic acids. ........ 19

Table 2. The percent recovery of phenolic acids when subjected to either acid hydrolysis (HCl) or alkaline hydrolysis (NaOH) prior to extraction. ........ 20

Table 3. Means in mg/gdrwt of the six phenolic acids extracted from infected and healthy *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. Means that show a significant effect of infection according to ANOVA are indicated by an asterisk. ........ 25

Table 4. Mean total phenolics in mg/gdrwt for healthy and infected plants sampled after three, four, and seven days. ........ 29

List of Figures


Figure 1. Total phenolics extracted from infected and healthy *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. ........ 24

Figure 2. The amount of protocatechuic acid extracted from healthy and infected plants collected at the south end of the Sunshine Skyway Bridge. ........ 26

Figure 3. The amount of parahydroxybenzoic acid (PHBA) extracted from healthy and infected plants collected at the south end of the Sunshine Skyway Bridge. ........ 26

Figure 4. The amount of vanillic acid extracted from healthy and infected plants collected at the south end of the Sunshine Skyway Bridge. ........ 27

Figure 5. The amount of caffeic acid extracted from healthy and infected plants collected at the south end of the Sunshine Skyway Bridge. ........ 27

Figure 6. Total phenolics extracted from healthy and infected plants sampled three, four, and seven days after clamping........ 29

Figure 7. Caffeic acid extracted from healthy and infected plants sampled three, four, and seven days after clamping. ........ 30

Figure 8. Vanillic acid extracted from healthy and infected plants sampled three, four, and seven days after clamping. ........ 31

Figure 9. Total phenolics extracted from healthy and infected plants grown under either high or low light conditions. ........ 32

Figure 10. Protocatechuic acid extracted from healthy and infected plants grown under either high or low light conditions........ 33

Figure 11. Caffeic acid extracted from healthy and infected plants grown under either high or low light conditions. ........ 33

Figure 12. Ferulic acid extracted from healthy and infected plants grown under either high or low light conditions........ 34

Figure 13. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity. ......35

Figure 14. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity. ......36

Figure 15. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity. ......36

Figure 16. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity. ......37

Figure 17. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity. ......37

Figure 18. Total phenolics extracted from healthy and infected plants grown at 25 and 35 ppt salinity, including previously collected data. ......39

Figure 19. Parahydroxybenzoic acid (PHBA) extracted from healthy and infected plants grown at 25 and 35 ppt salinity, including previously collected data. ......40

Figure 20. Vanillic acid extracted from healthy and infected plants grown at 25 and 35 ppt salinity, including previously collected data......41

Figure 21. Caffeic acid extracted from healthy and infected plants grown at 25 and 35 ppt salinity, including previously collected data......41

Figure 22. Caffeic acid extracted from healthy and infected plants grown under high and low light conditions, including previously collected data. ......42

Figure 23. Total phenolics extracted from healthy and infected plants grown under high and low light conditions, including previously collected data......48

The Effects of *Labyrinthula sp* Infection, Salinity, and Light on the Production of Phenolic Compounds in *Thalassia testudinum*.

Jennifer M. Sneed

ABSTRACT

In the fall of 1987, several areas of Florida Bay were severely affected by the sudden die-off of the seagrass *Thalassia testudinum* Banks ex Konig (turtle grass). Although the cause is still unknown, several factors were suggested as influencing the on-set of the die-off event including increased salinity, light stress due to self-shading, and disease. Blades of seagrass plants found in the area of die-off were infected by *Labyrinthula sp*, a pathogenic protist. A similar die-off occurred in another species of seagrass, *Zostera marina*, in the 1930's that was attributed to the pathogenic protist, *Labyrinthula zosterae*. *Zostera marina* produces inhibitory phenolic acids in response to infection by *L. zosterae*, a response that is diminished in plants exposed to low light and high temperature.

This study examined the differences in phenolic content of healthy and infected *T. testudinum* leaf blades in laboratory cultures to determine if *T. testudinum* produces a chemical defense against pathogens similar to that of *Z. marina.* The possible increased susceptibility of turtle grass to *Labyrinthula sp.* infection under high salinity and low light was also examined.

In culture, infection by *Labyrinthula sp.* induced a rapid, short-term production of total phenolics in *Thalassia testudinum* under normal, non-stressed conditions. The initial induction was followed by a sharp decline. The production of individual phenolic acids was not induced by infection. In contrast, the production of caffeic acid was inhibited by infection.

Environmental stress (low salinity and low light) caused a decrease in both total phenolics and several phenolic acids. Levels of PHBA, vanillic acid, and caffeic acid

decreased in low salinity (25ppt) treatments, and caffeic acid decreased in response to low light stress. There was an interaction between stress and infection that resulted in higher levels of phenolics in plants exposed to infection and stress compared to those exposed to stress alone. In culture, plants did not survive exposure to high salinity (45ppt) similar to that found in Florida Bay during the die-off event

# Introduction

Seagrass beds are important marine communities both ecologically and economically (Zieman, 1982). They act as nurseries and habitats for many ecologically and economically important species of fishes and invertebrates (Zieman, 1982). They also stabilize sediment, and filter estuarine waters (Zieman, 1982). *Thalassia testudinum* Banks ex Konig is the dominant seagrass found in subtropical/tropical estuaries in Florida and the Caribbean (Dawes, 1998).

Beginning in the fall of 1987, several areas of Florida Bay were severely affected by the sudden die-off of the seagrass *Thalassia testudinum* (turtle grass) (Robblee et al., 1991). Between 1987 and 1991 more than 4,000 ha had been completely denuded of *T. testudinum*, while another 23,000 ha had been affected (Robblee et al., 1991). Since 1991, seagrass beds in Florida Bay have continued to experience a decline in both density and biomass (Zieman, et al., 1999). This extensive die-off event was reminiscent of the widespread die-off of the seagrass *Zostera marina* (eelgrass) along the eastern coast of the United States and parts of Europe in the 1930's. The *Z. marina* die-off was attributed to the wasting disease, *Labyrinthula zosterae* a relative of which has been proposed to be involved in the die-off of *T. testudinum* in Florida Bay (Muehlstein et al., 1988; Robblee et al., 1991).

Although the long-term effects of the die-off are unknown, Thayer et al. (1994) proposed that it is unlikely to cause a permanent change in the seagrass composition of

the system. After periods of die-off, it is likely that the barren patches will recover relatively quickly due to rhizomatous growth of *T. testudinum* into the bare patches from contiguous beds (Thayer et al., 1994). However, the removal of large patches of *Thalassia* could result in: changes in bacterial densities, heterotrophic activity, dissolved organic carbon and nitrogen levels, algal blooms, increased turbidity, decreased light penetration, and resuspension of carbonate sediment (Durako, 1994).

Unlike the slow depletion of seagrass beds associated with eutrophication, the die-off in Florida Bay happened suddenly and occurred in areas far from pollution sources indicating that it was not a direct result of anthropogenic influence (Robblee et al., 1991). Although the cause is still unknown, several factors have been suggested to influence the on-set of the die-off event including increased salinity, light stress due to self-shading, increased water temperature, sulfide toxicity, and disease (Robblee et al., 1991). The die-off occurred in contiguous patches of dense vegetation, which lends merit to the possibility of a transmittable disease as a causative agent (Durako and Kuss, 1994).

## ***Labyrinthula***

The blades of seagrass plants found in the die-off areas exhibited black lesions typical of infection by *Labyrinthula sp*, a saprotrophic protist. *Labyrinthula* is the only genus in the monotypic family Labyrinthulaceae. After having been mistakenly classified in several phyla (Rhizpoda, Foraminifera, Mycetozoa, Chrysophyta, Gymnomyxa), labyrinthulids have been placed in an exclusive phylum Labyrinthulomycota along with thraustochytrids (previously placed in the phylum Oomycota) (Porter, 1990). *Labyrinthula* species are characterized by their spindle-

shaped cells that move along a membrane bound ectoplasmic network (Porter, 1990). Species of *Labyrinthula* have been isolated from a wide range of marine organisms including macroalgae, marine vascular plants and crustaceans as well as from marine sediment. It is found in nearly all species of seagrass and is thought to be specific to genera (Vergeer and den Hartog, 1993). Although the species found infecting *T. testudinum* has not yet been identified, the pathogen is a relative of *L. zosterae*, the causative agent of eelgrass wasting disease (Muehlstein et al., 1991; Short et al., 1987). *L. zosterae* has been credited with causing the massive die-off of *Z. marina* (eelgrass) in the 1930's (Muehlstein et al., 1991, Short et al., 1987). Under normal conditions, *L. zosterae* is restricted to the oldest leaves of the plant and can be relatively harmless (Vergeer and den Hartog, 1994). However, the pathogen can take over even the youngest leaves in diseased plants and cause considerable damage (Vergeer and den Hartog, 1994). According to Durako and Kuss (1994), infection by *L. sp.* decreases the photosynthetic capacity of *T. testudinum*, which can lead to hypoxia and sulfide toxicity, and the eventual death of the plant.

In a study of three areas in the Gulf of Mexico (Tampa Bay, Florida Bay, and the eastern edge of the Gulf of Mexico) Blakesley et al. (2002) found that *L. sp.* plays one of three roles depending on the condition of the seagrass community. This role is determined by five factors: salinity, seagrass density, pathogenicity of the strain of *L. sp.*, environmental stress, and the individual plant's resistance to disease (Blakesley et al., 2002). *L. sp.* thrives in areas of moderate to high salinity and does not survive well in salinities lower than 15ppt (Blakesley et al., 2002). Areas of high salinity and high seagrass density are optimal for this protist because the infection spreads throughout the

seagrass bed by blade-to-blade contact (Burdick et al., 1993). In a bed where salinity and density are high, but environmental stress is low, the disease acts as a "primary pathogen," causing only patchy die-off (Blakesley et al., 2002). When high salinity and high density are combined with high levels of environmental stress, the bed experiences more extensive die-off, similar to that seen in Florida Bay between 1987 and 1991 (Blakesley et al., 2002). In this situation, the *L. sp.* acts as a "secondary opportunistic pathogen." The activity of *L. sp.* is also affected by seasonality and is rarely found in Florida Bay between the months of January and July. It begins to be prevalent in early fall (September) and is at its peak in November (Blakesley et al., 2002). Further north along the western Gulf of Mexico coast the disease is active between April and September (Bowles and Bell, 2004).

**The Induction of Phenolic Defenses**

Plants often produce defensive secondary metabolites in response to both predators and diseases such as *Labyrinthula sp.* (McKey, 1979). It has been known for nearly a century that plants produce chemicals that enhance their resistance to both disease and herbivory (Harborne, 1993). In 1941, Muller and Borger introduced the idea that production of these chemicals could be induced at the time of attack (Harborne, 1993). Although there are examples of induced compounds from nearly every class of secondary metabolites, phenolic compounds are especially prevalent (McKey, 1979). Phenolics are aromatic compounds that are derived from the combination of three metabolic sections including the shikimate pathway section, phenylpropanoid section, and flavanoid section (Hrazdina, 1992). Their complex biosynthesis begins with the

formation of aromatic amino acids by the shikimate pathway (Hrazdina, 1991). Phenylalanine from the shikimate pathway then undergoes deamination via the enzyme phenylalanine ammonia-lyase (PAL) in the first step of the phenylpropanoid section of phenolic metabolism (Hrazdina, 1992). The phenylpropanoid section produces the cinnamic acids, the coumarins, and lignins (Hrazdina, 1992). P-coumaryl-CoA from the phenylpropanoid section is shuttled to the flavanoid section of phenolic metabolism giving rise to a diverse array of flavanoid compounds (Hrazdina, 1992). Because this complex pathway requires energetic input, the ability of an organism to induce the process only when needed has obvious benefits (Rhoades, 1979). Dyer et al. (1989) demonstrated that synthesis of the first enzyme in the shikimate pathway, 3-deoxyarabinoheptulosonate phosphate synthase (DAHP synthase), is induced by wounding in both potatoes and tomatoes. They also found an increase in PAL activity in response to wounding (Dyer et al., 1989). Both of these enzymes are critical in the initiation of phenolic biosynthesis (Hrazdina, 1992).

Although the physiological effects of phenolics are not well understood, there are a number of theories as to the mechanisms of deterrence for both herbivores and pathogens. Phenolic compounds are known to bind proteins, and therefore, may reduce the fitness of the attacker by making food indigestible (Swain, 1979). They are also responsible for the strengthening of cell walls (lignin, tannins) that makes it difficult for microbes to enter the cell and do damage (Swain, 1979). Because they are found universally in higher plants, phenolics are often investigated for their defensive roles. Also, as stated above, their biosynthetic pathways are well known and undergo induction in response to wounding and pathogens.

According to the Optimal Defense hypothesis proposed by Rhoades (1979), the production of secondary metabolites is energetically costly and, therefore, often suffers when a plant is required to shift the allocation of its resources in response to stress. In the case of the die-off of *T. testudinum* in Florida Bay, a number of abiotic factors changed preceding and during the die-off period, which may have caused a significant stress on the plants weakening their natural defense against disease. High salinity decreases the production of phenolics in terrestrial plants (Dunn et al., 1998). The salinity of Florida Bay was extremely high during the years of the die-off, rising in some parts to a high of 59 ppt in 1990 (Zieman et al., 1999). The average salinity of Florida Bay in 1990 was 46 ppt, which is well above the optimal salinity for healthy *T. testudinum* (Zieman et al., 1999; Berns, 2003; Lirman and Cropper, 2003). Light stress has also been implicated in the die-off because it occurred exclusively in dense seagrass beds that exhibit self-shading (Robblee et al., 1991).

The effects of stress on secondary metabolite production remains a controversial topic and the Optimal Defense hypothesis is only one of several theories. The Resource Availability and Carbon-Nutrient Balance hypotheses argue that the production of carbon based secondary metabolites, such as phenolics, increase in response to low nutrient availability due to an excess of carbon in relation to the amount of nitrogen present (Bryant et al, 1983; Bryant et al., 1985; Coley et al, 1985). The Growth-Differentiation Balance hypothesis extends this idea to include not only nutrient depletion, but also any stress that decreases growth while leaving photosynthetic capacity unaffected (Herms and Mattson, 1992).

Vergeer et al. (1995) found that infection by *L. zosterae* induces the production of phenolics in *Zostera marina*. Caffeic acid showed a significant increase in response to infection and also inhibited the growth of *L. zosterae* cultures (Vergeer and Develi, 1997). The production of phenolics by *Z. marina* was reduced in plants exposed to both low light and high temperature stress (Vergeer et al., 1995).

## Hypotheses

In this project, I hypothesized that infection by *Labyrinthula sp* would induce the production of phenolics in *Thalassia testudinum* similar to that seen in *Zostera marina*. I expected that *T. testudinum* samples exposed to *L. sp.,* both in the lab and in the field, would have higher levels of phenolics than those that were not exposed to *L. sp.*

Based on the Optimal Defense hypothesis, I further hypothesized that environmental stress, specifically light and salinity stress, would inhibit the induction of phenolics in response to infection (Rhoades, 1979).

### Question 1a (Field Collection).

Is infection by *Labyrinthula sp*. correlated with the increased production of phenolics in *Thalassia testudinum*?

- $H_{O1}$: Infected leaf blades of *Thalassia testu*dinum with *Labyrinthula sp*. have the same phenolic content as healthy leaf blades.
- $H_{A1}$: Infected leaf blades of *Thalassia testudinum* with *Labyrinthula sp*. have a greater concentration of phenolics than healthy leaf blades.

**Question 1b (Laboratory Perturbation).**

Does infection by *Labyrinthula sp*. induce the production of phenolics in *Thalassia testudinum*?

- $H_{O2}$: Infection by *Labyrinthula sp*. has no effect on the phenolic content of *Thalassia testudinum* leaf blades.
- $H_{A2}$: Infection by *Labyrinthula sp*. will cause an increase in the phenolic content of *Thalassia testudinum*.

**Question 2**

Does salinity or light affect the ability of *T. testudinum* to produce phenolics in response to infection?

- $H_{03}$: Salinity has no effect on the phenolic content of *T. testudinum* after infection by *Labyrinthula sp.*
- $H_{A3}$: Increase or decrease in salinity decreases the amount of phenolics produced in response to infection by *Labyrinthula sp.*
- $H_{04}$: The amount of light has no effect on the phenolic content of *T. testudinum* after infection by *Labyrinthula sp.*
- $H_{A4}$: Decrease in light decreases the amount of phenolics produced in response to infection by *Labyrinthula sp.*

## Experimental Design

### **Determining the Possible Correlation of Phenolic Production with *Labyrinthula Sp.* Infection in *Thalassia Testudinum***

Five short shoots with obvious *Labyrinthula sp*. infection on the third leaf and five short shoots without such symptoms were collected as described below. Only the third oldest short shoot from the rhizome apical meristem was used. Presence of *L. sp.* infection was determined visually by the presence of characteristic black lesions on the leaf blade. The third oldest leaf of each short shoot was separated from the short shoot, cleaned, and analyzed for phenolic content as described below. Phenolic content may vary between old and young leaves (Hillis, 1956, Sheen, 1969; Feeny, 1970; Rhoades and Cates, 1976; Gartlan et al., 1980). Sampling leaves of approximately the same age controls for differences in phenolic content caused by age. Each leaf blade was extracted and analyzed using the procedure originally outlined by Murphy and Stutte (1978) and later modified by Vergeer and Develi (1997) (see below). The extracts were analyzed to determine both the identity and quantity of individual phenolics present by using HPLC, as well as total phenolics by using the Folin-Denis assay (Mole and Waterman, 1987).

### **Determining the Effect of *Labyrinthula Sp.* Infection on the Phenolic Content in *Thalassia Testudinum*.**

The third and fourth oldest short shoots of sixty-three healthy plants were collected on February 21, 2004 off the coast of Islamorada, FL. Plants were transplanted

into six aquariums on February 22, 2004 (5 plants/aquarium). The third leaf of the third short shoot was used for all procedures. The plants were allowed to acclimate to the aquariums for twenty-nine days prior to treatment. After twenty-nine days, the third leaf of five short shoots in each of three aquariums were artificially infected by *L. sp*. by clamping a small section of an infected leaf blade to the healthy leaf with a small (1 cm) section of plastic Tygon tubing (1cm diameter) (Renn, 1936; Muehlstein et al., 1988). The third leaf of five short shoots in the other three aquariums had a small section of a healthy leaf blade clamped to them. Preparation of infected and healthy leaf blade sections is described below.

In order to determine whether or not infection by *L. sp.* induces the production of phenolics, five of the treated leaves and five of the control leaves were sampled 3 , 4, and 7 days after attachment of the leaf sections. Symptoms of *L. sp.* infection appear after a minimum of 72 hours after contact with *T. testudinum* (Blakesly, personal communication). If induction of phenolics is occurring, an increase in phenolic content was expected shortly after infection. The leaf blades were examined for the presence or absence of black lesions and then extracted and analyzed as stated above.

**Determining the Effects of Salinity and Light on the Ability of *T. Testudinum* to Produce Phenolics in Response to Infection by *Labyrinthula Sp.***

The third and fourth oldest short shoots of thirty healthy plants were collected on November 23, 2004 at the south end of the Sunshine Skyway Bridge, Tampa Bay, FL. The short shoots were transplanted into six aquariums (5 plants/aquarium). Collection protocol and aquarium conditions are described below. Each aquarium was filled with a combination of artificial seawater and natural seawater in an approximate 3:1 ratio and

adjusted to 35ppt (described below). The optimum salinity for growth and survivorship of *Thalassia testudinum* is between 30ppt and 40ppt (Berns, 2003; Lirman and Cropper, 2003). After approximately three weeks, the salinity in two tanks was adjusted to 25ppt, two at 35ppt, and two at 45ppt by adding distilled water for dilution or allowing evaporation to increase the salinity.

The transplanted plants were allowed to grow for approximately four weeks in the aquaria after the adjustment to experimental salinities. According to Dawes (1998), *T. testudinum* has a leaf turnover rate of 10-20 days; therefore, four weeks should be adequate time for the change in salinity to have an effect. After four weeks, *L. sp*. infected leaf sections were clamped to the third leaf of five short shoots in one of the aquarium in each salinity treatment. The remaining three aquariums had healthy sections of leaf blades clamped to the third leaf of five short shoots. After one week the treated and control leaves were sampled, extracted, and analyzed for phenolic content as described below.

The third and fourth oldest short shoots of twenty healthy plants were collected and transplanted into four aquariums (5 plants/aquarium). The salinity in all tanks was maintained at 35ppt. Two aquariums were exposed to high light (450 µmol photons/$m^2$/s at the surface of the water and 250 µmol photons/$m^2$/s at the surface of the sediment) and two to low light (200 µmol/$m^2$/s and 100 µmol/$m^2$/s respectively) for four weeks. Light was decreased in the low light treatment by covering the aquariums with a mesh shade cloth. Seagrass are shade plants and have a saturation irradiance of less than 200 µmol photon/$m^2$/s (Dawes, 1998). After four weeks, the plants in two aquariums (one in each treatment) were inoculated with *L. sp*. as described above. The plants in the other two

aquariums were clamped with healthy leaf section as described above. Again, the leaves were sampled after one week and analyzed for phenolic content.

## Methodology

**Seagrass Collection**

Healthy field samples were collected on December 16, 2003 and *L. sp.* infected field samples were collected on July 23, 2003 at the south end of the Sunshine Skyway Bridge, Tampa Bay, FL. During the summer when *L. sp.* infected samples were collected the infection was ubiquitous throughout the area, therefore, healthy samples had to be collected in the winter, when the disease was no longer present in the area. Infection was verified by visually identifying characteristic lesions and by identifying *L. sp.* cells in the leaves using a light microscope at 400X magnification. Plants used during the induction experiment were collected on February 21, 2004 off the coast of Islamorada, FL. Plants used during the light and salinity experiments were collected on November 23, 2004 at the south end of the Sunshine Skyway Bridge, Tampa Bay, FL.

The growing end of the rhizome was located by finding lines of *T. testudinum* growing away from the seagrass beds into bare patches. The sand was then fanned away from the rhizome with a back and forth hand motion. Once the rhizome was exposed, the rhizome apical meristem was identified, and the rhizome was broken between the fourth and fifth short shoot counting from the apical meristem. Roots were either broken by gently running the hand underneath the rhizome or pulled up with the rhizome. The rhizome was again broken between the second and third short shoots leaving a two short shoot section of rhizome containing the third and fourth short shoot. Plants were transported to the lab in a cooler filled with seawater collected on site.

**Laboratory Seagrass Cultures**

Ten-gallon aquariums were set up in an environmental growth chamber. Each tank contained approximately 12.5 lb of sand and 2.5 gallons of seawater collected near the rock jetty on the west side of the south end of the Sunshine Skyway Bridge, Tampa Bay, FL. Water was collected in 5 gallon carboys and kept in a cold room (4ºC) for at least three days prior to use. The remaining volume was filled with artificial seawater prepared by combining distilled water and Instant Ocean. Water in the aquariums was aerated continuously using airstones and filtered with 10 – 20 gallon capacity filters. Activated carbon was not used in the filters. The temperature in the growth chamber was set to 24ºC with 60% humidity. The lights were on a 12 hr light/dark cycle. Irradiance was measured using a LiCor Quantum/Radiometer/Photometer Model LI-185A with a LiCor LI-190S detector at both the surface level of the water and the surface level of the sediment. The irradiances were 450 µmol photons/$m^2$/s and 250 µmol photons/$m^2$/s, respectively. The salinity in the tanks was monitored using a Reichert 0-30 BRIX Refractometer and adjusted every two to three days by adding distilled water to each aquarium. The tanks were allowed to acclimate under these conditions for at least three days prior to the introduction of seagrasses. The third and fourth short shoots of each plant were collected as described above and transplanted into the laboratory aquariums within 24 hours of collection. Each aquarium contained five to seven plants.

**Preparation of Infected and Healthy Leaf Blade Sections**

Leaf blades were cut into 1cm long sections using a razor blade. Infected leaf blade sections were prepared by first autoclaving them for 15 minutes at 121ºC in a flask of 35ppt seawater collected from the aquariums and then placing them on plated cultures of *Labyrinthula*. Leaf blade sections were left on the *Labyrinthula* inoculated plates for at least seven days prior to use. Leaf blade sections were examined on the agar plate under the dissecting microscope to determine whether *Labyrinthula* cells were growing out from the leaf sections, away from the original culture. Several leaf blade sections were transferred to sterile SSA plates and examined for subsequent *Labyrinthula* growth to verify that transfer was occurring from the plated *Labyrinthula* culture to the leaf blade sections. Leaf blade sections were handled using forceps that were sterilized by placing them in isopropanol and then flaming them over a Bunsen burner. The lab bench was wiped clean with isopropanol prior to any manipulations of *Labyrinthula* cultures.

Healthy leaf blade sections were prepared by autoclaving them as described above and were stored in the sealed autoclaved container until use.

**Harvesting of Samples**

Sample leaves were cut from the plant at the base with a razor blade. They were rinsed in distilled water and set out to air dry until leaves appeared to curl up at the sides. While drying, general coloration of each sample was observed and they were visually examined for presence of *Labyrinthula* lesions. Any discoloration was noted as well as any apparent mechanical damage to the leaves. Leaves were then cut into small pieces with a scissors and placed into a BlueMax Jr. 15 ml polypropylene conical tube. Holes

were punched in the lid using a BD PrecisionGlide 20G1syringe needle to allow for removal of water during the freeze-drying process. Samples (inside the sample containers) were placed in a Styrofoam cooler with dry ice and then transferred to the freeze-drier once frozen.

## Extraction of Phenolics

In preparation for extraction the leaf blades were freeze-dried overnight using in a LABCONCO Freeze Dry System/Freezone 4.5 and then ground in liquid nitrogen using a mortar and pestle. Each sample was freeze-dried again for an hour, weighed, and separated into two portions (one for HPLC and one for Folin-Denis). The dried leaf material was stored in the freezer at –4ºC until further use.

### Extraction for HPLC Analysis

The major phenolics from *Thalassia testudinum* leaf blades were extracted using the method developed by Murphy and Stutte (1978) and later modified by Vergeer and Develi (1997). Dried plant material was homogenized in 5 ml of 2% acetic acid in a test tube in a boiling water bath for 10 minutes. The resulting solution was centrifuged in a Damon/ IEC Division IEC Clinical Centrifuge set at 5 for 10 minutes and the supernatant decanted. The homogenized material was hydrolyzed for one hour by adding 5 ml of 1 N HCl to the homogenized plant material and placing the test tube in a beaker of boiling water on a hot plate. Test tubes were covered with marbles to prevent evaporation loss during both homogenization and hydrolysis. Finally the solution was extracted three

times in ether and the extract was dried under nitrogen in an Organomation N-EVAP Analytic Evaporator.

Extracts were redissolved in 450 µl of butanol:methanol:acetic acid:water (1:5:2:92). Fifty µl of an internal standard (1 x $10^{-4}$M salicylic acid) were added to each sample prior to analysis.

### Extraction for the Folin-Denis Assay

Acetone (50%) was added to dried plant material and vortexed every 10 minutes for an hour. The samples were filtered through Whatman 4 filter paper and rinsed with 70% acetone. Five ml of ether were added to the acetone filtrate, vortexed, and discarded. The remaining acetone solution was dried in the freeze-drier.

## Analysis of Phenolic Acids: HPLC Analysis

Extracts were analyzed for both the quality and quantity of phenolics present using high-performance liquid chromatography (HPLC). A representative chromatogram is included in the appendix (Appendix 1). All HPLC analyses were performed using a Beckman System Gold at room temperature. Separation was performed on series of two C-18 reverse phase columns, a 100mm long Varian Microsorb 100-3 C18 and a 150mm long Grace/Vydac with a 5µm particle size. Samples were injected automatically by a Beckman System Gold auto sampler using a 100µl sample loop. The samples were separated isocratically for 13 minutes in an initial solvent of butanol:methanol:acetic acid:water (1:5:2:92) in 1.8X$10^{-2}$M ammonium acetate. This was followed by a 15-minute linear gradient separation ending with the final solvent, butanol:methanol:acetic

acid:water (2.5:12.5:2:83) in $1.8X10^{-2}M$ ammonium acetate. The separation continued in 100 % of the final solvent for 2 minutes. The column was equilibrated in the original solvent for 23 minutes between samples. Flow rate was set to 0.8 ml/minute. Peaks were detected using a Beckman 166 Ultraviolet (UV) Detector at a wavelength of 254nm.

According to Zapata and McMillan (1979), *Thalassia testudinum* contains seven phenolic acids: caffeic, ferulic, gentisic, p-coumaric, p-hydroxybenzoic, protocatechuic, and vanillic. For each compound listed above, the retention time was determined by running a standard through the HPLC separation described above. Standards of caffeic acid, ferulic acid, gentisic acid, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, and vanillic acid were obtained from Sigma and dissolved in butanol:methanol:acetic acid:water (1:5:2:92). These retention times were recorded and used to create standard curves and to identify sample peaks. Trial samples of plant extracts were spiked with standards to insure the correct identification of sample peaks (see Appendix 1 and Appendix 2).

Standard curves were prepared using solutions containing varying concentrations of each of the seven phenolic acids in butanol:methanol:acetic acid:water (1:5:2:92). Salicylic acid was added to all solutions as an internal standard. These curves were used to determine the quantity of each phenolic acid present in the leaf extract (Table 1).

Table 1. Equations of standard curves used for HPLC quantification of phenolic acids.

| Phenolic Acid | Equation of Standard Curve |
|---|---|
| Gentisic | $y = 25.8x - 2.00$ |
| Protocatechuic | $y = 1.66x - 0.452$ |
| PHBA | $y = 1.24x - 0.464$ |
| Vanillic | $y = 1.44x - 0.464$ |
| Caffeic | $y = 1.82x - 0.441$ |
| PCoumaric | $y = 5.47x - 0.375$ |
| Ferulic | $y = 1.81x - 0.495$ |

A standard solution containing all seven phenolic acids was prepared in butanol:methanol:acetic acid:water (1:5:2:92). This standard was run before each group of samples was separated to control for changes in environmental conditions that may affect the performance of the HPLC.

The amount of each phenolic acid lost during extraction was determined for two different extraction procedures, acid hydrolysis and alkaline hydrolysis. This was done to determine which extraction procedure extracted the greatest amount of each phenolic acid with the least amount variation. The results were then used to quantify accurately the amount of each compound found in the experimental samples.

To determine the amount of amount phenolic acid recovered from the extraction procedure a known amount (1ml of $1x10^{-4}$M) of each acid standard was added to a previously freeze-dried, weighed, and ground sample of plant material. These samples, along with control samples without the addition of standards, were then subjected to the extraction and analysis process described above for acid hydrolysis. Alkaline hydrolysis

followed the same homogenization procedure but was hydrolyzed in 5 ml of sodium hydroxide (2M) under nitrogen gas instead of 5ml of hydrochloric acid (1N). The alkaline hydrolyzed plant material was reacidified with HCl to pH of 2. It was dried and extracted in ether as described above.

The amount of phenolic acid present in the control was subtracted from that in the samples spiked with standards. The result is the amount of the original standard that remained after the extraction process. This amount was divided by the original amount of standard added to give the percent of standard recovered after extraction (Table 2).

Table 2. The percent recovery of phenolic acids when subjected to either acid hydrolysis (HCl) or alkaline hydrolysis (NaOH) prior to extraction.

| | NaOH | | | | | | |
|---|---|---|---|---|---|---|---|
| | gentisic | protocat | phba | vanillic | Caffeic | pcoumaric | ferulic |
| | 4.57% | 35.00% | 56.94% | 33.79% | 191.78% | 72.31% | 42.94% |
| | 3.87% | 15.55% | 27.27% | 20.31% | 7.41% | 10.79% | 21.41% |
| | 5.89% | 22.13% | 37.48% | 22.73% | 52.01% | 35.55% | 20.04% |
| | 1.10% | 18.39% | 23.21% | 19.73% | -3.93% | 12.81% | 21.15% |
| **Mean** | **3.86%** | **22.77%** | **36.23%** | **24.14%** | **61.82%** | **32.87%** | **26.39%** |
| Stand. Dev | 2.02% | 8.59% | 15.06% | 6.56% | 89.94% | 28.59% | 11.05% |

| | HCl | | | | | | |
|---|---|---|---|---|---|---|---|
| | gentisic | protocat | phba | vanillic | Caffeic | pcoumaric | ferulic |
| | 20.49% | 15.18% | 20.43% | 18.07% | 26.28% | 11.50% | 8.78% |
| | 14.57% | 14.89% | 18.96% | 14.46% | 27.31% | 11.32% | 5.90% |
| | 39.85% | 26.97% | 52.43% | 31.45% | 46.15% | 29.43% | 17.14% |
| | 33.12% | 20.23% | 36.42% | 24.79% | 39.41% | 22.23% | 15.74% |
| **Mean** | **27.01%** | **19.32%** | **32.06%** | **22.19%** | **34.79%** | **18.62%** | **11.89%** |
| Stand. Dev | 11.54% | 5.66% | 15.71% | 7.51% | 9.64% | 8.83% | 5.41% |

There was less variation in the amount of compound recovered using acid hydrolysis and therefore acid hydrolysis was chosen to use for the experimental samples.

Concentrations of phenolic acids present in the plant material were calculated by dividing the concentration found in the extract by the mean percent recovery value reported in Table 2.

## Analysis of Total Phenolics: Folin-Denis Assay

Total phenolics were measured using the Folin-Denis assay (Mole and Waterman, 1987). Folin-Denis reagent was made by combining 100g sodium tungstate (Matheson, Coleman, and Bell), 20g phosphomolybdic acid (Sigma), 50ml orthophosphoric acid (Sigma) and 750ml di $H_2O$ in a 2L flask and refluxing for 2hrs. The solution was allowed to cool and brought up to 1L with di $H_2O$. It was stored in a dark bottle in the refrigerator until use. A standard curve was created using tannic acid (Sigma) as a standard.

Extracts were reconstituted in 1ml di $H_2O$. This was combined with 60 ml di $H_2O$ and 5ml Folin-Denis reagent in a 100ml volumetric flask. After 3 minutes, 10ml saturated sodium carbonate were added to the solution, and the flask was brought to 100ml with di $H_2O$. The absorbance at 760nm was read using a Beckman Coulter DU 640 Spectrophotometer after 20 minutes.

## Labyrinthula Cultures

### Preparation of Agar Plates

Serum Seawater Agar (SSA) was prepared according to the recipe outlined by Porter (1990). The plates contained 1.2% agar on a sterilized seawater basis, 1% horse serum, 0.25g/L of streptomycin, 0.25g/L of penicillin, and 3mg/L germanium dioxide.

SSA was made in 500ml batches. Six grams of agar and 1.5mg of germanium dioxide were added to 450ml of 35ppt seawater (collected from aquariums). The solution was stirred continuously with a magnetic stir bar and brought to a boil on a hot/stir plate. The solution was autoclaved at 121ºC for 15 minutes. The solution was allowed to cool to 50ºC and then 5.0ml of horse serum (Gibco) and was added to the media using a Falcon serological, polystyrene, individually wrapped, calibrated pipette. A 50ml solution of antibiotics (125mg penicillin and 125mg streptomycin in di$H_2O$) was poured into a BD 60ml sterile syringe and filtered through a Fisher 25mm 0.2 micron syringe filter. Antibiotics were obtained from Sigma. The agar solution was swirled on the table and poured into sterile, 100 x 15mm, plastic Petri dishes. Plates were kept inside their original plastic bag in the refrigerator until use.

**Inoculation of Plates**

Pieces of *T. testudinum* with visible signs of *L. sp.* infection were collected and washed sequentially in 0.5% sodium hypochlorite, distilled water, and autoclaved seawater (Newell and Fell, 1982). They were then placed on SSA using flame-sterilized forceps and kept at room temperature. *L. sp* growth was visible after four days under a light microscope at 40X magnification. A continuous culture was kept using a flame-sterilized spatula to cut out a section of inoculated agar from one plate and transferring it to the surface of the agar on a clean plate. *Labyrinthula* cells grew away from the agar sections onto the new plate.

**Statistical Analysis**

All statistical analyses were performed and using SPSS for Windows version 11. Univariate analysis of variance (ANOVA) was used to test effects on total phenolics and multivariate ANOVA was used to test effects on individual phenolic acids. ANOVA assumes normality and homogeneity of variance but is robust to deviations from these assumptions (Zar, 1999). Effects were considered significant if the significance level was equal to or less than 0.05. All data were tested for normality prior to statistical testing, and those data sets found not to meet normality assumptions were transformed by calculating the natural log of the original value plus one. All data sets were normal unless otherwise reported.

$$X' = \ln(X+1)$$

All data were also tested for homogeneity of variance. Homogeneity of variance was noted for each data set, but no accommodations were made to correct for any lack of homogeneity of variance, as ANOVA is robust to deviations from this assumption (Zar, l999). Each data set had equal variance unless otherwise reported. Boxplots were created to visually examine trends in the data. Boxplots are a graphical representation of the median, interquartile range, maximum, minimum and outliers (Zar, 1999). Data presented in tables and figures are untransformed. Results of all ANOVAs are included as appendices.

## Results

**<u>Effects of *Labyrinthula sp.* Infection on Phenolic Content in Field Samples of *Thalassia testudinum.*</u>**

**Total Phenolics**

There was a significant effect of infection on total phenolics ($p < 0.001$). Infected samples collected from the field averaged 8.89mg (±1.715) of total phenolics per gram dry weight (gdrwt) of plant material. Those that were healthy averaged 25.4 ± 1.878 mg/gdrwt (Figure 1).

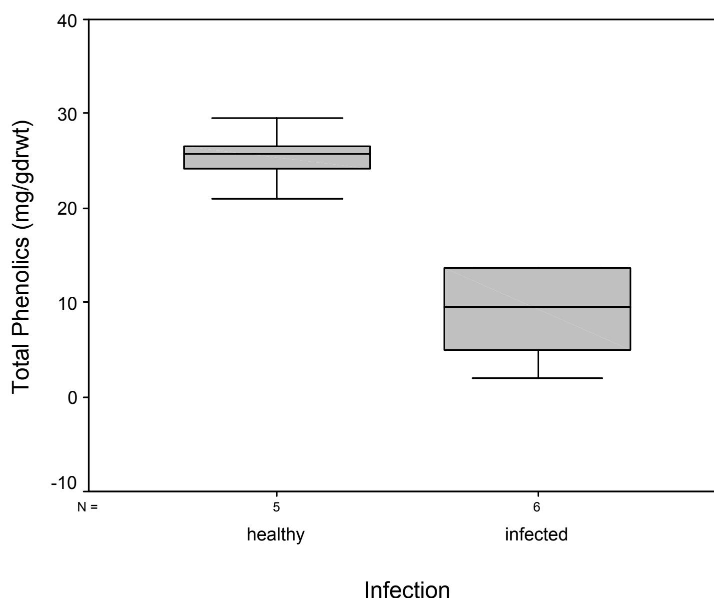


Figure 1. Total phenolics extracted from infected and healthy *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

### Individual Phenolic Acids

Individual phenolics were not normally distributed and were, therefore, transformed as stated above prior to analysis. Data for vanillic, pCoumaric and ferulic acids did not have homogeneity of variance. Gentisic acid was not detected in any samples and was, therefore, not reported. There was a significant effect of infection on protocatechuic acid ($p < 0.001$), parahydroxybenzoic acid (PHBA) ($p = 0.022$), vanillic acid ($p = 0.015$), and caffeic acid ($p = 0.020$). Amounts of all four of these acids were lower in infected plants than in healthy plants. The other two phenolic acids (pCoumaric acid and ferulic acid) exhibited no significant differences in response to infection (Table 3, Figures 2-5).

Table 3. Means in mg/gdrwt of the six phenolic acids extracted from infected and healthy *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. Means that show a significant effect of infection according to ANOVA are indicated by an asterisk. Healthy $N = 5$, infected $N = 6$.

| | Infection | |
|---|---|---|
| Phenolic Acid | healthy | infected |
| Protocatechuic | *0.960 | *0.040 |
| PHBA | *0.369 | *0.250 |
| Vanillic | *0.712 | *0.082 |
| Caffeic | *1.203 | *0.690 |
| pCoumaric | .209 | .636 |
| Ferulic | .103 | .031 |

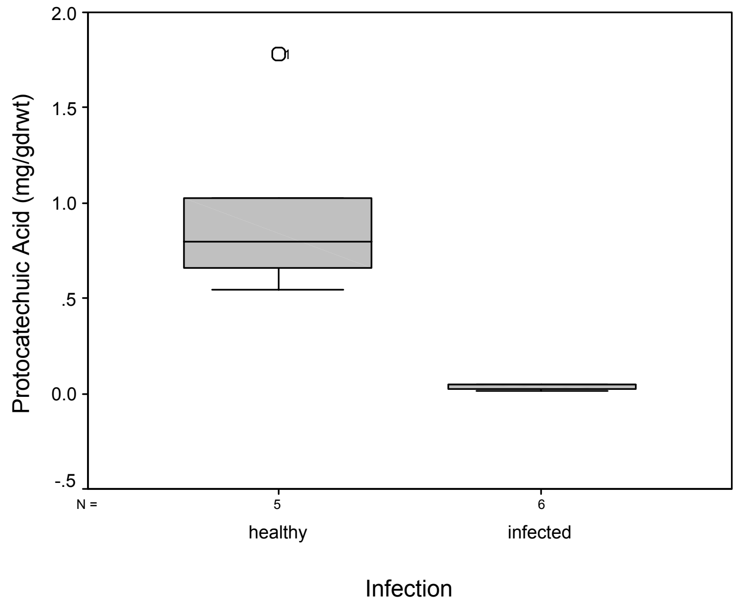


Figure 2. The amount of protocatechuic acid extracted from healthy and infected *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. The center line represents the median and the boxes represent the interquartile range. *N* values are listed at the bottom of the chart.

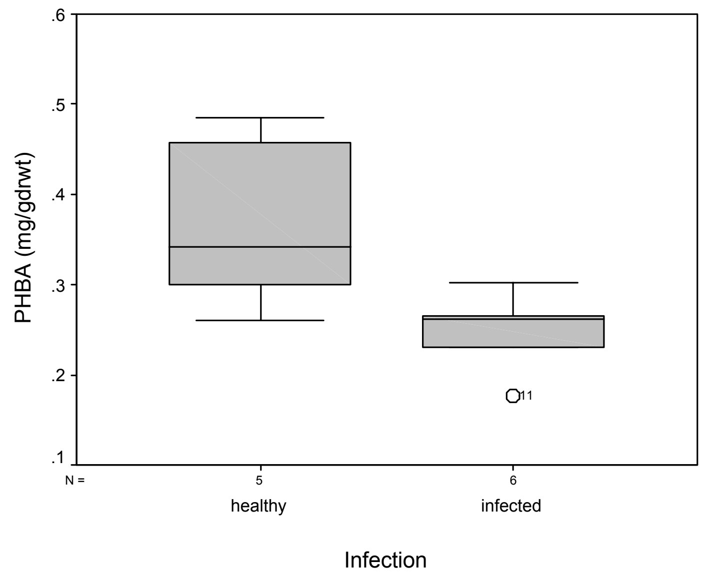


Figure 3. The amount of parahydroxybenzoic acid (PHBA) extracted from healthy and infected *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. The center line represents the median and the boxes represent the interquartile range. *N* values are listed at the bottom of the chart.

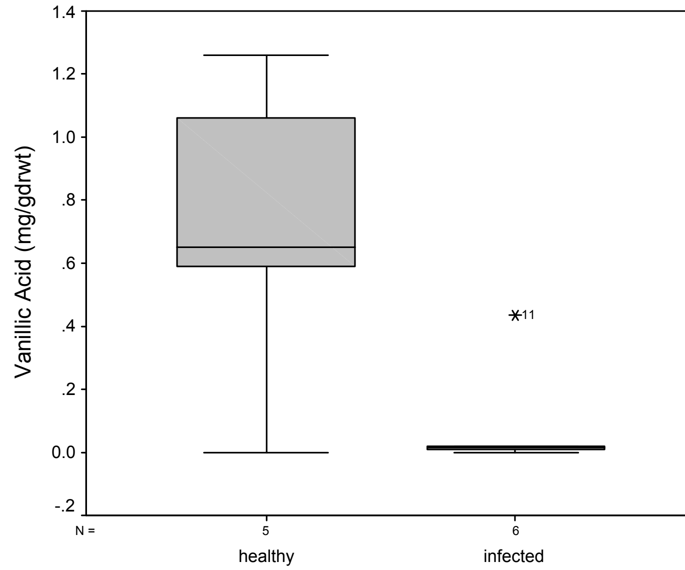


Figure 4. The amount of vanillic acid extracted from healthy and infected *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. The center line represents the median, the boxes represent the interquartile range and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

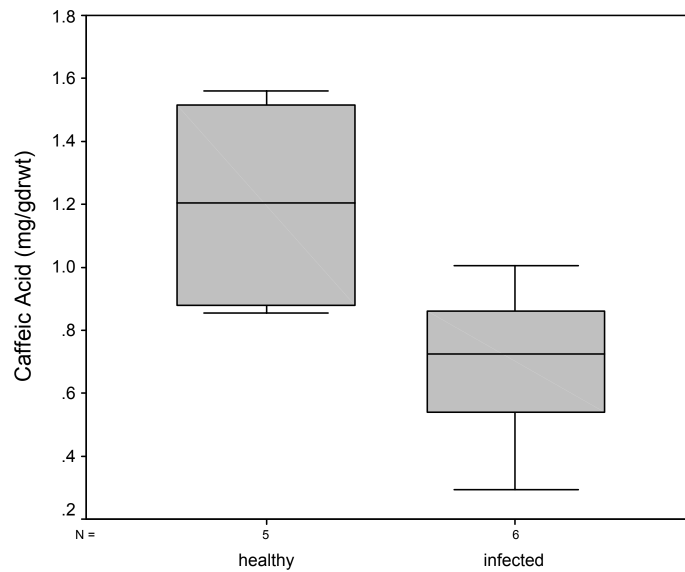


Figure 5. The amount of caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves collected at the south end of the Sunshine Skyway Bridge. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

## Effects of *Labyrinthula sp*. Infection on the Phenolic Content of *Thalassia testudinum* Three, Four, and Seven Days Post Infection.

Data for individual phenolics were not normally distributed and vanillic, pCoumaric, and ferulic acids did not have homogeneity of variance. Data for individual phenolics was transformed prior to analysis.

### Total Phenolics

According to a univariate analysis of variance, there was a significant effect of both time ($p = 0.020$) and time*infection ($p = 0.013$) on total phenolics. There was no significant effect of infection alone on total phenolics. The amount of total phenolics increased in both healthy and infected plants following clamping with either an infected section of leaf or a healthy section of leaf from day three to day four. The increased amount of total phenolics continued for those clamped with healthy sections of leaf from day four to day seven. However, in those plants that had infected leaf pieces clamped to them, the amount of phenolics decreased between day four and day seven (Table 4, Figure 6). When looking at each individual time, those plants that had infected leaf pieces clamped to them for three days had significantly higher amounts of total phenolics than those clamped with healthy leaf pieces for three days. Four days after clamping there was no significant difference between healthy and infected plants. After seven days, the amount of phenolics in infected plants was significantly lower than healthy plants (Table 4, Figure 6).

Table 4. Mean total phenolics in mg/gdrwt for healthy and infected *Thalassia testudinum* leaves sampled three, four, and seven days after clamping.

| Time | Healthy | Infected |
|---|---|---|
| 3 days | 17.772 | 30.119 |
| 4 days | 28.115 | 44.958 |
| 7 days | 42.092 | 17.668 |

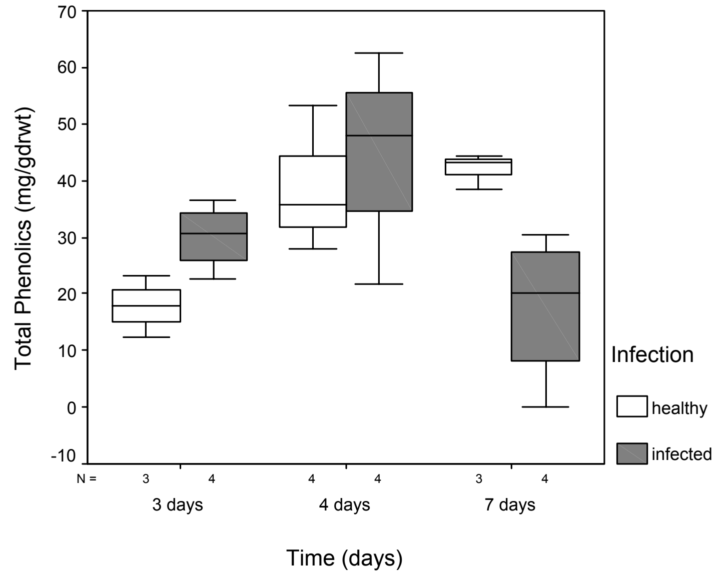


Figure 6. Total phenolics extracted from healthy and infected *Thalassia testudinum* leaves sampled three, four, and seven days after clamping. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

### Individual Phenolic Acids

Individual phenolic acids showed no significant effect of either time or time*infection. Infection alone had a significant effect on the amount of caffeic acid present ($p = 0.001$). Levels of caffeic acid in infected plants (2.026 ± .938 mg/gdrwt) were lower than healthy plants (5.197 ± 1.049 mg/gdrwt) at day three, day four (3.239 ± .938 mg/gdrwt and 4.771 ± .938 mg/gdrwt, respectively), and day seven (1.654 ± .938 mg/gdrwt and 5.386 ± .938 mg/gdrwt, respectively) (Figure 7). Although not significant

according to MANOVA, vanillic acid also exhibited a pattern of decreased concentration in infected plants (0.722 ± .446 mg/gdrwt) as compared to healthy plants (1.873 ± .446 mg/gdrwt) after seven days (Figure 8).

Gentisic acid was not detected in any samples and was, therefore, not reported.

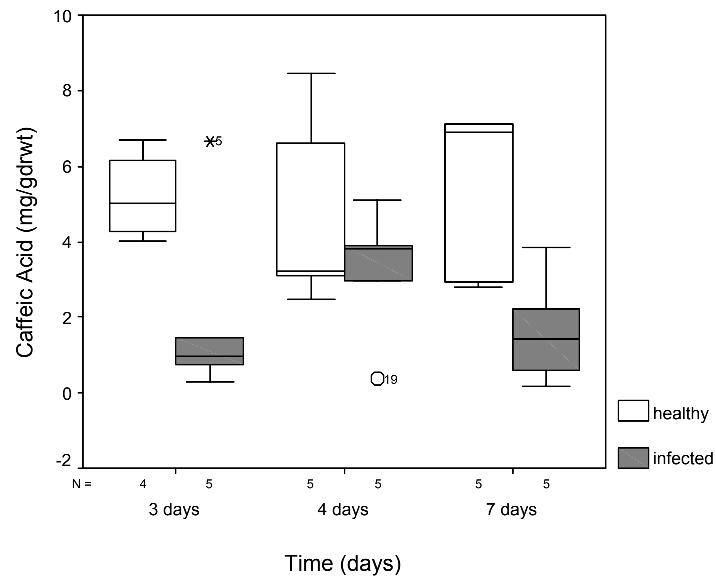


Figure 7. Caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves sampled three, four, and seven days after clamping. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

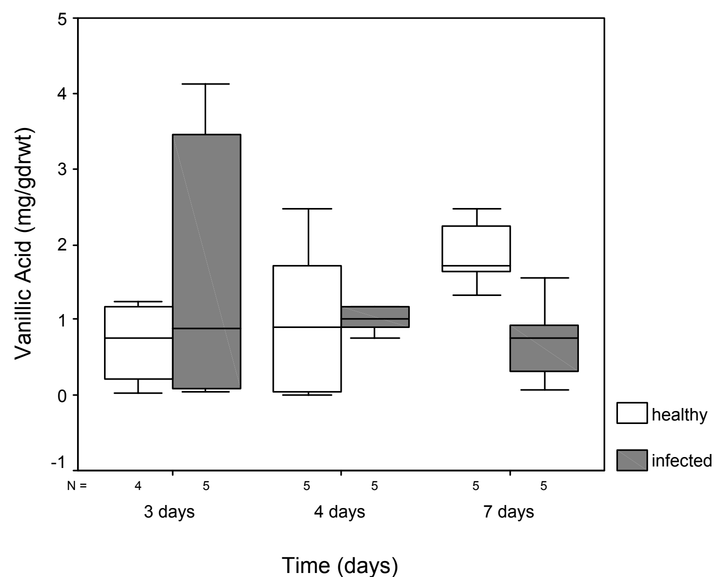


Figure 8. Vanillic acid extracted from healthy and infected *Thalassia testudinum* leaves sampled three, four, and seven days after clamping. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

## Effects of *Labyrinthula sp*. Infection and Light Level on the Phenolic Content of *Thalassia testudinum*

All light data were transformed because they were not normally distributed. Total phenolics, pCoumaric acid, and ferulic acid did not have homogeneity of variance.

### Total Phenolics

There was no significant effect of infection, light or infection*light on the production of total phenolics. There were seemingly lower levels of total phenolic in infected plants grown under low light conditions than healthy plants grown under low light condition but these differences were not statistically significant (Figure 9).

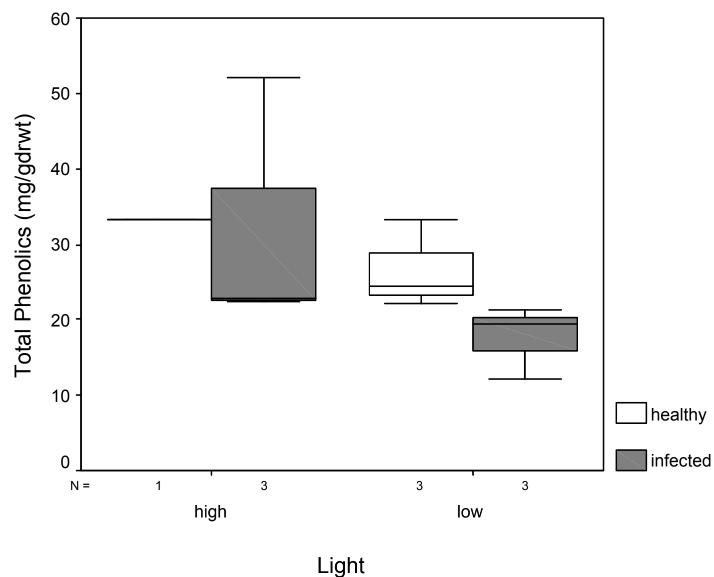


Figure 9. Total phenolics extracted from healthy and infected *Thalassia testudinum* leaves grown under either high or low light conditions. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

**Individual Phenolic Acids**

There were also no significant effects of light, infection, or light*infection on any of the individual phenolic acids. However, some trends emerged. Protocatechuic, caffeic, and ferulic acids exhibited higher levels in infected plants than healthy plants under the low light condition (Figures 10-12).

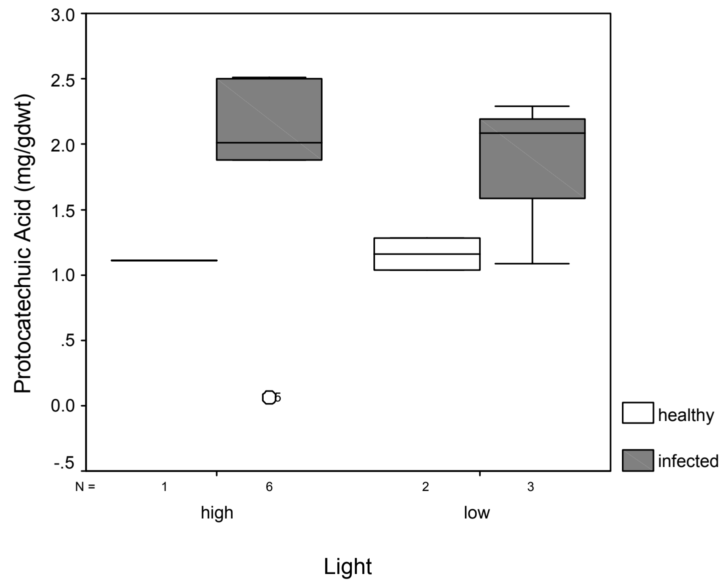


Figure 10. Protocatechuic acid extracted from healthy and infected *Thalassia testudinum* leaves grown under either high or low light conditions. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

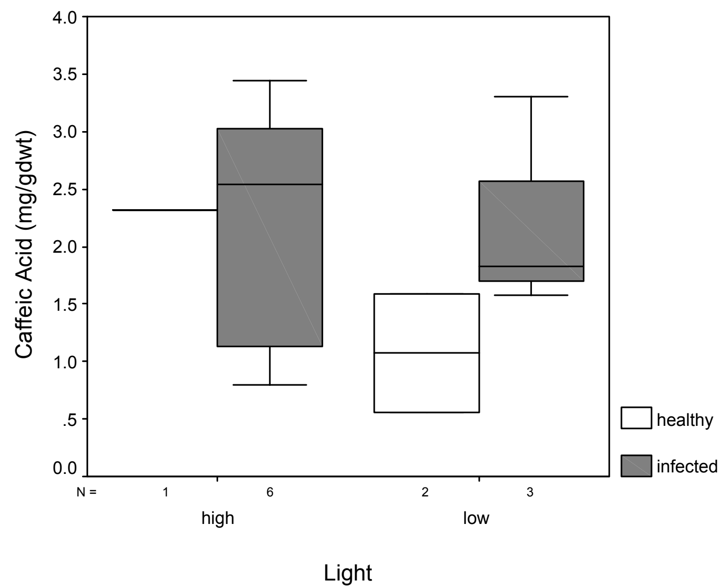


Figure 11. Caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves grown under either high or low light conditions. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

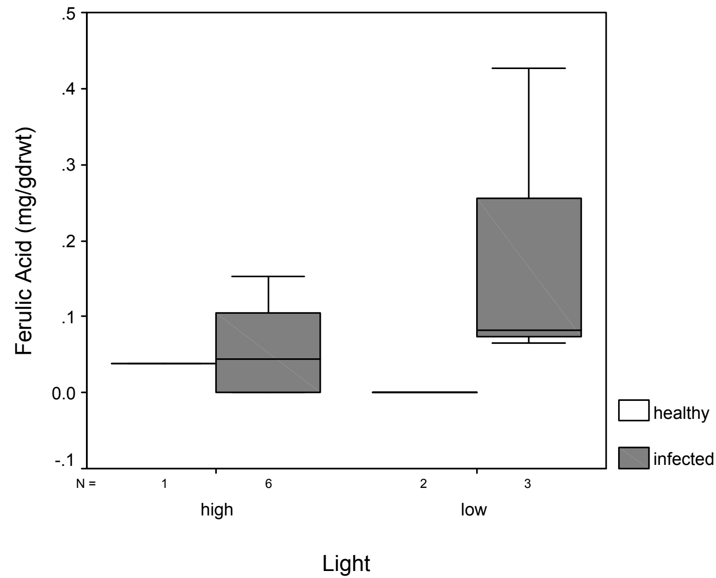


Figure 12. Ferulic acid extracted from healthy and infected *Thalassia testudinum* leaves grown under either high or low light conditions. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

## **<u>Effects of *Labyrinthula sp*. Infection and Salinity on the Phenolic Content of *Thalassia testudinum*</u>**

### **Total Phenolics**

There was no significant effect of salinity, infection, or salinity*infection on total phenolics. The level of total phenolics was lower in healthy plants (0.377 ± 9.276 mg/gdrwt) than infected plants (30.025 ± 7.574 mg/gdrwt) at 25ppt (Figure 13) although these were not significant according to a MANOVA. The amount of total phenolic in healthy plants at 25 ppt was also lower than the amount of total phenolics in healthy plants at 35 ppt (33.2 ± 13.118 mg/gdrwt) (Figure 13).

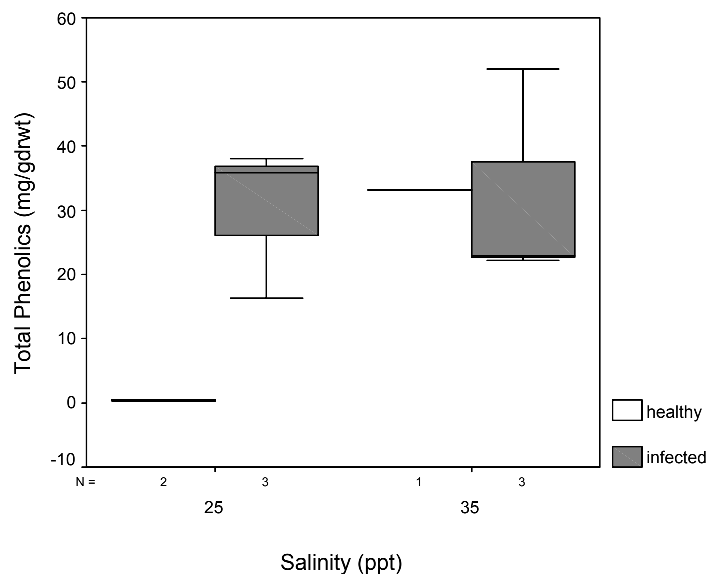


Figure 13. Total phenolics extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

**Individual Phenolic Acids**

There were no significant effects of salinity, infection, or salinity*infection on any of the individual phenolic acids, however, protocatechuic, PHBA, vanillic, and caffeic acids all showed trends toward higher levels in infected plants than healthy plants at 25ppt (Figures 14-17).

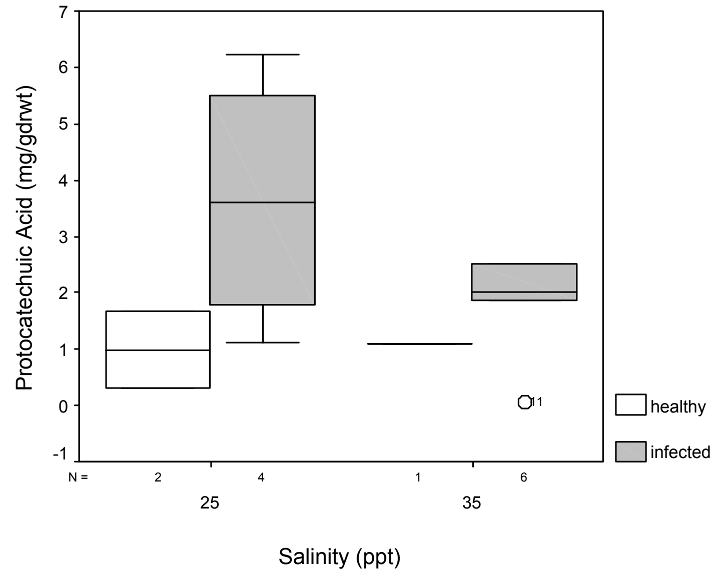


Figure 14. Protocatechuic acid extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

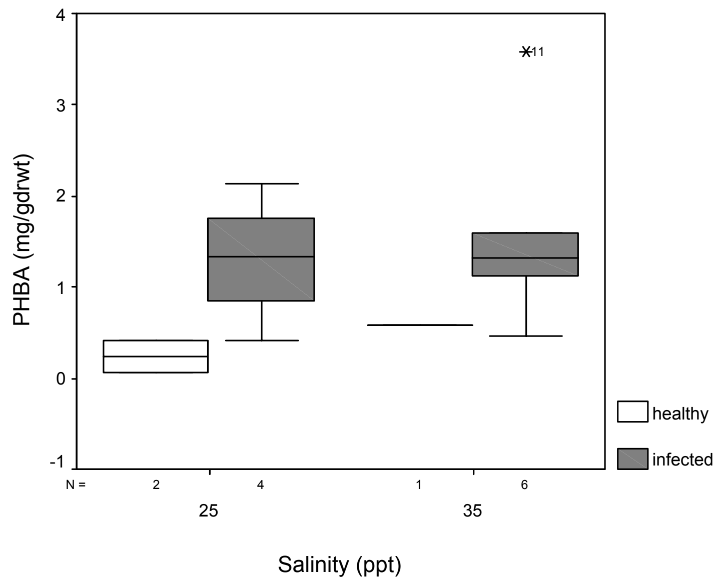


Figure 15. PHBA extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

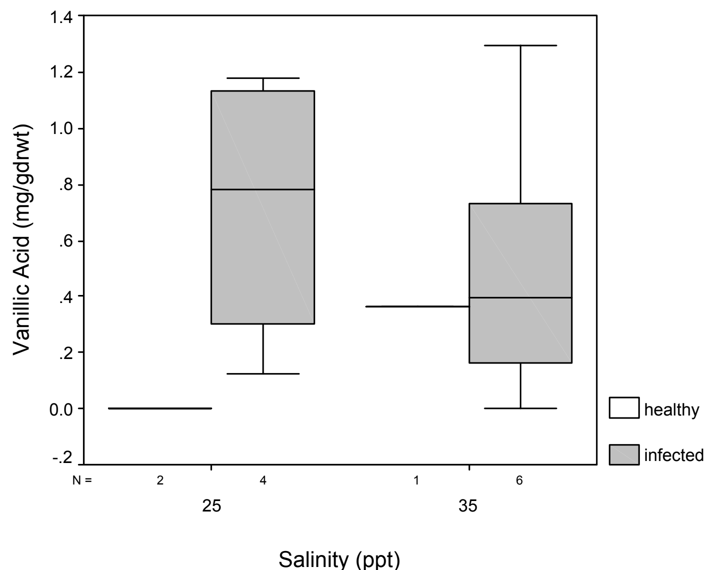


Figure 16. Vanillic acid extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

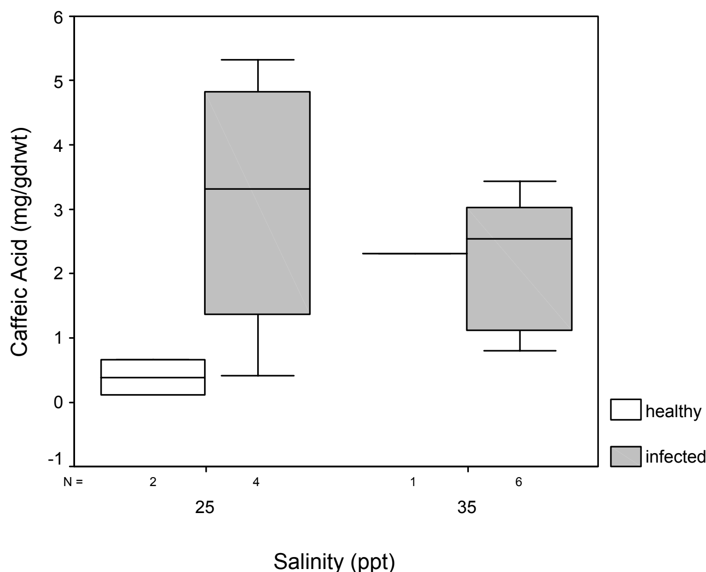


Figure 17. Caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

**Combination of Time Data with Salinity and Light Data**

The samples used for the high light treatment were kept at 35ppt salinity. These samples, therefore, were also used as the 35ppt salinity treatment in the salinity experiment. High light is the normal condition as is 35ppt salinity. Only one healthy sample in the 35ppt/high light treatment remained clamped during the experiment, leaving only one healthy sample for analysis. In order to increase the sample size for this treatment, data from a previous experiment were added to both the healthy and infected data in the 35ppt/high light treatment. The plants used in the previous experiment were grown in the lab under the same conditions as those used in the salinity and light experiments (35ppt salinity, high light, seven day exposure to clamping). Although, the plants used for the previous experiment were collected from a different geographical location than those used for the salinity and light experiments and on a different date, an ANOVA comparison of the two data sets showed that they are not significantly different.

When those data were included with the data collected during the salinity and light experiments, there was a significant effect of infection*salinity on total phenolic levels ($p = 0.006$), PHBA ($p = 0.046$), vanillic ($p = 0.005$), and caffeic acids ($p = 0.003$). There was also a significant effect of infection*light on caffeic acid ($p = 0.020$). Data was transformed due to lack of normality for individual phenolics, but not total phenolics. Protocatechuic, caffeic, and ferulic acids lacked homogeneity of variance.

**Salinity**

At 25ppt healthy samples contained less total phenolics (0.377 ± 8.739 mg/gdrwt) than infected samples (30.025 ± 7.136 mg/gdrwt). At 35 ppt healthy samples contained

higher levels of total phenolics (39.869 ± 6.180 mg/gdrwt) than did infected samples (23.987 ± 4.671 mg/gdrwt) (Figure 18).

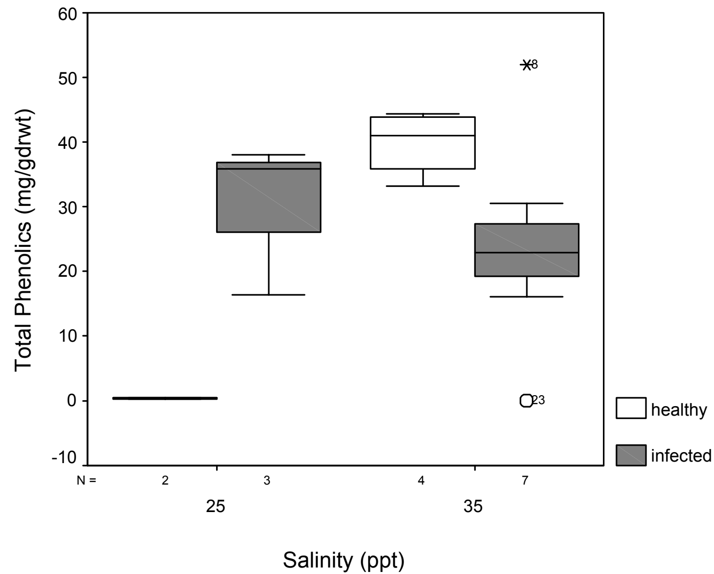


Figure 18. Total phenolics extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity, including previously collected data. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

There was a significant effect of infection*salinity on PHBA (p = 0.046), vanillic (p = 0.005), and caffeic acids (p = 0.003). At 25ppt there was less PHBA in healthy plants (0.241 ± .525 g/gdrwt) than in infected plants (1.210 ± .224) (Figure 19). There was also a larger amount of vanillic acid in infected plants at 25ppt (0.716 ± 0.285 mg/gdrwt) than in healthy plants at the same salinity (0.000 ± 0.403 mg/gdrwt). However, at 35ppt there was less vanillic acid in infected plants (0.599 ± 0.172 mg/gdrwt) than in healthy plants (1.621 ± 0.233 mg/gdrwt) (Figure 20). Healthy plants also contained less caffeic acid (0.398 ± 1.237 mg/gdrwt) than infected plants (3.087 ±

0.875 mg/gdrwt) at 25ppt and more in healthy (4.873 ± 0.714 mg/gdrwt) than infected (1.976 ± 0.528 mg/gdrwt) at 35ppt (Figure 21).

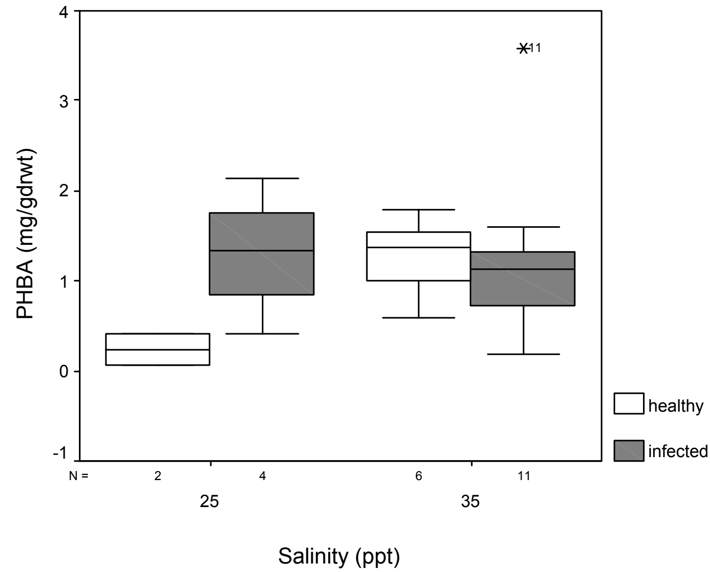


Figure 19. Parahydroxybenzoic acid (PHBA) extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity, including previously collected data. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

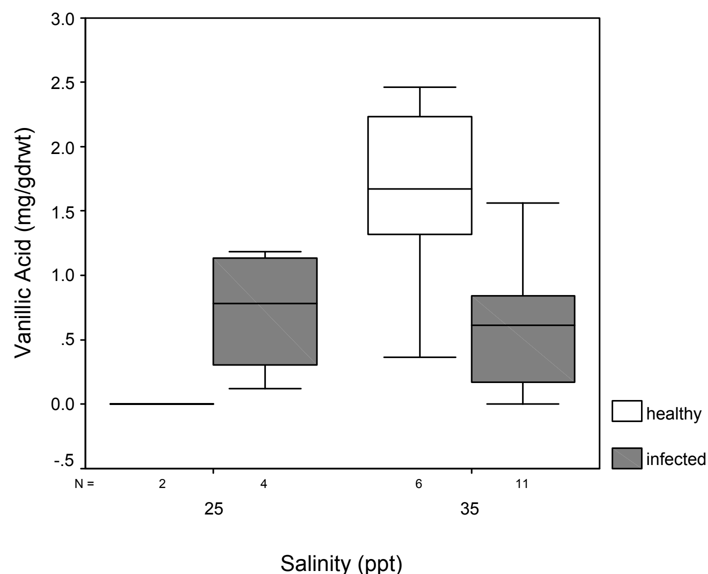


Figure 20. Vanillic acid extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity, including previously collected data. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

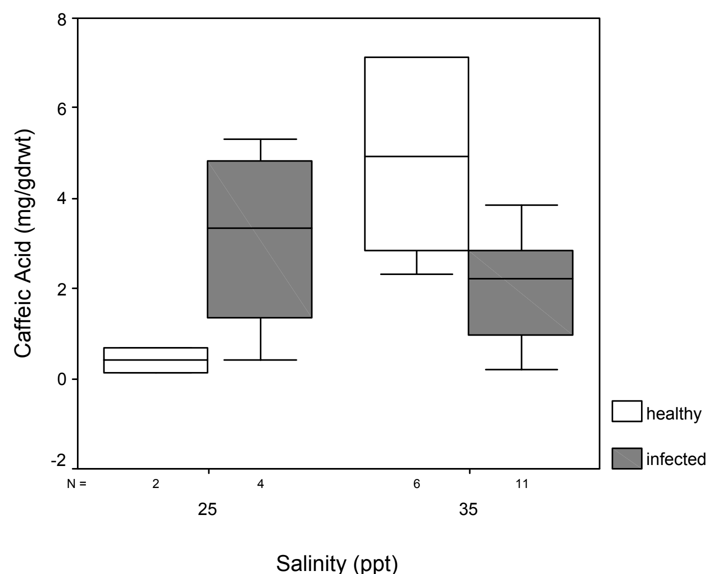


Figure 21. Caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves grown at 25 and 35 ppt salinity, including previously collected data. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

**Light**

There was no effect of light, infection or light*infection on total phenolics (Figure 23). There was a significant effect of light*infection on caffeic acid (p = 0.020). Healthy plants grown in high light had higher levels of caffeic acid (4.873 ± 0.653 mg/gdrwt) than infected (1.976 ± 0.482 mg/gdrwt) while the opposite was true for plants grown in low light (1.069 ± 1.131 and 2.231 ± 0.932 mg/gdrwt, respectively) (Figure 22).

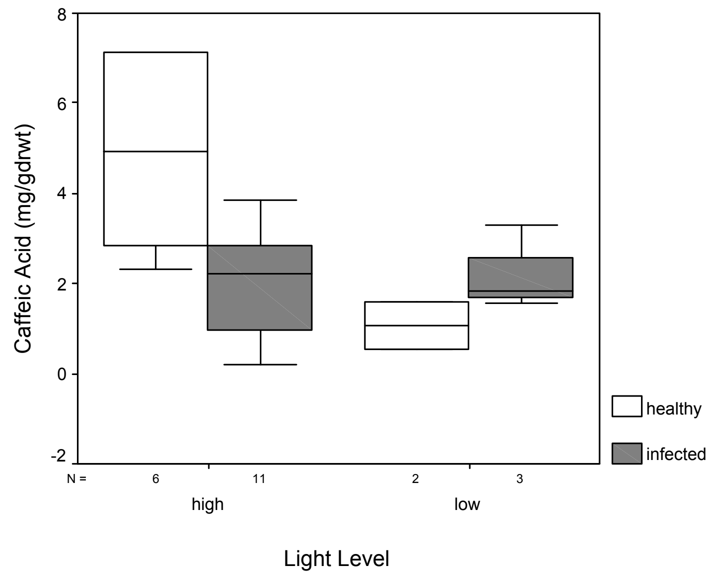


Figure 22. Caffeic acid extracted from healthy and infected *Thalassia testudinum* leaves grown under high and low light conditions, including previously collected data. Boxes represent the interquartile range and whiskers represent maximum and minimum values.

# Discussion

The presence of a pathogen such as *Labyrinthula sp.* often induces the production of defensive metabolites (Harbourne, 1993). This has been demonstrated in both terrestrial and marine plants, including another species of seagrass, *Zostera marina*, which produces increased amount of phenolics in response to *Labyrinthula zosterae* (Dyer et. al, 1989; Pare et. al., 1991, Pare et. al., 1992; Vergeer and Develi, 1997). In this study, there appeared to be an initial induction of phenolic production in *Thalassia testudinum* in response to *L. sp.* infection under normal (non stressful) conditions. This initial induction was followed by a sharp decline in phenolic production (Table 2, Figure 6).

The induction pattern described above was altered when plants were exposed to environmental stress (low salinity and low light) (Figures 18-21). Control (healthy) plants responded differently than infected plants to each factor. Plants that did not receive the infection exhibited a decrease in phenolic production, both total and individual, in response to low light and low salinity. Plants that received the infection treatment had similar levels of phenolics regardless of stress. This resulted in infected plants having higher levels of phenolics than healthy under environmentally stressful conditions, suggesting that there is an induction of phenolics in response to the combination of infection and stress, but neither by themselves. In healthy plants, low light and low salinity caused a decrease in phenolic production, however, the presence of

the protist infection either inhibited this reduction or induced increased phenolic production that masked any decrease caused by the stress condition. It may be that the induced increase in phenolics exhibited after three days is being delayed due to stress and, therefore, those plants that are stressed are exhibiting induction in response to infection after seven days.

These results were contrary to what was expected based on the Optimal Defense hypothesis (Rhoades, 1979). Although the Optimal Defense hypothesis proposes that under stress a plant's energetic output will be spent on the production of primary metabolites and, therefore, production of defensive secondary metabolites will decrease, we would expect that the added stress of infection would further reduce the production of secondary metabolites.

When a plant is presented with multiple stressors, these stressors, both abiotic and biotic, may act synergistically or antagonistically (Nilsen, E.T. and Orcutt, D.M., 1996). Koch and Erskine (2001) found that turtle grass mortality occurred only in the presence of high sulfide, high temperature, and high salinity combined. Any of these stresses individually did not cause mortality. It appears that there is an interaction of stressors occurring here that needs to be further investigated.

## Induction of Phenolic Compounds in *Thalassia testudinum* in Response to *Labyrinthula sp.* Infection.

### Field Study

Infected samples collected from the field had significantly lower levels of both total phenolics and several individual phenolic acids than those that lacked signs of infection. However, because the infected samples were collected during the summer and

healthy during the winter it is not possible to determine whether the difference is a result of infection or season. It is essential to measure phenolic levels at different times of the year to determine the effect, if any, that seasonality has on their production.

**Laboratory Perturbation**

Laboratory experiments controlled for differences in seasonality. There was a significant difference in total phenolics content in response to infection and the nature of the difference was affected by time (Figure 6).

The increase in total phenolic production after clamping in control (healthy) treatments indicates that clamping itself caused an increase in the production of phenolics (Figure 6). The infected plants mirrored the post clamping increase in total phenolics at day three and day four but showed a decreased amount of total phenolics after day seven. The interaction of time and infection indicates that the response is being induced by the infection.

The initial increase in total phenolics indicates a defense response to the presence of *L. sp*. The induction of phenylalanine ammonia lyase (PAL) activity has been shown to occur rapidly (reaching a maximum within six hours) in response to wounding and to subsequently decline (Dyer, et al., 1989). Activation of PAL in the cactus *Cephalocereus senilis* was greatest between twelve and twenty-four hours after exposure to chitin (Pare et al., 1992). There appears to be a similar rapid, short-term response occurring here.

The reduction in phenolics at day seven suggests that phenolic production is a short-term response and that in the long-term infection is suppresses the production of phenolics. This suppression may be the result of a decrease in photosynthetic capacity. Infection by *L. sp*. has been shown to decrease photosynthetic capacity in *T. testudinum*

(Durako and Kuss, 1994). Durako and Kuss (1994) concluded that the decrease in photosynthetic capacity was a direct result of the reduction of photosynthetic tissue caused by the destruction of leaf material by *L. sp*. However, the infected plants harvested in this experiment had very small, if any, lesions visible, even after seven days. This suggests that the reduction in phenolics may be caused by something other than reduced photosynthetic capacity.

Caffeic acid was the only individual phenolic acid to show a significant response, and this compound also decreased in response to infection (Figure 7). There was no significant interaction of time and infection on caffeic acid; the effect was of infection alone. Unlike total phenolics, the level of caffeic acid was initially lower in infected plants and remained so for each time period. It is unlikely that there would have been enough reduction in photosynthetic tissue after three days to cause this decrease in caffeic acid. As stated above, the leaves did not visually appear to have large areas of damaged tissue.

These findings are contrary to those found in *Zostera marina*. Both total phenolic content and caffeic acid increased in response to infection by *Labyrinthula zosterae* in *Zostera* plants (Vergeer et al., 1995, Vergeer and Develi, 1997). Vergeer et. al. (1995) found increased amount of total phenolics in plants that had been subject to infection for four weeks. Although there was an increase in total phenolics at day three in this study, subsequent decline already at day seven is contrary to the results found by Vergeer et. al (1995).

## Effects of Environmental Stress on the Induction of Phenolic Compounds

The results discussed here are those that were determined by combining data from the induction experiment with data from the light and salinity stress experiments (see results). It should be kept in mind that plants used in the induction experiment were collected in February in Islamorada, FL (Florida Bay) and those used in the light and salinity experiments were collected in November from Tampa Bay. Although statistical tests showed no significant differences in the data collected at the two different locations, only further experiments addressing geographical and seasonal variation can determine if these conditions have skewed the results.

### Light

Light has been shown to increase phenolic production and accumulation in plants (McClure, 1979). Vergeer et al. (1995), found that low light intensities caused a decrease in the amount of total phenolics in *Zostera marina*. This is concurrent with the decrease in total phenolics in plants grown under low light. It also supports the idea that a decrease in photosynthesis results in lower production of phenolics. Although there was no *significant* effect of light, infection, or light*infection on total phenolics, both healthy and infected plants grown under low light treatments had a lower amount of total phenolics compared to the healthy plants in the high light treatment (Figure 23). Under high light treatments, infected plants had lower levels of phenolics than healthy plants. The level of phenolics in infected plants under high light was similar to the levels of both infected and healthy plants grown under low light (Figure 23). This supports the idea that phenolic levels in infected plants may be decreased due to lower photosynthetic activity.

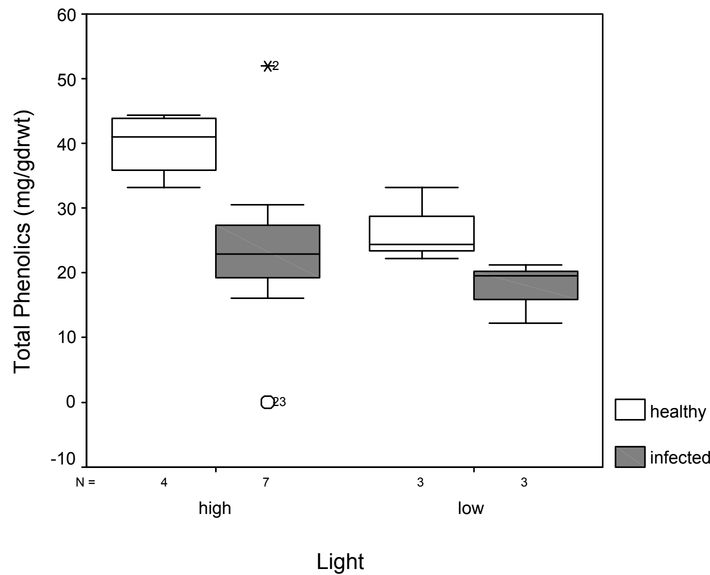


Figure 23. Total phenolics extracted from healthy and infected *Thalassia testudinum* leaves grown under high and low light conditions, including previously collected data. The center line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values. *N* values are listed at the bottom of the chart.

Individual phenolic acids had varied responses to changes in light level, all but p-coumaric acid having either the same or higher levels in infected plants than healthy plants at low light. They all had either the same or lower levels in infected plants than healthy plants at high light. The only significant effect of light level was on caffeic acid, which was lower in infected than healthy in high light, but higher in infected than healthy in low light. However, an examination of Figure 22 shows that the amount of caffeic acid present in infected plants does not appear to be different in high and low light. This significance is a result of a difference in the amount of caffeic acid in healthy plants grown under high and low light. There is a greater amount of caffeic acid in healthy plants at high light than healthy plants at low light. Since phenolic levels decrease under

low light conditions (Vergeer et al., 1995), it appears that the production of phenolics is being reduced by both low light and infection.

### Salinity

The response to salinity stress was similar to that seen when plants were exposed to light stress. Healthy plants exposed to low salinity (25ppt) had lower amounts of both total phenolics and individual phenolic acids than infected plants at low salinity, and either treatment at normal salinity (35ppt) (Figures16-19). Again, it appears that environmental stress is causing the suppression of phenolic production in control plants. The concentration of phenolics in infected plants does not appear to change as a result of salinity stress (Figures 16-19). In relation to healthy plants, infected plants had lower concentrations of phenolics at normal salinity (35ppt) and higher at low salinity (25ppt). Although Vergeer et. al., (1995) found no significant effect of salinity on the production of phenolics in *Zostera marina,* decreases in phenolic production in response to high salinity stress have been found in terrestrial plants (Dunn, 1998) Overall growth and productivity decrease substantially in *Thalassia* when exposed to extremely low salinities (< 12ppt) and even moderately low salinity (25ppt) affects growth and productivity negatively (Doering and Chamberlain, 2000; Lirman and Copper, 2003).

Plants grown in high salinity (45ppt) did not survive and, therefore, were not included in the analysis. It is interesting to note that plants did not survive exposure to 45ppt salinity in this experiment, and Lirman and Cropper (2003) reported the lowest growth rate of *Thalassia* at 45ppt when looking at growth rates over the salinity range of

5ppt to 45ppt. The average salinity in Florida Bay during the die-off event was 46ppt with extremes reaching as high as 59ppt (Zieman et. al., 1999).

**Future Research**

The initial increase and subsequent decrease in phenolic production indicates that there is a rapid, short-term induction of phenolics. The samples were harvested three four and seven days post infection, assuming that three days would be the minimum exposure time to produce a response. This assumption was based on *L. sp.* taking 72 hours (three days) to infect healthy *T. testudinum* leaves (Blakesley, personal communication). It would be beneficial to examine phenolic levels present after a shorter amount of time and at smaller time intervals.

There were large variances in the amounts of individual phenolic acids present which, along with the low sample sizes may have decreased the ability to determine significant effects. Increasing the sample size would enhance future studies.

Also, it is important to study the between and within plant variation in phenolic levels. There were large variances between samples within a single treatment as well as between samples used to determine the percent recovery of compounds from the extraction process. Phenolic levels have been shown to vary significantly between individual plants of the same species (Castells et al., 2002).

Phenolics levels can also differ significantly within the same plant (Hillis, 1956; Sheen, 1969; Feeny, 1970; Rhoades and Cates, 1976; Gartlan et al., 1980). Younger leaves have been found in some cases to contain higher levels of phenolic compounds than the older leaves. (Hillis, 1956; Sheen, 1969; Rhoades and Cate, 1976; Gartlan et

al.,1980). Under normal conditions, *L. sp.* is much more commonly found on older leaves and it would be interesting to know if there is a difference in phenolic levels between young and old leaves (Vergeer and den Hartog, 1994).

**Conclusions**

1. Under normal, non-salinity or light-stressed, conditions there was a short-term increase in the production of total phenolics in response to infection followed by a decrease in phenolic production.
2. Both low salinity and low light caused a decrease in the production of phenolics in healthy plants.
3. There was an interaction between environmental stress and infection causing phenolic levels to be higher in plants that were exposed to infection and stress as compared to those exposed to stress alone.

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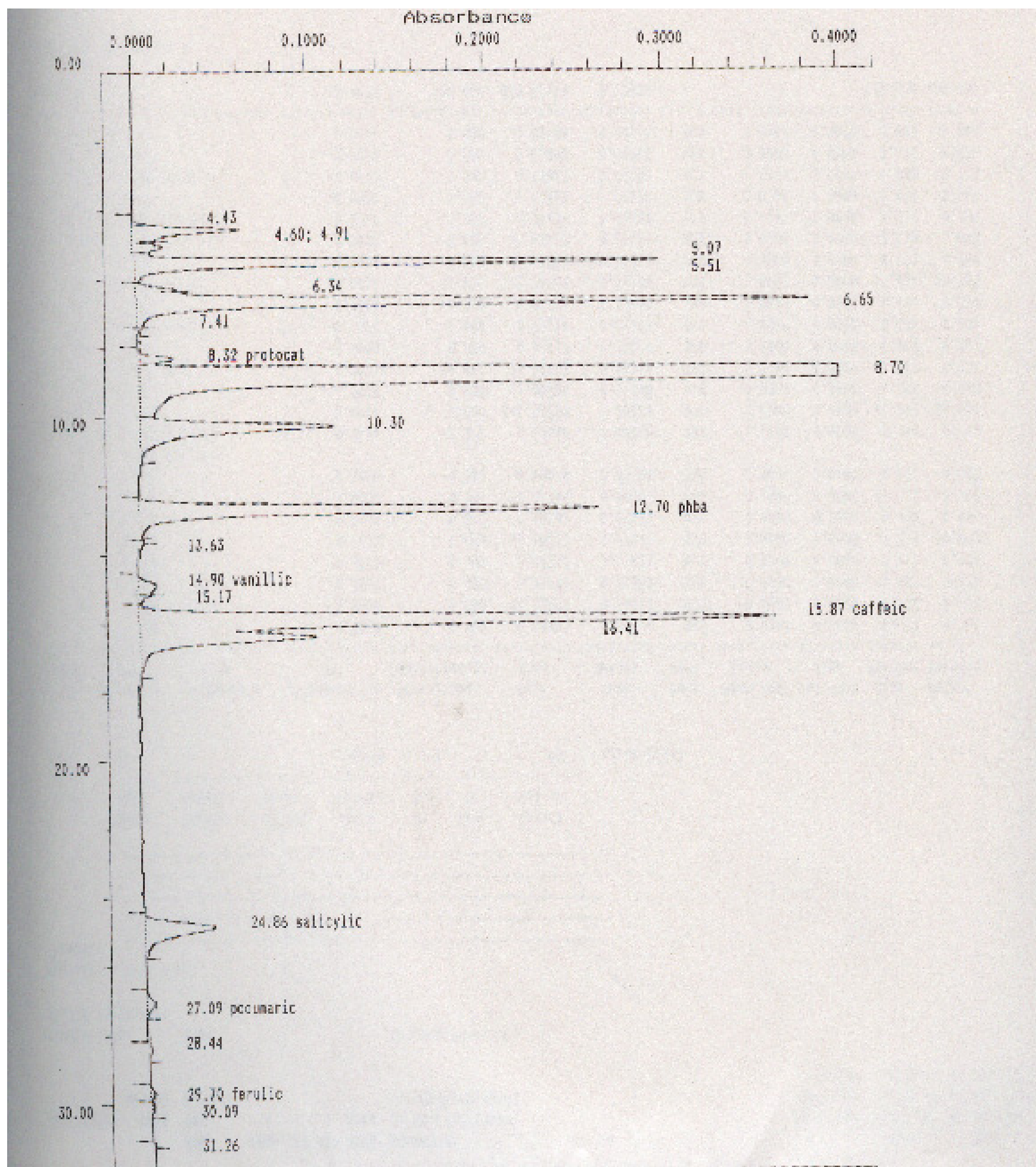
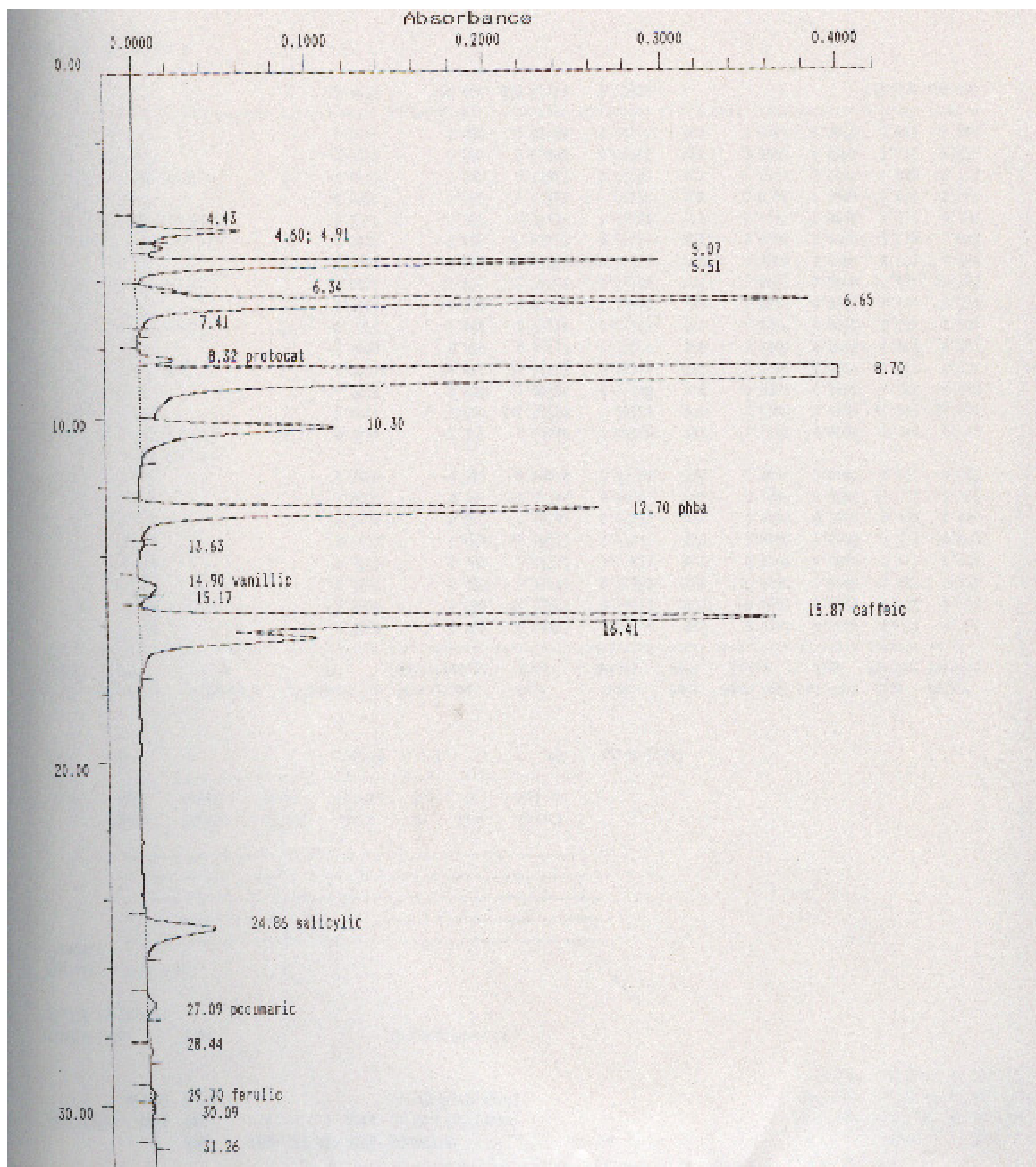
Appendix 1: HPLC chromatogram of gentisic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, and ferulic acid standards.



Appendix 2: HPLC chromatogram of *Thalassia testudinum* extract.



Appendix 3: Univariate ANOVA: The effects of *Labyrinthula sp.* infection on the total phenolic content in field samples of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

Dependent Variable: PHENOLIC

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 743.696[a] | 1 | 743.696 | 42.164 | .000 |
| Intercept | 3206.710 | 1 | 3206.710 | 181.805 | .000 |
| INFECTIO | 743.696 | 1 | 743.696 | 42.164 | .000 |
| Error | 158.744 | 9 | 17.638 | | |
| Total | 3858.951 | 11 | | | |
| Corrected Total | 902.440 | 10 | | | |

a. R Squared = .824 (Adjusted R Squared = .805)

Appendix 4: Multivariate ANOVA: The effects of *Labyrinthula sp.* infection on the individual phenolic acid content in field samples of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | 1.021[a] | 1 | 1.021 | 42.774 | .000 |
| | PHBA | 2.197E-02[b] | 1 | 2.197E-02 | 7.577 | .022 |
| | Vanillic | .505[c] | 1 | .505 | 9.065 | .015 |
| | Caffeic | .192[d] | 1 | .192 | 7.923 | .020 |
| | pCoumaric | 5.954E-02[e] | 1 | 5.954E-02 | .357 | .565 |
| | Ferulic | 1.194E-02[f] | 1 | 1.194E-02 | 3.456 | .096 |
| Intercept | Protocatechuic | 1.295 | 1 | 1.295 | 54.259 | .000 |
| | PHBA | .780 | 1 | .780 | 268.873 | .000 |
| | Vanillic | .888 | 1 | .888 | 15.931 | .003 |
| | Caffeic | 4.577 | 1 | 4.577 | 189.027 | .000 |
| | pCoumaric | .739 | 1 | .739 | 4.427 | .065 |
| | Ferulic | 4.329E-02 | 1 | 4.329E-02 | 12.533 | .006 |
| INFECTIO | Protocatechuic | 1.021 | 1 | 1.021 | 42.774 | .000 |
| | PHBA | 2.197E-02 | 1 | 2.197E-02 | 7.577 | .022 |
| | Vanillic | .505 | 1 | .505 | 9.065 | .015 |
| | Caffeic | .192 | 1 | .192 | 7.923 | .020 |
| | pCoumaric | 5.954E-02 | 1 | 5.954E-02 | .357 | .565 |
| | Ferulic | 1.194E-02 | 1 | 1.194E-02 | 3.456 | .096 |
| Error | Protocatechuic | .215 | 9 | 2.387E-02 | | |
| | PHBA | 2.610E-02 | 9 | 2.900E-03 | | |
| | Vanillic | .502 | 9 | 5.572E-02 | | |
| | Caffeic | .218 | 9 | 2.422E-02 | | |
| | pCoumaric | 1.502 | 9 | .167 | | |
| | Ferulic | 3.108E-02 | 9 | 3.454E-03 | | |
| Total | Protocatechuic | 2.339 | 11 | | | |
| | PHBA | .810 | 11 | | | |
| | Vanillic | 1.783 | 11 | | | |
| | Caffeic | 4.855 | 11 | | | |
| | pCoumaric | 2.345 | 11 | | | |
| | Ferulic | 8.260E-02 | 11 | | | |
| Corrected Total | Protocatechuic | 1.236 | 10 | | | |
| | PHBA | 4.807E-02 | 10 | | | |
| | Vanillic | 1.007 | 10 | | | |
| | Caffeic | .410 | 10 | | | |
| | pCoumaric | 1.561 | 10 | | | |
| | Ferulic | 4.302E-02 | 10 | | | |

a. R Squared = .826 (Adjusted R Squared = .807)

b. R Squared = .457 (Adjusted R Squared = .397)

c. R Squared = .502 (Adjusted R Squared = .446)

d. R Squared = .468 (Adjusted R Squared = .409)

e. R Squared = .038 (Adjusted R Squared = -.069)

f. R Squared = .277 (Adjusted R Squared = .197)

Appendix 5: Univariate ANOVA: Effects of *Labyrinthula sp*. infection on the total phenolic content of *Thalassia testudinum* three, four, and seven days post infection.

**Tests of Between-Subjects Effects**

Dependent Variable: PHENOLIC

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2569.791[a] | 5 | 513.958 | 4.299 | .011 |
| Intercept | 21825.571 | 1 | 21825.571 | 182.570 | .000 |
| TIME | 1201.241 | 2 | 600.620 | 5.024 | .020 |
| INFECTIO | 16.430 | 1 | 16.430 | .137 | .716 |
| TIME * INFECTIO | 1368.586 | 2 | 684.293 | 5.724 | .013 |
| Error | 1912.741 | 16 | 119.546 | | |
| Total | 26948.814 | 22 | | | |
| Corrected Total | 4482.532 | 21 | | | |

a. R Squared = .573 (Adjusted R Squared = .440)

Appendix 6: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection on individual phenolic acid content of *Thalassia testudinum* three, four, and seven days post infection.

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | .644[a] | 5 | .129 | .221 | .950 |
| | PHBA | .313[b] | 5 | 6.255E-02 | .948 | .469 |
| | Vanillic | 1.089[c] | 5 | .218 | 1.106 | .384 |
| | Caffeic | 4.647[d] | 5 | .929 | 3.556 | .016 |
| | pCoumaric | .542[e] | 5 | .108 | .399 | .845 |
| | Ferulic | .163[f] | 5 | 3.263E-02 | .782 | .573 |
| Intercept | Protocatechuic | 24.674 | 1 | 24.674 | 42.243 | .000 |
| | PHBA | 14.227 | 1 | 14.227 | 215.617 | .000 |
| | Vanillic | 13.309 | 1 | 13.309 | 67.599 | .000 |
| | Caffeic | 55.809 | 1 | 55.809 | 213.544 | .000 |
| | pCoumaric | 2.786 | 1 | 2.786 | 10.250 | .004 |
| | Ferulic | .541 | 1 | .541 | 12.952 | .002 |
| INFECTION | Protocatechuic | 5.009E-02 | 1 | 5.009E-02 | .086 | .772 |
| | PHBA | .215 | 1 | .215 | 3.262 | .084 |
| | Vanillic | 1.546E-02 | 1 | 1.546E-02 | .079 | .782 |
| | Caffeic | 3.885 | 1 | 3.885 | 14.864 | .001 |
| | pCoumaric | 2.441E-02 | 1 | 2.441E-02 | .090 | .767 |
| | Ferulic | 3.280E-02 | 1 | 3.280E-02 | .786 | .385 |
| TIME | Protocatechuic | .339 | 2 | .169 | .290 | .751 |
| | PHBA | 7.894E-03 | 2 | 3.947E-03 | .060 | .942 |
| | Vanillic | .123 | 2 | 6.135E-02 | .312 | .735 |
| | Caffeic | .190 | 2 | 9.515E-02 | .364 | .699 |
| | pCoumaric | .340 | 2 | .170 | .625 | .544 |
| | Ferulic | 6.744E-02 | 2 | 3.372E-02 | .808 | .458 |
| INFECTION * TIME | Protocatechuic | .287 | 2 | .143 | .246 | .784 |
| | PHBA | 8.663E-02 | 2 | 4.331E-02 | .656 | .528 |
| | Vanillic | .954 | 2 | .477 | 2.423 | .111 |
| | Caffeic | .577 | 2 | .289 | 1.104 | .348 |
| | pCoumaric | .182 | 2 | 9.112E-02 | .335 | .719 |
| | Ferulic | 6.016E-02 | 2 | 3.008E-02 | .721 | .497 |
| Error | Protocatechuic | 13.434 | 23 | .584 | | |
| | PHBA | 1.518 | 23 | 6.598E-02 | | |
| | Vanillic | 4.528 | 23 | .197 | | |
| | Caffeic | 6.011 | 23 | .261 | | |
| | pCoumaric | 6.252 | 23 | .272 | | |
| | Ferulic | .960 | 23 | 4.173E-02 | | |
| Total | Protocatechuic | 38.469 | 29 | | | |
| | PHBA | 16.044 | 29 | | | |
| | Vanillic | 19.295 | 29 | | | |
| | Caffeic | 65.697 | 29 | | | |
| | pCoumaric | 9.514 | 29 | | | |
| | Ferulic | 1.688 | 29 | | | |
| Corrected Total | Protocatechuic | 14.079 | 28 | | | |
| | PHBA | 1.830 | 28 | | | |
| | Vanillic | 5.617 | 28 | | | |
| | Caffeic | 10.658 | 28 | | | |
| | pCoumaric | 6.794 | 28 | | | |
| | Ferulic | 1.123 | 28 | | | |

a. R Squared = .046 (Adjusted R Squared = -.162)

b. R Squared = .171 (Adjusted R Squared = -.009)

c. R Squared = .194 (Adjusted R Squared = .019)

d. R Squared = .436 (Adjusted R Squared = .313)

e. R Squared = .080 (Adjusted R Squared = -.120)

f. R Squared = .145 (Adjusted R Squared = -.041)

Appendix 7: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the total phenolic content of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

Dependent Variable: LTOTAL

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .556[a] | 3 | .185 | 1.646 | .276 |
| Intercept | 86.670 | 1 | 86.670 | 769.755 | .000 |
| INFECTION | .128 | 1 | .128 | 1.136 | .327 |
| LIGHT | .289 | 1 | .289 | 2.571 | .160 |
| INFECTION * LIGHT | 4.611E-02 | 1 | 4.611E-02 | .410 | .546 |
| Error | .676 | 6 | .113 | | |
| Total | 106.422 | 10 | | | |
| Corrected Total | 1.232 | 9 | | | |

a. R Squared = .452 (Adjusted R Squared = .177)

Appendix 8: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the individual phenolic acid content of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | .122[a] | 3 | 4.069E-02 | .282 | .837 |
| | PHBA | .323[b] | 3 | .108 | 1.161 | .383 |
| | Vanillic | 2.508E-02[c] | 3 | 8.361E-03 | .116 | .948 |
| | Caffeic | .333[d] | 3 | .111 | .934 | .468 |
| | pCoumaric | .502[e] | 3 | .167 | .290 | .831 |
| | Ferulic | 3.981E-02[f] | 3 | 1.327E-02 | 1.437 | .302 |
| Intercept | Protocatechuic | 6.130 | 1 | 6.130 | 42.538 | .000 |
| | PHBA | 3.118 | 1 | 3.118 | 33.621 | .000 |
| | Vanillic | 1.191 | 1 | 1.191 | 16.500 | .004 |
| | Caffeic | 8.668 | 1 | 8.668 | 72.984 | .000 |
| | pCoumaric | 1.597 | 1 | 1.597 | 2.772 | .134 |
| | Ferulic | 3.301E-02 | 1 | 3.301E-02 | 3.575 | .095 |
| INFECTION | Protocatechuic | .115 | 1 | .115 | .799 | .397 |
| | PHBA | .128 | 1 | .128 | 1.383 | .273 |
| | Vanillic | 4.035E-03 | 1 | 4.035E-03 | .056 | .819 |
| | Caffeic | 7.218E-02 | 1 | 7.218E-02 | .608 | .458 |
| | pCoumaric | 3.459E-03 | 1 | 3.459E-03 | .006 | .940 |
| | Ferulic | 1.684E-02 | 1 | 1.684E-02 | 1.824 | .214 |
| LIGHT | Protocatechuic | 2.451E-03 | 1 | 2.451E-03 | .017 | .899 |
| | PHBA | 1.772E-02 | 1 | 1.772E-02 | .191 | .674 |
| | Vanillic | 1.971E-02 | 1 | 1.971E-02 | .273 | .615 |
| | Caffeic | .115 | 1 | .115 | .969 | .354 |
| | pCoumaric | 3.225E-02 | 1 | 3.225E-02 | .056 | .819 |
| | Ferulic | 2.776E-03 | 1 | 2.776E-03 | .301 | .598 |
| INFECTION * LIGHT | Protocatechuic | 2.858E-04 | 1 | 2.858E-04 | .002 | .966 |
| | PHBA | 5.631E-02 | 1 | 5.631E-02 | .607 | .458 |
| | Vanillic | 7.427E-05 | 1 | 7.427E-05 | .001 | .975 |
| | Caffeic | .137 | 1 | .137 | 1.154 | .314 |
| | pCoumaric | .453 | 1 | .453 | .787 | .401 |
| | Ferulic | 1.094E-02 | 1 | 1.094E-02 | 1.185 | .308 |
| Error | Protocatechuic | 1.153 | 8 | .144 | | |
| | PHBA | .742 | 8 | 9.274E-02 | | |
| | Vanillic | .577 | 8 | 7.216E-02 | | |
| | Caffeic | .950 | 8 | .119 | | |
| | pCoumaric | 4.610 | 8 | .576 | | |
| | Ferulic | 7.387E-02 | 8 | 9.234E-03 | | |
| Total | Protocatechuic | 11.663 | 12 | | | |
| | PHBA | 7.345 | 12 | | | |
| | Vanillic | 2.425 | 12 | | | |
| | Caffeic | 14.888 | 12 | | | |
| | pCoumaric | 8.175 | 12 | | | |
| | Ferulic | .175 | 12 | | | |
| Corrected Total | Protocatechuic | 1.275 | 11 | | | |
| | PHBA | 1.065 | 11 | | | |
| | Vanillic | .602 | 11 | | | |
| | Caffeic | 1.283 | 11 | | | |
| | pCoumaric | 5.112 | 11 | | | |
| | Ferulic | .114 | 11 | | | |

a. R Squared = .096 (Adjusted R Squared = -.243)

b. R Squared = .303 (Adjusted R Squared = .042)

c. R Squared = .042 (Adjusted R Squared = -.318)

d. R Squared = .259 (Adjusted R Squared = -.018)

e. R Squared = .098 (Adjusted R Squared = -.240)

f. R Squared = .350 (Adjusted R Squared = .106)

Appendix 9: Univariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the total phenolic content of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

Dependent Variable: TOTAL

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 1518.823[a] | 3 | 506.274 | 2.942 | .138 |
| Intercept | 4254.851 | 1 | 4254.851 | 24.727 | .004 |
| INFECTION | 384.414 | 1 | 384.414 | 2.234 | .195 |
| SALINITY | 572.218 | 1 | 572.218 | 3.325 | .128 |
| INFECTION * SALINITY | 427.498 | 1 | 427.498 | 2.484 | .176 |
| Error | 860.378 | 5 | 172.076 | | |
| Total | 7819.100 | 9 | | | |
| Corrected Total | 2379.202 | 8 | | | |

a. R Squared = .638 (Adjusted R Squared = .421)

Appendix 10: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the individual phenolic acid content of *Thalassia testudinum.*

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | 13.283[a] | 3 | 4.428 | 1.922 | .197 |
| | PHBA | 3.067[b] | 3 | 1.022 | 1.285 | .338 |
| | Vanillic | .698[c] | 3 | .233 | 1.090 | .402 |
| | Caffeic | 9.658[d] | 3 | 3.219 | 1.448 | .293 |
| | pCoumaric | 9.410[e] | 3 | 3.137 | .589 | .637 |
| | Ferulic | 6.886E-03[f] | 3 | 2.295E-03 | .263 | .850 |
| Intercept | Protocatechuic | 29.821 | 1 | 29.821 | 12.942 | .006 |
| | PHBA | 7.136 | 1 | 7.136 | 8.966 | .015 |
| | Vanillic | 1.296 | 1 | 1.296 | 6.074 | .036 |
| | Caffeic | 33.706 | 1 | 33.706 | 15.160 | .004 |
| | pCoumaric | 10.146 | 1 | 10.146 | 1.905 | .201 |
| | Ferulic | 1.398E-02 | 1 | 1.398E-02 | 1.601 | .238 |
| INFECTION | Protocatechuic | 5.956 | 1 | 5.956 | 2.585 | .142 |
| | PHBA | 2.183 | 1 | 2.183 | 2.742 | .132 |
| | Vanillic | .375 | 1 | .375 | 1.756 | .218 |
| | Caffeic | 3.591 | 1 | 3.591 | 1.615 | .236 |
| | pCoumaric | 7.080 | 1 | 7.080 | 1.330 | .279 |
| | Ferulic | 4.121E-03 | 1 | 4.121E-03 | .472 | .509 |
| SALINITY | Protocatechuic | 1.500 | 1 | 1.500 | .651 | .440 |
| | PHBA | .192 | 1 | .192 | .241 | .635 |
| | Vanillic | 1.090E-02 | 1 | 1.090E-02 | .051 | .826 |
| | Caffeic | .596 | 1 | .596 | .268 | .617 |
| | pCoumaric | .526 | 1 | .526 | .099 | .760 |
| | Ferulic | 3.701E-04 | 1 | 3.701E-04 | .042 | .841 |
| INFECTION * SALINITY | Protocatechuic | 1.944 | 1 | 1.944 | .844 | .382 |
| | PHBA | 3.564E-03 | 1 | 3.564E-03 | .004 | .948 |
| | Vanillic | .178 | 1 | .178 | .834 | .385 |
| | Caffeic | 3.959 | 1 | 3.959 | 1.781 | .215 |
| | pCoumaric | .523 | 1 | .523 | .098 | .761 |
| | Ferulic | 1.211E-03 | 1 | 1.211E-03 | .139 | .718 |
| Error | Protocatechuic | 20.737 | 9 | 2.304 | | |
| | PHBA | 7.163 | 9 | .796 | | |
| | Vanillic | 1.920 | 9 | .213 | | |
| | Caffeic | 20.010 | 9 | 2.223 | | |
| | pCoumaric | 47.925 | 9 | 5.325 | | |
| | Ferulic | 7.858E-02 | 9 | 8.732E-03 | | |
| Total | Protocatechuic | 96.991 | 13 | | | |
| | PHBA | 29.163 | 13 | | | |
| | Vanillic | 5.579 | 13 | | | |
| | Caffeic | 93.978 | 13 | | | |
| | pCoumaric | 87.421 | 13 | | | |
| | Ferulic | .119 | 13 | | | |
| Corrected Total | Protocatechuic | 34.020 | 12 | | | |
| | PHBA | 10.231 | 12 | | | |
| | Vanillic | 2.618 | 12 | | | |
| | Caffeic | 29.668 | 12 | | | |
| | pCoumaric | 57.335 | 12 | | | |
| | Ferulic | 8.547E-02 | 12 | | | |

a. R Squared = .390 (Adjusted R Squared = .187)

b. R Squared = .300 (Adjusted R Squared = .066)

c. R Squared = .267 (Adjusted R Squared = .022)

d. R Squared = .326 (Adjusted R Squared = .101)

e. R Squared = .164 (Adjusted R Squared = -.114)

f. R Squared = .081 (Adjusted R Squared = -.226)

Appendix 11: Univariate ANOVA: Effects of *Labyrinthula sp.* infection and light level on the total phenolic content of *Thalassia testudinum* with the addition of previous data.

**Tests of Between-Subjects Effects**

Dependent Variable: TOTAL

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 991.217[a] | 3 | 330.406 | 2.582 | .098 |
| Intercept | 11020.792 | 1 | 11020.792 | 86.118 | .000 |
| INFECTION | 583.963 | 1 | 583.963 | 4.563 | .052 |
| LIGHT | 364.582 | 1 | 364.582 | 2.849 | .115 |
| INFECTION * LIGHT | 44.799 | 1 | 44.799 | .350 | .564 |
| Error | 1663.643 | 13 | 127.973 | | |
| Total | 15101.729 | 17 | | | |
| Corrected Total | 2654.861 | 16 | | | |

a. R Squared = .373 (Adjusted R Squared = .229)

Appendix 12: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and light level on the individual phenolic acid content of *Thalassia testudinum* with the addition of previous data.

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | .108[a] | 3 | 3.611E-02 | .115 | .950 |
| | PHBA | .144[b] | 3 | 4.803E-02 | .578 | .637 |
| | Vanillic | 1.067[c] | 3 | .356 | 3.881 | .027 |
| | Caffeic | 2.412[d] | 3 | .804 | 4.348 | .018 |
| | pCoumaric | .386[e] | 3 | .129 | .263 | .851 |
| | Ferulic | .133[f] | 3 | 4.447E-02 | 1.204 | .337 |
| Intercept | Protocatechuic | 12.039 | 1 | 12.039 | 38.403 | .000 |
| | PHBA | 6.646 | 1 | 6.646 | 79.940 | .000 |
| | Vanillic | 4.504 | 1 | 4.504 | 49.151 | .000 |
| | Caffeic | 18.882 | 1 | 18.882 | 102.144 | .000 |
| | pCoumaric | 3.473 | 1 | 3.473 | 7.120 | .016 |
| | Ferulic | .176 | 1 | .176 | 4.765 | .043 |
| INFECTION | Protocatechuic | 2.399E-02 | 1 | 2.399E-02 | .077 | .785 |
| | PHBA | 1.651E-04 | 1 | 1.651E-04 | .002 | .965 |
| | Vanillic | .194 | 1 | .194 | 2.117 | .163 |
| | Caffeic | 5.324E-02 | 1 | 5.324E-02 | .288 | .598 |
| | pCoumaric | .141 | 1 | .141 | .288 | .598 |
| | Ferulic | 2.704E-05 | 1 | 2.704E-05 | .001 | .979 |
| LIGHT | Protocatechuic | 2.541E-03 | 1 | 2.541E-03 | .008 | .929 |
| | PHBA | .135 | 1 | .135 | 1.622 | .219 |
| | Vanillic | .207 | 1 | .207 | 2.256 | .150 |
| | Caffeic | .670 | 1 | .670 | 3.622 | .073 |
| | pCoumaric | 8.246E-03 | 1 | 8.246E-03 | .017 | .898 |
| | Ferulic | 1.045E-02 | 1 | 1.045E-02 | .283 | .601 |
| INFECTION * LIGHT | Protocatechuic | .107 | 1 | .107 | .342 | .566 |
| | PHBA | 2.275E-02 | 1 | 2.275E-02 | .274 | .607 |
| | Vanillic | .265 | 1 | .265 | 2.892 | .106 |
| | Caffeic | 1.201 | 1 | 1.201 | 6.495 | .020 |
| | pCoumaric | .380 | 1 | .380 | .779 | .389 |
| | Ferulic | 9.744E-02 | 1 | 9.744E-02 | 2.638 | .122 |
| Error | Protocatechuic | 5.643 | 18 | .314 | | |
| | PHBA | 1.496 | 18 | 8.313E-02 | | |
| | Vanillic | 1.649 | 18 | 9.163E-02 | | |
| | Caffeic | 3.327 | 18 | .185 | | |
| | pCoumaric | 8.781 | 18 | .488 | | |
| | Ferulic | .665 | 18 | 3.694E-02 | | |
| Total | Protocatechuic | 23.916 | 22 | | | |
| | PHBA | 13.020 | 22 | | | |
| | Vanillic | 9.692 | 22 | | | |
| | Caffeic | 36.514 | 22 | | | |
| | pCoumaric | 14.157 | 22 | | | |
| | Ferulic | 1.063 | 22 | | | |
| Corrected Total | Protocatechuic | 5.751 | 21 | | | |
| | PHBA | 1.641 | 21 | | | |
| | Vanillic | 2.716 | 21 | | | |
| | Caffeic | 5.739 | 21 | | | |
| | pCoumaric | 9.166 | 21 | | | |
| | Ferulic | .798 | 21 | | | |

a. R Squared = .019 (Adjusted R Squared = -.145)

b. R Squared = .088 (Adjusted R Squared = -.064)

c. R Squared = .393 (Adjusted R Squared = .292)

d. R Squared = .420 (Adjusted R Squared = .324)

e. R Squared = .042 (Adjusted R Squared = -.118)

f. R Squared = .167 (Adjusted R Squared = .028)

Appendix 13: Univariate ANOVA: Effects of *Labyrinthula sp.* infection and salinity on the total phenolic content of *Thalassia testudinum* with the addition of previous data.

**Tests of Between-Subjects Effects**

Dependent Variable: TOTAL

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2159.093[a] | 3 | 719.698 | 4.711 | .021 |
| Intercept | 7245.763 | 1 | 7245.763 | 47.434 | .000 |
| INFECTION | 154.530 | 1 | 154.530 | 1.012 | .334 |
| SALINITY | 912.758 | 1 | 912.758 | 5.975 | .031 |
| INFECTION * SALINITY | 1690.509 | 1 | 1690.509 | 11.067 | .006 |
| Error | 1833.042 | 12 | 152.753 | | |
| Total | 14923.740 | 16 | | | |
| Corrected Total | 3992.135 | 15 | | | |

a. R Squared = .541 (Adjusted R Squared = .426)

Appendix 14: Multivariate ANOVA: Effects of *Labyrinthula sp*. infection and salinity on the individual phenolic acid content of *Thalassia testudinum* with the addition of previous data.

**Tests of Between-Subjects Effects**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Corrected Model | Protocatechuic | 1.189[a] | 3 | .396 | 1.128 | .363 |
| | PHBA | .596[b] | 3 | .199 | 2.084 | .136 |
| | Vanillic | 1.612[c] | 3 | .537 | 5.532 | .007 |
| | Caffeic | 3.456[d] | 3 | 1.152 | 4.878 | .011 |
| | pCoumaric | 1.136[e] | 3 | .379 | .687 | .571 |
| | Ferulic | .128[f] | 3 | 4.261E-02 | 1.237 | .324 |
| Intercept | Protocatechuic | 15.030 | 1 | 15.030 | 42.779 | .000 |
| | PHBA | 6.404 | 1 | 6.404 | 67.184 | .000 |
| | Vanillic | 3.399 | 1 | 3.399 | 34.987 | .000 |
| | Caffeic | 18.137 | 1 | 18.137 | 76.790 | .000 |
| | pCoumaric | 4.152 | 1 | 4.152 | 7.541 | .013 |
| | Ferulic | .110 | 1 | .110 | 3.195 | .090 |
| INFECTION | Protocatechuic | .510 | 1 | .510 | 1.451 | .243 |
| | PHBA | .266 | 1 | .266 | 2.790 | .111 |
| | Vanillic | 3.731E-05 | 1 | 3.731E-05 | .000 | .985 |
| | Caffeic | 6.401E-02 | 1 | 6.401E-02 | .271 | .609 |
| | pCoumaric | .868 | 1 | .868 | 1.576 | .225 |
| | Ferulic | 9.874E-03 | 1 | 9.874E-03 | .287 | .599 |
| SALINITY | Protocatechuic | 4.581E-02 | 1 | 4.581E-02 | .130 | .722 |
| | PHBA | .285 | 1 | .285 | 2.991 | .100 |
| | Vanillic | .702 | 1 | .702 | 7.222 | .015 |
| | Caffeic | 1.242 | 1 | 1.242 | 5.257 | .033 |
| | pCoumaric | 3.727E-02 | 1 | 3.727E-02 | .068 | .798 |
| | Ferulic | 4.457E-02 | 1 | 4.457E-02 | 1.294 | .270 |
| INFECTION * SALINITY | Protocatechuic | .799 | 1 | .799 | 2.274 | .148 |
| | PHBA | .435 | 1 | .435 | 4.560 | .046 |
| | Vanillic | 1.000 | 1 | 1.000 | 10.292 | .005 |
| | Caffeic | 2.668 | 1 | 2.668 | 11.294 | .003 |
| | pCoumaric | .462 | 1 | .462 | .840 | .371 |
| | Ferulic | 4.841E-02 | 1 | 4.841E-02 | 1.405 | .250 |
| Error | Protocatechuic | 6.676 | 19 | .351 | | |
| | PHBA | 1.811 | 19 | 9.532E-02 | | |
| | Vanillic | 1.846 | 19 | 9.716E-02 | | |
| | Caffeic | 4.488 | 19 | .236 | | |
| | pCoumaric | 10.463 | 19 | .551 | | |
| | Ferulic | .655 | 19 | 3.445E-02 | | |
| Total | Protocatechuic | 29.616 | 23 | | | |
| | PHBA | 14.229 | 23 | | | |
| | Vanillic | 9.942 | 23 | | | |
| | Caffeic | 39.337 | 23 | | | |
| | pCoumaric | 18.233 | 23 | | | |
| | Ferulic | .985 | 23 | | | |
| Corrected Total | Protocatechuic | 7.864 | 22 | | | |
| | PHBA | 2.407 | 22 | | | |
| | Vanillic | 3.458 | 22 | | | |
| | Caffeic | 7.944 | 22 | | | |
| | pCoumaric | 11.598 | 22 | | | |
| | Ferulic | .782 | 22 | | | |

a. R Squared = .151 (Adjusted R Squared = .017)

b. R Squared = .248 (Adjusted R Squared = .129)

c. R Squared = .466 (Adjusted R Squared = .382)

d. R Squared = .435 (Adjusted R Squared = .346)

e. R Squared = .098 (Adjusted R Squared = -.045)

f. R Squared = .163 (Adjusted R Squared = .031)