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Defensive Roles And Factors That Affect The Production Of Monoterpenes in Morella Cerifera

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DEFENSIVE ROLES AND FACTORS THAT AFFECT THE PRODUCTION OF MONOTERPENES IN *MORELLA CERIFERA*

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DEDICATION

This dissertation is dedicated to my children (Prince, Chinma (Nancy), Franklin, and Nnenna Anoruo) and to my parents (Mr. and Mrs. Sunday Ndimele Ukauwa). Your steadfast and unconditional love and support inspired and encouraged me to accomplish this great milestone in my life. To my cousin, Dr. Nwabueze Ehiobuche, your incessant support, encouragement, and love throughout this whole process is greatly appreciated. Thank you and l love you all.

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ABSTRACT

Plants as sessile organisms encounter myriad biotic and abiotic challenges in their habitats. Pathogen and herbivore attack are among the prominent biotic challenges that plants face in their environment. Plants respond to these attacks by using chemical compounds including monoterpenes that are constitutively and inductively produced in some plants, and stored inside glands on their leaves. Among the abiotic factors that influence the production of defense compounds, especially monoterpenes are light intensity and nutrient availability.

The objectives of this dissertation are to 1) comprehend the defensive role of monoterpenes, specifically investigating the associational defense of a non-odorous species (*Ilex vomitoria*) co-occurring with other odorous species (*Morella cerifera and Iva frutescens*), along the marsh edge at Goat Island, Belle Baruch Hobcow Barony in Georgetown, South Carolina, 2) to evaluate the effects of *Morella cerifera-Frankia* symbiotic association on leaf monoterpene production, and the relationship between nitrogen availability and rate of nitrogen fixation by *Frankia*, 3) to determine the combined effect of light intensity and nutrient availability on monoterpene production in *Morella cerifera.* . Results indicated that leaf damage was significantly higher in both monocultures of *I. vomitoria* and *M. cerifera*. However, predation was significantly lower in the mixed species culture. The lowest level of predation was observed in the three species combination of *I. vomitoria*, *M. cerifera*, and I. *frutescens.* Although the nitrogen fixation rate within the 1/4 strength Hoagland inoculated treatment group was higher than

full strength Hoagland inoculated, and the un-inoculated groups, the observed difference was not statistically significant F (3, 16) = 1.447, p = .266. Analysis of average monoterpene concentration revealed a statistically significant difference for the four treatment groups, (F (3, 12) = 34.11, p < .001). There was a positive significant correlation between nitrogen fixation rate and monoterpene production in the full strength Hoagland inoculated (FS H) treatment group (r = 0.81, p < 0.01). Additionally, a statistically significant difference in monoterpene concentration was observed between the plants in the native marsh edge and forest interior, $F_{1,8}$ = 200.45, p < 0.000005. The fertilized and unfertilized treatments within the forest interior were also significantly different. Monoterpene production was highest among the plants growing under high intensity and high nitrogen soil concentration, therefore highlighting the sygergistic influence of light and nutrient availability on plant defense mechanism.

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ASSOCIATIONAL DEFENSE OF *ILEX VOMITORIA*, *MORELLA CERIFERA*,AND *IVA FRUTENSCENS*

1.1 ABSTRACT

Prevention/reduction of tissue damage of a palatable or less chemically defended species from phytophagous predators by virtue of association with non-palatable or chemically defended neighbors has been observed in some plant communities. *Ilex vomitoria (Aquifoliaceae)*, (a non-odorous species) *Iva frutescens (Asteraceae)*, and *Morella cerifera (Myricaceae)* (both odorous species) inhabit the transition zone of shrub/forest edge of salt marshes along the South Eastern coast of the United States. This study investigated level of herbivory as measured by leaf damage within experimental monocultures of either *Ilex vomitoria* or *Morella cerifera* and mixed species stands of *I. vomitoria* neighbored by either *M. cerifera* or *M. cerifera* and *I. frutescens*. Similar mixed species stands with *M. cerifera* neighbored by either *I. vomitoria* or *I. vomitoria* and *I. frutescens* were also established. All three species were transplanted from Goat Island at the Belle Baruch Hobcow Barony in Georgetown, South Carolina and grown under greenhouse conditions. The plants were enclosed in 1.2 x 1.2 m wire mesh cages with two *Schistocerca americana* (*Orthoptera*) introduced as native predators in each cage. The two *S. americana* were removed after 36 hours and a VistaMetrix® image analyzer was used to measure total leaf area loss. Results indicated that leaf damage was significantly higher in both monocultures of *I. vomitoria* and *M. cerifera*. However,

predation was significantly lower in the mixed species culture. The lowest level of predation was observed in the three species combination of *I. vomitoria*, *M. cerifera*, and I. *frutescens.*

1.2 INTRODUCTION

In plant biology the principal paradigm often referred to as "the dilemma of plants" is the trade-off between growth, reproduction, and defense (Herms and Mattson, 1992, Ballare et al., 2012 Pierik et al., 2014). Volatile organic compounds produced by plants, especially monoterpenes, play a crucial role in plant ecology (Pierik et al., 2014). Plant-plant interactions with their environment are often mediated by these volatile compounds, and have evolved over time. Many studies have implicated plant volatiles in playing major roles in defense against herbivores and attraction of pollinators (Schoohhoven et al., 2005, Raguso, 2008, Kessler and Halitschke, 2009, Dicke and Baldwin, 2010, Dicke and Loreto, 2010, Bruce and Pickett, 2011, Lucas-Barbosa et al. 2011).

Other studies have also reported involvement of plant volatiles in interactions of plants with their neighbors within the communities (Dicke and Bruin, 2001, Baldwin et al., 2006, Heil and Karban 2010, Glinwood et al., 2011). Plants exhibit two types of defenses in their habitats: 1) direct defenses, mediated by plant characteristics that affect the herbivore's behavior and function (e.g. hairs, trichomes, thorns, spines, and thicker leaves) or production of toxic compounds (terpenoids, alkaloids, anthocyanins, phenols, and quinones) that either kill or impede the development of the herbivores (Motifer and Boland, 2012; Pierik et al., 2014), and 2) indirect defenses which boost the effectiveness

of natural enemies of herbivores either through provision of alternative food sources or production of volatile organic compounds (VOCs) that attract enemies of their herbivorous victim (Vet and Dicke, 1992; Dicke and Baldwin, 2010; Hilker and Meiners, 2011).

Plants respond to attack from herbivores by using chemical compounds that are constitutively produced or induced and stored inside glands or trichomes on the leaves or in other leaf cells (Turlings et al., 1995; Kessler and Baldwin 2002; Dicke 2009). These constitutive and induced defense compounds have been reported to affect herbivore settling, feeding, oviposition, growth and development, fecundity and/or fertility (Bernays and Chapman, 1994; Baldwin and Preston 1999; Pare and Tumlinson, 1999; Walling 2000, 2001).

The effectiveness of a plant defense is dependent on the structural, physiological and biochemical characteristics of the individual plant, as well as that of its neighbors (Rautioa et al., 2008; Miller et al., 2007). Volatile organic compounds produced systemically and inducibly upon attack by herbivores have been shown to deter or prevent future predation (Pare and Tumlinson, 1999; Niinemets et. al., 2004; Rose and Tumlinson, 2005). The susceptibility of a palatable or less chemically defended plant to herbivory may be altered by the spatial arrangement of unpalatable or well chemically defended plants around it (Marie et al., 2006). Such associational defense has been reported in several studies (Baldwin and Preston, 1999; Walling 2000; Bergvail et al., 2006; Miller et. al., 2006., Miller et. al., 2007). Conversely, the resistance of a chemically well defended plant to phytophagous predators may in some cases be compromised by cohabitation with palatable or less chemically defended neighbors. This

phenomenon is known as associational susceptibility (Stiling et al., 2003; Marie et al., 2006) and has been reported by Hamback et al. (2000). The degree of protection of an individual plant thus can be influenced by the palatability as well as the defensive characteristics of its neighbors (Bergvail et. al., 2006; Miller et. al., 2007).

Several factors can contribute to this ecological phenomenon: identity of the focal plant; identity of the neighboring plants, proximity of the neighbors, host preference and characteristics of the phytophagous predator (Marie et al 2006; Atsatt & O'Dowd 1976; White & Whitman 2000; Vehvilainen et al., 2006). Studies focusing on the interaction between species composition with a given plant community and rate of herbivory or predation have been conducted mainly on agricultural crops and small shrubs and other perennials (Marie et al., 2006, Stiling et al., 2003, Miller et al., 2007, Hamback et al., 2000). *Ilex vomitoria*, *Iva frutescens*, and *Morella cerifera* inhabit the transition zone of shrub/forest edge of salt marshes along the South Eastern coast of the United States. These three species coexist on numerous Atlantic Coast Barrier Islands (Wijnholds and Young 2000). *Morella cerifera* (L.) (Myricaceae) and *Iva frustescens* L. (Asteraceae) are both odorous plants containing numerous compounds mostly belonging to the monoterpene and sesquiterpene group of metabolites (Degenhardt and Lincoln, 2006; Cheynier et al., 2013). Both species produce systemic and herbivore induced volatile organic compounds (VOCs). Degendhardt and Lincoln (2006) identified approximately 99 different compounds of the leaf volatiles of *Iva frustescens*. *Iva frustescens* and *Morella cerifera* share several volatile compound in common such as α–pinene, β-pinene, β-carophyllene, β-eudesmol, α-trans-bergamotene, α-phellandrene, terpinolene, and γ-curcurmine (Degenhardt and Lincoln, 2006; Sylvester et al., 2005). However, *Morella*

cerifera and *Iva frustescens* also produce compounds that are specific and unique to the individual species. Among some of the compounds produced by *Iva frustescens,* but not present in *Morella cerifera,* are sabinene, garmacrene, and β-farnescene. Likewise, some of the compounds specifically produced by *Morella cerifera,* but not by *I. frustescens,* include α-thujene, α-ocimene, β-ocimene, limonene, and myrcene (Degenhardt and Lincoln, 2006; Sylvester et al., 2005). *Ilex vomitoria* Aiton (Aquifoliaceae), a non odorous plant, which co-occurs with these species in the field, lacks these VOCs but contains a suite of other types of terpenoids, saponins, polyphenols and glycosides, as well as alkaloids, including caffeine (Hao et al., 2015). The relative abundance of the volatile organic compounds produced by *Morella cerifera* and *Iva frustescens*, which are known to both prevent and deter herbivory (Baldwin and Preston 1999, Walling, 2000; Bergvail et al., 2006; Miller et. al., 2007), is expected to provide a higher level of protection to *Morella cerifera* and *Iva frustescens,* compared to *Ilex vomitoria* which lacks these compounds (Bergvail et. al., 2006; Miller et. al., 2007; Pierik et al., 2014).

This laboratory study investigated the level of predation as measured by leaf damage amongst a monoculture of either the non-odorous species *Ilex vomitoria,* or the odorous species *Morella cerifera*, versus leaf damage in a mixed species culture of a non-odorous species *I. vomitoria* neighbored by either *M. cerifera* or by *M. cerifera* and *I. frutescens,* which are both odorous species. We tested the hypothesis that *Ilex vomitoria* growing in a monoculture will encounter higher level of phytophagous predation by a highly polyphagocious insect compared to those growing in mixed species culture with odorous species. The selected insect is routine to these coastal communities. We

predicted that the lowest level of herbivory on *I. vomitoria* would be observed in the three species mixed culture of *I. vomitoria*, *M. cerifera,* and *Iva frutescens.*

1.3 METHODS AND MATERIALS

Plant species

Ilex vomitoria (a non-odorous species), and two odorous species, *Iva frutescens*, and *Morella cerifera* inhabit the transition zone of shrub/forest edge of salt marshes along the South Eastern coast of the United States. All three species coexist on numerous Atlantic Coast Barrier Islands (Wijnholds and Young 2000). They were transplanted into 20 cm plastic pots from Goat Island at the Belle Baruch Hobcaw Barony in Georgetown, South Carolina, USA (33.32N, 79.20W) in late spring, and grown under greenhouse conditions. Day/night time temperatures were approximately 27/21°C respectively. The plants were watered daily or as needed, and fertilized with Miracle Grow all purpose fertilizer (NPK 20:20:20) biweekly prior to their use in the experiment.

Greenhouse Study

Branches of three plant species (*M. cerifera, I. frutescens,* and *I. vomitoria*) were grouped as follows :

Group 1 –three branches of *Morella* representing a monoculture of a species with the same odorous compounds.

Group 2 –one branch of *Morella* and two branches of *Iva* representing a mixed species culture producing partially related odorous compounds i.e. *Morella* co-occurring with other odorous neighbors.

Group 3 –two branches of *Morella* and one branch of *Ilex* representing odorous species co-occurring with non-odorous neighbors.

Group 4 –3 branches of *Ilex* representing a monoculture of a non-odorous species.

Group 5- 1 branch of *Iva*, 1 branch of *Morella*, and 1 branch of *Ilex* representing two odorous species co-occurring with non-odorous neighbors.

A completely randomized design was used in placement of the cages in the greenhouse. The species were enclosed in 1.2 x 1.2 m wire mesh cages with two *Schistocerca americana* (Drury, 1770) (Orthoptera, Acrididae), a generalist grasshopper herbivore, introduced as natural predators into each cage. The experiment consisted of the five treatments listed above with four replicates per treatment for a total of twenty cages. The branches within each cage were inserted into one liter glass bottles filled with water and covered with aluminum. To ensure that the leaves were un-chewed and each cage contained the same number of leaves for each species combination, the leaves were counted and thoroughly inspected. Each combination within the cage was replicated four times. The *S. americana* were removed 36 hours after introduction into the cages for each trial, and replaced with new sets of insects to eliminate the effects of learned behavior in feeding choice selection. The leaves of each species were excised thereafter and measured for total leaf area loss with a VistaMetrix® image analyzer. Area of bite marks were measured and total leaf area loss calculated using the formula below:

$$[(\text{AREA OF LEAF} - \text{AREA OF LEAF EATEN})/\text{AREA OF LEAF}] \times 100$$

Rearing of *Schistocerca americana*

Eggs of *Schistocerca americana* were obtained from the Agricultural Research Laboratory, Insect Rearing Lab, Sydney, Minesota. Eggs were incubated at 30 °C for approximately two weeks. Once hatched the *S. americana* were reared in plastic storage containers fitted with screen door wire mesh (1.6 mm openings) at the top and bottom.

The insects were kept at room temperature with a high intensity lamp approximately 25.4 cm above the cage to maintain a day time temperature of 25.6 - 26.7 °C. They were kept on a 14/10 hour day/night time light cycle. Their diet consisted of romaine lettuce and wheat germ. Moist cotton balls were placed in petri dishes inside the rearing cage to maintain moisture. The cotton balls were changed every other day for proper sanitation inside the cage. The insects were allowed to grow to the fourth instar before they were used in this study.

Statistical Analysis

An inferential statistical technique, one-way analysis of variance (ANOVA) was performed to compare the means of the un-chewed leaf areas of the species between treatments within the cages. The necessary data assumptions required for ANOVA such as normality in data, and homogeneity of variances were confirmed (normality: Kolmogorov-Smirnov and Shapiro-Wilk tests, homogeneity of variance: Levene's test), therefore no transformation of data was done. Tests of significance for difference in un-chewed percentages of leaves for the three species by treatment combinations were also conducted using post hoc Tukey multiple comparison tests. The last column of Table 1 presents the test results from ANOVA for all the three species. The results of the post hoc

Tukey multiple comparison tests are indicated by alphabet notations in first column of Table 1. Data analysis was conducted with SPSS Statistics 20.

1.4 RESULTS

The mean percentage of un-chewed leaf area (signifying level of protection) indicates that the non-odorous species (*I. vomitoria*) was most protected from herbivory when in combination with two odorous species *(Iva-Ilex-Morella*) (mean percentage un-chewed area = 99%) (Table 1.1, Fig. 1.1). The next best protection (mean percentage un-chewed area = 94%) for non-odorous *Ilex* was observed when it was neighbored by one odorous plant (*Ilex- Morella- Ilex*), and it was least protected (mean percentage un-chewed area = 89%) if it was planted in a monoculture (all non-odorous: *Ilex- Ilex- Ilex*). All of these differences were statistically significant from one another (Table 1.1).

The odorous species (*Morella*) was found to be most protected (highest percentage un-chewed) (mean percentage un-chewed area = 98%) when neighbored by two non-odorous plants (*Ilex- Morella - Ilex*) and least protected (mean percentage un-chewed area = 92%) when planted in a monoculture of all odorous species (*Morella- Morella - Morella*). Additionally, the odorous species *Morella* was found to be better protected (mean percentage un-chewed area = 95%) when planted in all odorous combination but surrounded by other odorous species (*Iva – Morella - Iva*) than in a *Morella* monoculture. It was slightly better protected (mean percentage un-chewed area = 97%) when planted with one non-odorous and one other odorous plant (*Iva-Morella- Ilex*). The level of

damage to leaves in the monoculture of *Morella* was significantly greater than that in all other treatments (Table 1.1).

The second odorous species (*Iva*) was less protected (mean percentage un-chewed area = 98%) when planted with itself plus a second odorous species (*Iva-Morella-Iva*) than when co-occurring with one odorous and one non-odorous species (*Iva-Morella-Ilex*) (mean percentage un-chewed area = 99%) (Fig. 1.1). These differences however were not statistically significant.

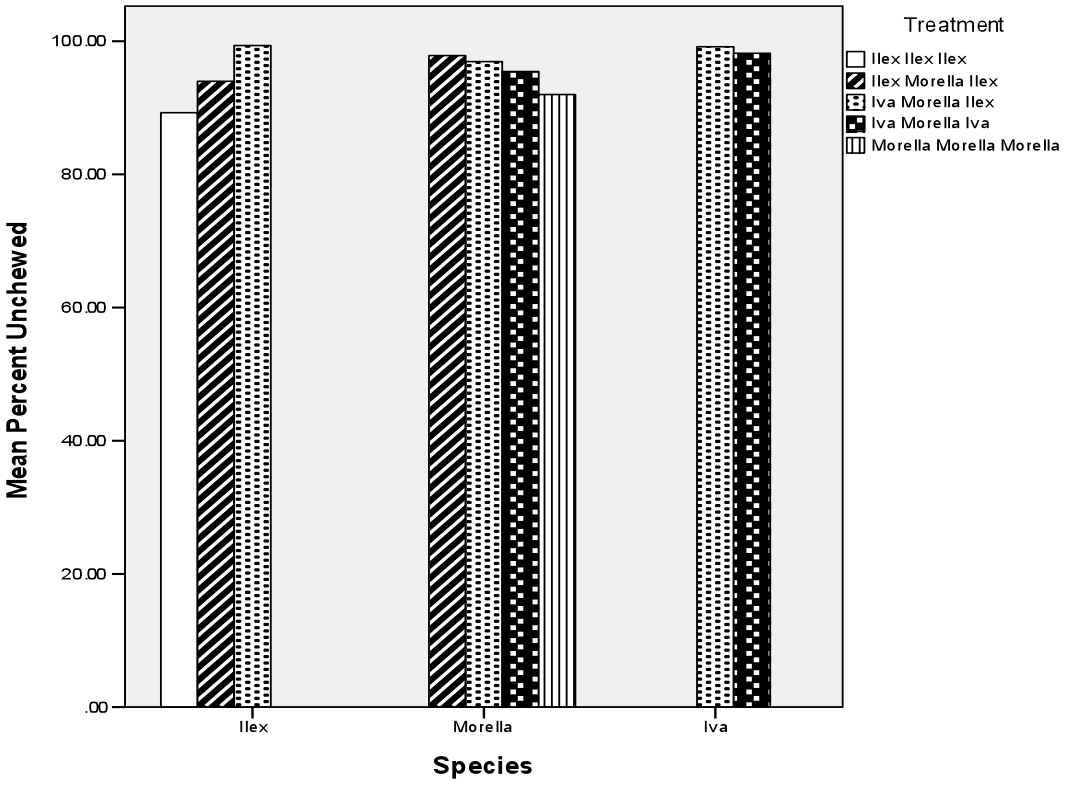

Figure 1.1: Average un-chewed percentages of leaves by species and treatments, representing the level of protection for the non-odorous and odorous species.

Table 1.1: Descriptive statistics of mean percent of un-chewed area of leaves of the three species by treatment types along with ANOVA *F* test of differences in means.

Species and Treatments	Descriptive Statistics						ANOVA *F* Test
	N	Mean	Median	Min	Max	SD	
Ilex							
Ilex Ilex Ilex[a]	4	89	89	86	94	3.59	$F(2, 9) = 22.07$, $p < 0.001$
Ilex Morella Ilex[b]	4	94	94	93	95	0.95	
Iva Morella Ilex[c]	4	99	99	99	99	0.20	
Morella							
Ilex Morella Ilex[a]	4	98	97	95	99	1.97	$F(3, 12) = 13.78$, $p < 0.001$
Iva Morella Ilex[a]	4	97	97	96	97	1.44	
Iva Morella Iva[a]	4	95	96	94	97	1.09	
Morella Morella Morella[b]	4	92	92	92	93	0.73	
Iva							
Iva Morella Ilex[a]	4	99	99	98	99	0.53	$F(1, 6) = 4.38$, $p = 0.081$
Iva Morella Iva[a]	4	98	98	97	99	0.78	

Note: [a, b, c]: Different letters present significant difference from post hoc Turkey test.

1.5 DISCUSSION

The results for *I. vomitoria* clearly support the associational resistance/defense theory that chemically defended or unpalatable plants reduce herbivore damage to palatable

plant species within their vicinity (Hamback et al., 2000; Stiling et al. 2003; Bergvall et al., 2005; Miller et al., 2007). Miller et al.(2007) and Stiling et al., 2003) reported that the degree of herbivory on a focal plant is dependent not only on physical, physiological, and chemical characteristics of the focal plant but also on its neighbors. Other studies have reported similar findings (Dicke and Bruin, 2001; Baldwin et al., 2006; Heil and Karban 2010; Glinwood et al., 2011). The high percentage of undamaged leaves observed in the mixed species culture where *I. vomitoria,* a non-odorous species, was neighbored by *M. cerifera* and *I. frustescens*, is a testament to associational defense. It is also evident that species diversity plays a vital role in plant-herbivore interaction. The observed higher percentage of damaged leaves in *M. cerifera* monoculture may be due to herbivore's inability to locate optimal or preferred food choice when only defended leaves are present (Fig. 1.1).

1.6 CONCLUSION

Results indicate that leaf damage was significantly higher in monoculture stands of both *I. vomitoria* and *M. cerifera* and predation on leaves of *I. vomitoria* was significantly lower in the mixed species combinations of *M. cerifera* and *I. vomitoria*, *M. cerifera I. vomitoria*, and *I. frutescen* (Table 1.1). These results are consistent with our hypotheses that species occurring in a monoculture in any given habitat are more likely to encounter higher level of phytophagous predation compared to those growing in mixed species culture. The higher levels of herbivory observed in both the *Ilex vomitoria* and *Morella cerifera* monoculture combinations attest to these predictions. Similarly, the

lower levels of phytophagous predation detected in the three species mixed culture further affirm the prediction (Table 1.1).

The combined synergistic effects of the related volatile organic compounds produced by both *M. cerifera,* and *Iva frutescens* may have contributed to the observed lower percentage of leaf damage in the mixed species culture combinations within the cages, compared to the monoculture species combinations. Finally, although *Ilex vomitoria* is a non-odorous species, and incurred significantly lower level of predation in the presence of the two odorous species, the unique phytochemicals it produced provided some level of protection from herbivory in the monoculture setting.

These observations support the fact that species diversity not only plays an important role in ecosystem dynamics and health, but is also important in chemically mediated intra- and inter-species interactions within an ecosystem. The three plant species selected for this study naturally co-exist on numerous Atlantic Coast Barrier Islands, and the experimental treatments may mimic what could be obtainable in the natural environment or field conditions.

CHAPTER 2

INTERACTION BETWEEN SOIL NITROGEN AVAILABILITY, *FRANKLIA* INOCULATION, RATE OF NITROGEN FIXATION, AND MONOTERPENE PRODUCTION

2.1 ABSTRACT

This study determined the rate of nitrogen fixation and monoterpene production in seedlings of *Morella cerifera (Myricaceae)* inoculated with *Frankia*, a nitrogen fixing actinomycete. Germinated seedlings of *Morella cerifera* were planted into 3.79L plastic pots in surface sterilized sand medium. Two groups of 10 plants each were inoculated with *Frankia* spores and fertilized with two levels of nitrogen (1/4 strength and full strength Hoagland). Two additional groups of 10 plants each were un-inoculated but also received two levels of nitrogen fertilization (1/4 and full strength Hoagland). The un-inoculated plants were separated from the inoculated group in a separate growth chamber. After growth of seven weeks under the treatment conditions, an acetylene reduction assay was used to measure the rate of nitrogen fixation, and monoterpene production was evaluated using GC-MS. Although the nitrogen fixation rate within the 1/4 strength Hoagland inoculated treatment group was higher than full strength Hoagland inoculated, and the un-inoculated groups, the observed difference was not statistically significant F (3, 16) = 1.447, p = .266. Analysis of average monoterpene concentration revealed a statistically significant difference for the four treatment groups, (F (3, 12) = 34.11, p < .001). There was a positive significant correlation between nitrogen fixation rate and

monoterpene production in the full strength Hoagland inoculated (FS H) treatment group ($r = 0.81$, $p < 0.01$). No overall significant negative correlation between nitrogen supply and monoterpene production was observed.

2.2 INTRODUCTION

Monoterpene production can be affected by biotic and abiotic conditions within a species habitat (Karban and Myers, 1989). Among the abiotic factors that influence monoterpene production are high light intensity and temperature (Wang and Lincoln, 2004), and soil nutrient availability (Mihaliak and Lincoln 1985, 1989, Lerdual et al., 1995). High light intensity and temperature, low nutrient availability, heat shock, and water stress lead to increased monoterpene production (Wang and Lincoln, 2004). Mihaliak and Lincoln (1985, 1989) reported increased mono- and sesquiterpenes, and lower levels of herbivory in *Hetherotheca subaxillaris* growing in low nitrogen supply conditions. Nutrients (carbon and nitrogen) which are normally used for vegetative growth and reproduction can be diverted to defense in response to herbivore attack (Baldwin and Preston, 1999). Studies conducted by Burney et al., (2012) investigating the effects of stimulated browsing and nutrient availability on terpenoid synthesis of three tree species indicated prioritization of resources towards production of terpenoids under increased herbivory and limited nutrient availability.

Plants apparently channel more carbon to the synthesis of carbon-based volatile organic compounds to reduce tissue losses to herbivory under nitrogen limiting conditions (Mihaliak and Lincoln, 1985 and 1989, Coviella et al., 2000, Walling 2000). Mihaliak and Lincoln (1985, 1989) and Coviella et al., (2000) have shown that high

carbon/nitrogen ratio is positively correlated with elevated monoterpene production, and inversely correlated with growth rate. Thus, nitrogen availability not only influences the photosynthetic rate of a species, it may also mediate allocation of carbon to anti-herbivore leaf chemicals (Mihaliak and Lincoln 1985, 1989, Coviella et al. 2000). Herbivory by insects and other animals decreases the photosynthetic capability of plant species, thus inhibiting metabolic functions such as growth and production of secondary metabolites (Mabry and Wayne, 1997, Vourc'h et al., 2003).

Nutrient availability has been suggested to aid plants in recuperating from herbivory by adding to the total resource budget. Addition of nitrogen to the soil has been reported to enhance the overall photosynthetic capacity of the Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings, by increased production of chloroplastic cells, as well as photosynthetic rates (Manter et al., 2005). The enhanced soil nitrogen consequently increases products of photosynthesis which are converted to the precursors of amino acids used for both plant growth and biosynthesis of defensive compounds (Tingey et al. 1980, Buchanan et al., 2000, Niinemets et al. 2002). Since the precursors of both amino acids and terpenoids have same origin (glucose), their biosynthesis may be in direct competition. However, increased nitrogen availability added exogenously or via nitrogen fixation by symbiotic microbes such as *Frankia* can counteract this problem.

Allocation of the carbon towards either amino acid or terpenoid biosynthesis may be based on the carbon/nutrient balance hypothesis (CNBH) (Blanch et al. 2007; Burney et al., 2012). The carbon/nutrient balance hypothesis posits that an increase in available nutrient (such as nitrogen fertilization) would reduce the production of secondary

metabolites, thereby channeling the resources to plant growth (Penuelas and Estiare 1998, Blanch et. al. 2007). This suggests that available carbon (by-product of photosynthesis) is preferentially channeled toward production of amino acids, rather than terpenoids. Resource allocation towards the biosynthesis of defensive secondary metabolites at the expense of plant growth to deter herbivory may play a pivotal role in the survival of the plant species in their habitats (Burney and Jacobs 2011; Burney et al., 2012).

Actinorhizal plants are distinguished by their ability to form nodules in symbiosis with nitrogen fixing actinomycetes of the genus *Frankia* (Alkermans et al., 1992, Mirza et al., 2009). Actinorhizal species such as *Morella cerifera* can obtain up to 90% of their nitrogen requirement from symbiotic association with actinomycetes, and are therefore uniquely successful pioneer plants that often establish themselves on nutrient –limited or degraded soil, as well as soils impacted by catastrophic events (Dawson, 1986; Domenach et al., 1989; Roy et al., 2007). Several studies have reported the positive effects of inoculation of actinorhizal plants with *Frankia* on plant establishment and overall growth and performance, and this has become a recommended practice to improve the successful establishment of forestry crops (Huss-Danell and Frej, 1986, Ridgeway et al., 2004; Yamanaka & Okabe, 2006). Understanding the role of nitrogen availability in the soil on the nitrogen fixing capacity of an actinomycete (*Frankia*) in association with an actinorhizal plant (*Morella cerifera*) is crucial in plant defense allocation. We investigated the effects of nitrogen availability (through N fertilization and *Frankia* inoculation) on nitrogen fixing capacity of *Morella cerifera* and evaluated the effects on monoterpene production.

2.3 MATERIALS AND METHODS

Seed Germination and Plant Growth

Seeds of *Morella cerifera* were collected along the marsh edge at Goat Island, Hobcaw Barony of Belle W. Baruch Foundation at the end of the growing season. The seeds were scarified using steel wool to quicken germination. The scarified seeds were surfaced sterilized in 10% sodium hypochlorite for 5 minutes and rinsed twice with distilled water prior to broadcasting in germination trays containing a sterilized sand, vermiculite, and perlite mixture (3:2:1). Approximately 5 weeks after broadcasting the seeds, they were transplanted into 3.79 L plastic pots. All seedlings were maintained at 14/10 hour night/day photoperiod at daytime and nighttime temperatures of 27ºC and 22ºC respectively in the growth chamber.

Inoculation of Seedlings with *Frankia* spores

Spores from excised *Frankia* nodules obtained from established *Morella cerifera* plants growing along the marsh edge at Goat Island, Hobcaw Barony of Belle W. Baruch Foundation were used to inoculate the seedlings according to published protocols (Reddell and Bowen, 1985, Tian et al. 2001). Briefly, individual nodules isolated from roots of *Morella cerifera* were surfaced sterilized in 30% H_2O_2 for 20 minutes, rinsed three times in sterile distilled water and homogenized in a blender with sterile 1% saline solution. An initial volume of 5 mL of the saline solution used in homogenizing the nodules was diluted to 30 mL. Subsequently, 6 mL of the suspension was removed and diluted to 60 mL with sterile distilled water, to be used as inocula for the seedlings. Three

mL of the final dilution of the inoculum was injected into pots containing the seedlings at several locations near the rhizosphere.

Seedling Growth

The rooted seedlings of *Morella cerifera* were transplanted into sterilized soil. Once roots were established, equal number of plants were planted in 1 gallon plastic containers and maintained at 14/10 hour night/day photoperiod, and day/nighttime temperatures of 27ºC and 22ºC respectively in the growth chamber. The treatment groups were inoculated with *Frankia* according to techniques described in Tian et al., 2001, and treated with Hoagland solution (Hoagland and Arnon, 1941) as shown below. The plants were grown for seven weeks under treatment conditions until sampled.

Group1 = (10 plants) *Morella cerifera* with quarter-strength Hoagland and *Frankia*

Group 2 = (10 plants) *Morella cerifera* with quarter-strength Hoagland and without *Frankia*

Group 3 = (10 plants) *Morella cerifera* with full-strength Hoagland and without *Frankia*

Group 4 = (10 plants) *Morella cerifera* with full-strength Hoagland and *Frankia*

Approximately 30 mL of Hoagland solution was added to each pot once per week. All plants were watered with water to maintain adequate soil moisture.

Acetylene Reduction Assay

The rate of nitrogen fixation was measured using the acetylene reduction assay as described by Staal et al. 2001. Briefly, 4 x 2 cm metal soil corers were used to retrieve aliquots of nodulated soil samples around the rhizophere of the soil, and enclosed in an air tight 40mL glass vessel. Five plants were randomly selected from each treatment group and four core soil samples were removed from each pot, for a total of 20 samples per treatment. The samples collected were used to measure the concentration of ethylene which was consequently used to calculate the amount of nitrogen fixed per plant within the respective treatment groups. The acetylene reduction assay is based on nitrogenase (N_2ase)-catalyzed reduction of C_2H_2 to C_2H_4, and gas chromatographic isolation of C_2H_2 and C_2H_4.

Distilled water (10 mL) and 1.5 mL acetylene was injected into the air tight glass vessel, to approximately a concentration 10% by volume. The glass vessels were then incubated under environmental chamber conditions, 14 h day, and 10 h night cycle for 48 hours.

Small samples of (250 uL) of head space gas from the vials were withdrawn and injected into a Gas Chromatograph (Shimadzu Scientific Instrument Inc.) for measurement of ethylene. The injection port temperature was set at 200° F and column set at 80° F. The concentration of ethylene measured was used to calculate the amount of nitrogen fixed per plant within the respective treatment groups.

Measurement of Monoterpene Leaf Concentration

Two grams of fresh leaves from each plant (5 per treatment group) in the four treatment groups were collected from the growth chamber and ground in pentane (GC-MS grade, Burdick and Jackson, Muskegon, Missouri) using a polytron (Brinkmann Inc., Westbury, New York) and 0.2 mg n-tridecane was added as an internal standard. All leaf extracts were centrifuged for five minutes at 3600 rpm. The supernatant was subsequently concentrated under flowing nitrogen. Monoterpene quantity and composition was evaluated by combined gas chromatography-mass spectroscopy using a Hewlett Packard 5890 series II gas chromatograph equipped with an HP-5 methyl-silicone 30 m x 0.25 mm capillary column and helium was used as a carrier gas. The injector temperature was 275°C and injection volumes were 2 mL. The temperature program is comprised of an initial hold at 50°C for 3 min, consequently reaching a final temperature of 220°C at a rate of 5°C/min, and finally held at maximum temperature for 20 min. The total concentration of monoterpenes eluting during the first 8 minutes are expressed as milligrams per gram of fresh leaf.

Statistical Analysis

Nitrogen Fixation

Descriptive statistics of the peak area measurements and amount of nitrogen fixed per hour by treatment groups were examined using SPSS Statistics 20. One-way analysis of variance (ANOVA) was carried out to compare mean level of nitrogen fixation across the treatments. Post hoc pairwise comparisons were also performed using Tukey's HSD

technique if needed. Prior to conducting the repeated measures ANOVA, the normality, and homogeneity of variances were tested with standard statistical techniques and no considerable violation of these assumptions were found.

Monoterpene Concentration

Inferential statistical technique, one-way analysis of variance (ANOVA) was carried out to compare mean level of monoterpene concentration across the treatments. IBM SPSS Statistics 20.0 was used to conduct the statistical analysis. Post hoc pairwise comparisons were also performed using Tukey's HSD technique. Normality and homogeneity of monoterpene concentration data was checked and found satisfactory.

2.4 RESULTS

An analysis of the amount of nitrogen fixed per hour by *Frankia* by treatment groups indicated that the inoculated groups with quarter and full strength Hoagland (1/4 H, FSH) had mean nitrogen fixed per hour (0.2135 and 0.0216 nmol/mL/hr) respectively (Table 2.1). The average amount of nitrogen fixed for un-inoculated ¼ H and FS H were .0073 and .0065 mmol/mL/hr respectively (Table 2.1, Figure 2.1). There was no significant difference among the average nitrogen fixation rates at termination for the four treatment groups (ANOVA: F (3, 16) = 1.447, p = .266).

The inoculated treatments (1/4 H and FS H) produced higher average monoterpene concentrations than un-inoculated treatment groups. The inoculated 1/4 H treatment showed the highest average monoterpene concentration (mean = 3.75 mg/g fresh weight, SD = .17) followed by inoculated FS H (Mean = 3.04, SD = .30), un-

inoculated FS H (mean = 2.45, SD = .18); and un-inoculated 1/4 H produced the least (mean = 2.19, SD = .23) (Table 2.2).

The overall ANOVA F test indicated a statistically significant difference in average monoterpene concentration at termination among the four treatments, F (3, 16) = 48.01, $p < .001$ (Table 2.2). The un-inoculated treatments were not significantly different from one another, whereas both the inoculated treatments (1/4 H & FS H) produced significantly higher monoterpene amounts than the un-inoculated treatments (Tukey HSD post hoc multiple comparisons test, Table 2.2). The inoculated 1/4 H treatment produced significantly higher monoterpene than the inoculated FS H treatment (Table 2.2, Figure 2.1). Results also indicated that the only significant correlation between nitrogen fixation rate and monoterpene production was for the FS H group, a strong significant positive correlation ($r = 0.81$, $p < 0.01$) (Table 2.3, Figure 2.1).

Table 2.1: Descriptive statistics nitrogen fixation rate (nmol/mL/hr) by *Frankia* by treatments.

Treatments	N	Mean	SD	Min	Max	ANOVA *F*-Test
Un-Inoculated 1/4 H	5	0.0073	.001	.053	.0088	$F(3, 16) = 1.447$, $p = .266$
Inoculated 1/4 H	5	0.2135	.376	.063	.8840	
Un-Inoculated FS H	5	0.0065	.002	.044	.0088	
Inoculated FS H	5	0.0216	.002	.020	.0241	

1/4 H = 1/4 Strength Hoagland Solution; FS H = Full Strength Hoagland Solution.

Table 2.2: Descriptive statistics of monoterpene concentration (mg/g) by treatments

Treatments	N	Mean	SD	Min	Max	ANOVA F-Test
Un-Inoculated 1/4 H	5	2.19[b]	.63	1.11	3.04	$F(3, 12) = 34.11, p < .001$
Inoculated 1/4 H	5	3.75[a]	.52	2.51	4.55	
Un-Inoculated FS H	5	2.45[b]	.62	1.27	3.56	
Inoculated FS H	5	3.04[c]	.53	2.00	4.06	

1/4 H = 1/4 Strength Hoagland Solution; FS H = Full Strength Hoagland Solution; [a, b, c]: Different letters indicate significant difference from post hoc Tukey HSD test at 5% significance level.

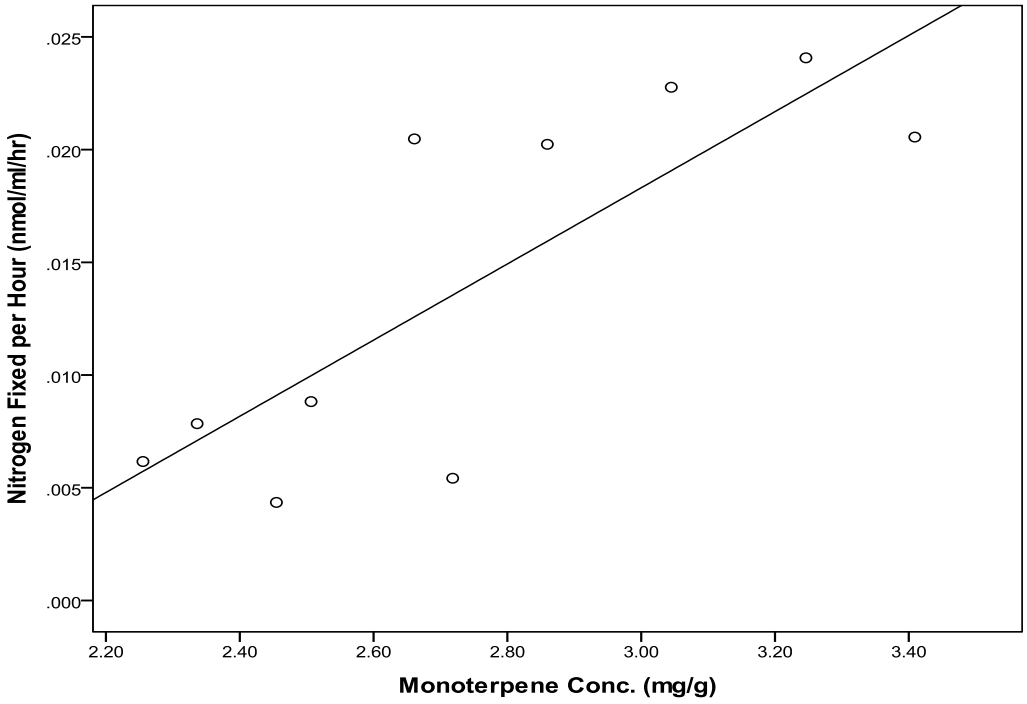

Figure 2.1. Correlation between leaf monoterpene concentration and nitrogen fixation rate. ($r = 0.81, p < 0.01$) for all analyses.

Table 2.3: Correlation between nitrogen fixation rate and monoterpene production.

Groups (n**)**	**Pearson's Correlation coefficient (*p*-value)**
Overall (20)	0.30 (0.20)
Inoculated (10)	0.12 (0.75)
Un-Inoculated (10)	-0.12 (0.75)
FS H (10)	0.81 (< 0.01)*
¼ H (10)	0.29 (0.42)
Inoculated FS H (5)	0.39 (0.51)
Inoculated ¼ H (5)	-0.23 (0.32)
Un-Inoculated FS H (5)	-0.20 (0.75)
Un-Inoculated ¼ H (5)	0.30 (0.63)

$r = 0.81, p < 0.01$

Foliar Monoterpene Composition and Concentration in High and Low Nitrogen Fertilization

GC-MS analysis of sampled leaf monoterpene content indentified the following compounds: α-pinene, camphene, β-sabinene, β-pinene, myrcene, α-terpinene, limonene, 1,8-cineole, β-ocimene, γ-terpinene, terpinolene, terpinen-4-ol, linalool, borneol, and α-

terpineol, camphene, tricyclene, α -carene, limonene, 3-phellandrene, and terpinolene, γ-muurolene, γ-carophyllene, guaiene, 2-carene, α- carophyllene, phenol,2,4,-bis(1,1-dimethylethye), valencene, selina-3,7(11) – diene, humenlene, elemene, γ- elemene, viridiflorene, β- carophyllene, isoledene, gurjunene, himachalene, guaiene (cis- β), α-patchoulene, β-cadinene, and phellandrene. The sum of all monoterpenes eluting in the first 8 minutes of the GC-MS analysis is reported here.

Although the concentration of monoterpenes varied among the treatment groups, the compositions of were relatively similar. Analysis of average monoterpene concentration revealed a statistically significant difference among the four treatments, F (3, 16) = 48.01, $p < .001$ (Table 2.2, Figure 2.1). Pearson correlation analysis showed a positive significant correlation between nitrogen fixation rate and monoterpene production in the full strength Hoagland (FS H) treatment group ($r = 0.81$, $p < 0.01$) (Table 2.3). Negative correlations, though not significant were observed in the un-inoculated full strength Hoagland (r=-0.20, p=0.75) (¼ /FS Hoagland), and inoculated ¼ Hoagland treatment groups (r=-0.23, p=0.32), (Table 2.3, Figure 2.1).

2.5 DISCUSSION

Morella cerifera's symbiotic association with *Frankia* influences its nitrogen fixing capacity. Results of the two levels of nitrogen fertilization with and without inoculation with *Frankia* showed increased nitrogen fixing capacity of *M. cerifera* and monoterpene production in association with *Frankia (*Tables 2.1 & 2.2, Figures 2.1 & 2.2). The response of nitrogen fixing capacity was highly variable in the ¼ HS inoculated

treatment, which showed the greatest response, and thus not significant, although the mean was 1.5 orders of magnitude greater than that of both un-inoculated treatments and an order of magnitude greater than that of the HS inoculated treatment (Fig. 2.1). The response in terms of monoterpene production in association with *Frankia* was highly significant and followed the same pattern of greatest response in the ¼ HS inoculated, next in the F HS inoculated, and minimal in both un-inoculated treatments (Table 2.2).

Several studies reported enhanced nitrogen fixing capacity of actinorhizal plants including *M. cerifera* that have symbiotic associations with *Frankia* species (Dawson 1986; Domenach et al., 1989; Penuelas and Estiare 1998; Roy et al., 2007). Our results support the findings of these studies. *M. cerifera* and other actinorhizal plants may obtain up to 90% of their nitrogen requirement from symbiotic association with actinomycetes such as *Frankia (*Alkermans et al., 1992; Mirza et al., 2009). This mutualistic association plays a critical role in the physiological development of *M. cerifera*, and also in plant-herbivore interactions in the environment. Typically, species that have these symbiotic associations with microbes divert the energy that is supposed to be invested in nitrogen fixation towards other physiological processes such growth and reproduction (Benson & Silvester, 1993; Mirza et al., 2009).

Other studies have evaluated the relationship between soil nitrogen availability and monoterpene production and the majority of them have reported a negative relationship between soil nitrogen content and monoterpene production (Mihaliak and Lincoln 1985, 1989; Tang et al., 1993; Lerdual et al., 1995, Coviella et al. 2000, Walling 2000; Wang and Lincoln 2004; Blanch et al., 2007; Burney and Jacobs 2012). Results of

our study support those reported previously, particularly in reference to higher monoterpene production under the low nitrogen conditions (specifically in the quarter strength Hoagland treatment). However, the correlation analysis of the relationship between inoculated and un-inoculated full strength Hoagland treatment group and monoterpene concentration in this study contradicted the previous findings. The positive correlation noted in this treatment group, may be the due to the symbiotic interaction between *M. cerifera* and *Frankia*.

The fact that there was no statistically significant difference between inoculated treatment groups with the ¼ strength and full strength Hoagland solution amended soil, indicates that *Frankia* inoculation enhanced the nitrogen fixing capacity of *M. cerifera*, irrespective of soil nitrogen content. It also implies that *M. cerifera* as with other actinorhizal species can successfully establish, develop, and be defended in soils with low nitrogen content, and even severely degraded soils when associated with *Frankia* and other actinomycetes (Huss-Danell and Frej, 1986; Ridgeway et al., 2004; Yamanaka & Okabe, 2006). The un-inoculated group had a lower rate of nitrogen fixation (Table 2.1) and a significantly lower monoterpene concentration (Table 2.2), again supporting the conclusion that *Frankia*'s symbiotic association with *M. cerifera* positively affected its nitrogen fixing capacity (Figure 2.3). The higher monoterpene concentration observed in the inoculated group additionally highlights the potential role of *Frankia* in *M. cerifera's* chemically mediated defense. Even more significant is the fact that the treatment group grown in low nitrogen amended soil and inoculated with *Frankia* spores had higher concentrations of monoterpenes compared to the same group without *Frankia*.

According to the carbon/nutrient balance hypothesis (CNBH), plants preferentially direct available resources towards production of amino acids and other primary metabolites to maximize growth, rather than to production of defensive secondary metabolites under conditions of high nutrient availability. However, when faced with severe herbivory, the resources are reshuffled towards production of defensive secondary metabolites (Blanch et. al 2007, Burney and Jacobs 2012). The re-prioritization of resource allocation negatively affects plant growth. The co-existence of *M. cerifera* and *Frankia* therefore may play an important role in the balancing of resource allocation towards growth and defense. Nitrogen fixation by *Frankia* even under low nitrogen conditions may eliminate the need for plants to re-allocate resources towards defense, rather than to growth. Interaction between actinorhizal plants and nitrogen fixing actinomycetes play a crucial role in the chemical defense of these groups of plants. Resource allocation towards biosynthesis of defensive secondary metabolites at the expense of plant growth to deter herbivory play a pivotal role in the survival, establishment and development of species in their habitats (Reddel and Bowen; 1985, Mirza et al., 2009).

Studies by Mihaliak and Lincoln (1985, 1989), Coviella et al., (2000) confirmed that nitrogen availability not only influences the photosynthetic rate of a species, it also mediates allocation of carbon to anti-herbivore leaf chemicals. Additionally, Tingey et al., (1980), Buchanan et al., (2000), Niinemets et al., (2002) also reported that augmented soil nitrogen consequently increases products of photosynthesis which are converted to the precursors of amino acids used for both plant growth and biosynthesis of defensive compounds. Enhanced nitrogen availability through biological nitrogen fixation by

Frankia will apparently have a positive effect on the growth and defense of *M. cerifera* in the environment, due to their symbiotic association.

2.6 CONCLUSIONS

Inoculation of *M. cerifera* seedlings with *Frankia* spores positively influenced its nitrogen fixing capability and monoterpene production, especially under low soil nitrogen conditions. Results indicated that inoculated plants fertilized with two levels of Hoagland solution (¼ and full strength) showed no statistically significant difference in nitrogen fixation rate compared to the un-inoculated groups. Monoterpene production was statistically significantly different in the inoculated groups (1/4 and full strength Hoagland), compared to the un-inoculated groups. However, the concentrations of monoterpene were significantly higher in the group inoculated with *Frankia* spores and fertilized with ¼ Hoagland solutions than the other treatment groups. A correlation analysis contradicted the C: N hypothesis because our results indicated a positive correlation between the full strength Hoagland treatment group and monoterpene concentration. This finding supports the premise that actinorhizal plants and actinomycetes are crucial to the establishment, development, defense and overall health of these species in their habitats. The mutualistic association between *M. cerifera* and *Frankia* will undoubtedly influence the effects of limited nitrogen availability on its growth, development, and defense.

CHAPTER 3

EFFECTS OF LIGHT INTENSITY AND NITROGEN AVAILABILITY ON MONOTERPENE PRODUCTION

3.1 ABSTRACT

A field study was conducted to evaluate the combined effects of nitrogen fertilization and high light intensity on monoterpene production in *Morella cerifera*. The light intensity and nutrient supply of plots in two habitats (marsh edge and adjacent forest interior) were altered using artificial shading and nitrogen fertilization, and leaf monoterpene concentration was analyzed after eight weeks. A statistically significant difference in monoterpene concentration was observed between the plants in the native marsh edge and forest interior, $F_{1,8} = 200.45$, $p < 0.000005$. The fertilized and unfertilized treatments within the forest were significantly different, consistent with the importance of N availability (high N > low N). The treatments within the marsh edge suggested that light was more important than N but that there was an effect of N (Tukey HSD test: high light and high N > high light and low N > low light and high N = low light and low N).

3.2 INTRODUCTION

The synthesis of terpenes and other secondary metabolites is influenced by both environmental conditions and genetic deposition of the species (Hamilton et al. 2001,

Ormeno et al. 2008). The biotic factors reported to modify terpene (particularly monoterpene) production in plants include inter/intra species competition (Ormeño et al. 2007a, b), pollinators (Caissard et al. 2004, Schoohhoven et al., 2005, Raguso, 2008; Kessler and Halitschke, 2009; Dicke and Baldwin, 2010; Dicke and Loreto, 2010; Bruce and Pickett, 2011; Lucas-Barbosa et al., 2011), herbivores, viruses, bacteria, and fungi (Panizzi et al., 1993; Pasqua et al., 2002; Lahlou and Berrada 2003; Giordani et al., 2004). Abiotic factors such as ultraviolet radiation (Zavala and Ravetta 2002), drought (Delfine et al. 2005), high temperatures and light intensity (Flesh et al. 1992, Wang and Lincoln, 2001; Wassner and Ravetta, 2005), ozone (Kainulainen et al., 2000), and nutrients (Mihaliak and Lincoln 1985, 1989, Tang et al. 1993, Lerdual et al. 1995, Wang and Lincoln, 2004, Ormeño et al. 2008) also alter terpene production.

Results of studies conducted on the effects of nutrient availability indicated that enhanced soil nitrogen increased monoterpene production in needles of *Pinus sylvestris* (Kainulainen et al. 2000), leaves of *P. halepensis* (Ormeño et al. 2007c) and *Eucalyptus* species (Close et al. 2004). Other studies however reported either no variation or reduction in monoterpene concentration due to increased soil nitrogen availability in *Pinus sylvestris L.*, *Eucalytus globus and nitens*, and *Eucalytus polybractea* (Kainulainen et al. 1996, Heyworth et al. 1998, Close et al. 2004; King et al. 2004). Wang and Lincoln (2004) reported a positive correlation between leaf monoterpene concentration and light intensity in *M. cerifera*. A positive correlation between high light intensity and monoterpene production was also reported by Burbott and Loomis (1967) for *Menta piperita*. In a previous greenhouse study, a statistically significant reduction in herbivory was observed in a mixed species combination of odorous species (*Morella*

cerifera/Iva frutescens), both known to produce higher levels of monoterpenes, compared to a monoculture of a non-odorous species (*Ilex vomitoria*) (Anoruo and Lincoln, 2016). Anoruo (Chapter two) also reported a positive correlation between *Frankia* inoculation/nitrogen fertilization and monoterpene production in *Morella cerifera*.

Studies on the combined effects of fertilization (nitrogen enhancement) and habitat variation under native conditions are limited. This study was designed as a field test of the greenhouse study that examined the interaction between nitrogen availability and monoterpene production. The objective of this study was therefore to evaluate the effects of nitrogen fertilization and habitat variation (high and low light conditions) on monoterpene production in *Morella cerifera*.

3.3 Materials and Methods

Field habitats of *M. cerifera*

The study was conducted at Goat Island site, Hobcaw Barony, Belle W. Baruch Foundation (33°20′N, 79°15′W), Georgetown, South Carolina, USA. *Morella cerifera* inhabit the transition zone of shrub/forest edge of salt marshes along the South Eastern coast of the United States. *Morella cerifera*'s population primarily occurred in the zonation assemblage where odorous species predominated and transition from salt marsh to island pine forest. *Morella cerifera* inhabit two distinct habitats, where the canopy is exposed to full irradiance (marsh edge) and beneath a dense canopy of predominantly *Pinus taeda* stands, with very limited irradiance and shaded conditions. *Morella cerifera*

plants were selected along the marsh edge where high light conditions predominated and within the heavily shaded forest interior, and exposed to treatments as described below.

Field Study

Five blocks measuring 20 m X 20 m each were established along the upper salt marsh edge at Goat Island, Hobcaw Barony of Belle W. Baruch Foundation, Georgetown SC. Twenty *Morella cerifera* shrubs were selected and assigned to four treatments as shown below such that each block contains four plants, with each plant representing a treatment in a randomized complete block design (RCB). The RCB was chosen to eliminate minor micro habitat variations that are often found in field conditions. The same five block experimental design was adopted for the upland pine forest (forest interior) except that only two treatments (1 and 2) were used because high light conditions could not be achieved at this site, therefore precluding treatments 3 and 4 (ten plants total: one per treatment per block). The shading of the plants along the marsh edge was achieved by erecting wooden structures with treated lumber (1.2 m x 3.0 m) around the selected plants, to allow a canopy of black shade cloth (Park Seed Wholesale Inc. Greenwood, SC) that reduced irradiance up to 80% above the plants. This light condition is similar to that observed in the forest interior. Randomly selected plots within the two habitats were exposed to treatment for 8 weeks during the spring growth period (April 23rd to June 23rd) as shown below:

Marsh Edge

Treatment 1: Un-fertilized & shaded - low carbon & low nitrogen

Treatment 2: Fertilized & shaded- high nitrogen & low carbon

Treatment 3: Fertilized & non-shaded – high nitrogen & high carbon

Treatment 4: Un-fertilized & non-shaded – high carbon & low nitrogen (native condition).

Forest Interior

Treatment 1:Un-fertilized & shaded – low nitrogen & low carbon (native condition)

Treatment 2: Fertilized & shaded - high nitrogen & low carbon

The treatments were initiated at the beginning of the growing season and leaves were sampled after 8 weeks. All sampled leaves were excised from those grown under the treatment conditions.

Light intensity Measurement

Light intensities above the *M. cerifera* canopy within the marsh edge and forest interior were measured at approximately 12:00 p.m. on a cloudless day in late April with an Intergrating Quantum Radiometer Photometer (LI-188B), and a pyranometer sensor (LI-200SB) (LI-COR, Lincoln, Nebraska). Midday light intensity in the marsh edge (characterized by high full irradiance), and forest interior (shady or low irradiance) measured 875 W m^{-2} and 160 W m^{-2}) respectively. Light intensity measurement in the

artificially shaded environment was 140 W m^{-2}, which mimics the conditions in the shady native forest habitat.

Fertilization of Plants

Granular ammonium sulfate (21-0-0-24S) was used as source of nitrogen supply and applied at the rate of 4.9kg N/92 m^2 on April 26th. The granular fertilizer was spread around the perimeter of the root zone of the trees (approximately 30 cm from the stem). Fertilization application was done on a day with an 80% prediction of rainfall, and it rained as predicted.

Measurement of Monoterpene Leaf Concentration

Leaves of *Morella cerifera* (new growth produced under treatment conditions) from the two habitats were collected, immediately placed on ice in a cooler, and transported to the lab for monoterpene analysis. Two grams of fresh leaves from plants (5) in each treatment group were collected from the two habitats and ground in pentane (GC-MS grade, Burdick and Jackson, Muskegon, Missouri) using a polytron (Brinkmann Inc., Westbury, New York) and 0.2 mg n-tridecane was added as an internal standard. All leaf extracts were centrifuged for five minutes at 3600 rpm. The supernatant was subsequently concentrated under flowing nitrogen. Monoterpene quantity and composition was evaluated by combined gas chromatography-mass spectroscopy using a Hewlett Packard 5890 series II gas chromatograph equipped with an HP-5 methyl-silicone 30 m x 0.25 mm capillary column and helium was used as a carrier gas. The injector temperature was 275°C and injection volumes were 2 mL. The temperature

program is comprised of an initial hold at 50°C for 3 min, consequently reaching a final temperature of 220°C at a rate of 5°C min^{-1}, and finally held at maximum temperature for 20 min. There were four replicates per treatment group. The total concentration of monoterpenes eluting during the first 8 minutes are expressed as milligrams per gram of fresh leaf.

Statistical Analysis

To compare the levels of monoterpene concentration of *M. cerifera* within the two habitats (unmanipulated marsh edge and unmanipulated forest interior), a one-way ANOVA was used. Two-way ANOVAs were used to analyze the block and treatment effects of the marsh and forest experiments. Because the block term was not significant, it was dropped from the model and the model was rerun as a one-way ANOVA. The data were checked for normality and variance heteroskedasticity.

3.4 RESULTS

An assessment of variation in leaf monoterpene concentration among native marsh edge and forest interior habitats showed that the average level of monoterpene concentration in the marsh edge characterized by high light intensity (mean = 2.44, SD = 0.08, N =5) was higher than that of the forest interior characterized by low light conditions (mean = 1.64, SD = 0.09, N = 5) (Figure 3.1). This difference was highly statistically significant (one-way ANOVA: treatment: $F_{1,8}$ = 200.45, p < 0.000001). Block was not significant in the two-way ANOVA so the model was collapsed to a one-way ANOVA (block: $F_{4,4}$ = 0.11, p >0.95).

Descriptive statistics of monoterpene concentration by levels of light intensity (shade, no shade) and nitrogen availability (± fertilizer) are presented in Table 3.1. The levels of monoterpene concentration for the four light intensity-nitrogen concentration levels in the marsh were statistically highly significantly different (one-way ANOVA: treatment: F (3, 16) = 36.06, $p < 0.000001$ (Figure 3.2). Block was not significant in the two-way ANOVA so the model was collapsed to a one-way ANOVA (two-way ANOVA: block: $F_{4,12} = 1.17$, $p > 0.35$). All treatments were significantly different from one another except for the high N low light and low N low light treatments (Tukey HSD a posteriori test, $p < 0.05$). It is apparent from the results that the observed significant difference in monoterpene concentration between low and high light intensity treatments is more pronounced than the difference between fertilized and not fertilized treatments with the same light intensity (Figure 3.2, Table 3.1).

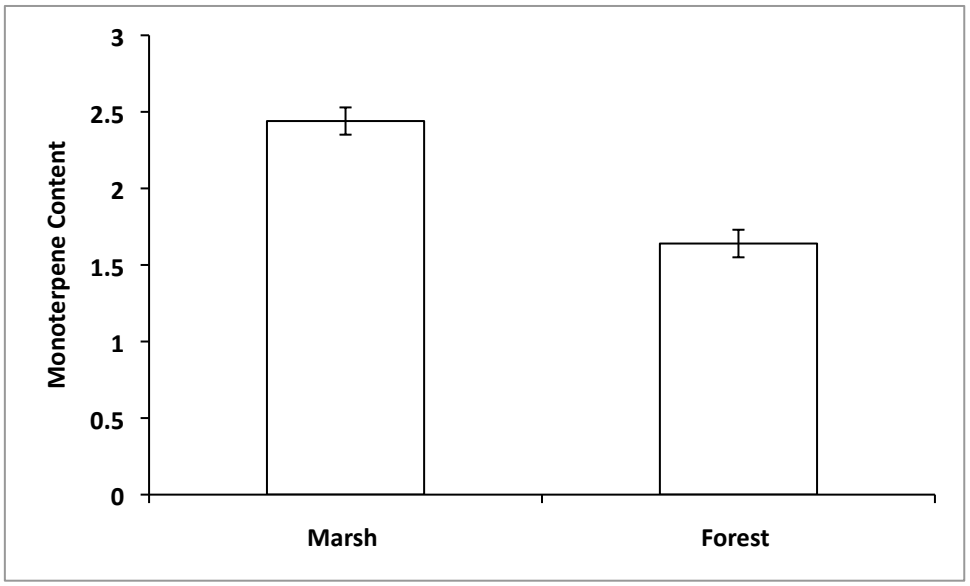

Figure 3.1: Monoterpene content (mg/g of fresh leaf weight) in two native habitats, marsh edge and forest interior (characterized by high light intensity and low light intensity, respectively). Monoterpene content of leaves is significantly different between the two native habitats. Error Bars: standard deviations.

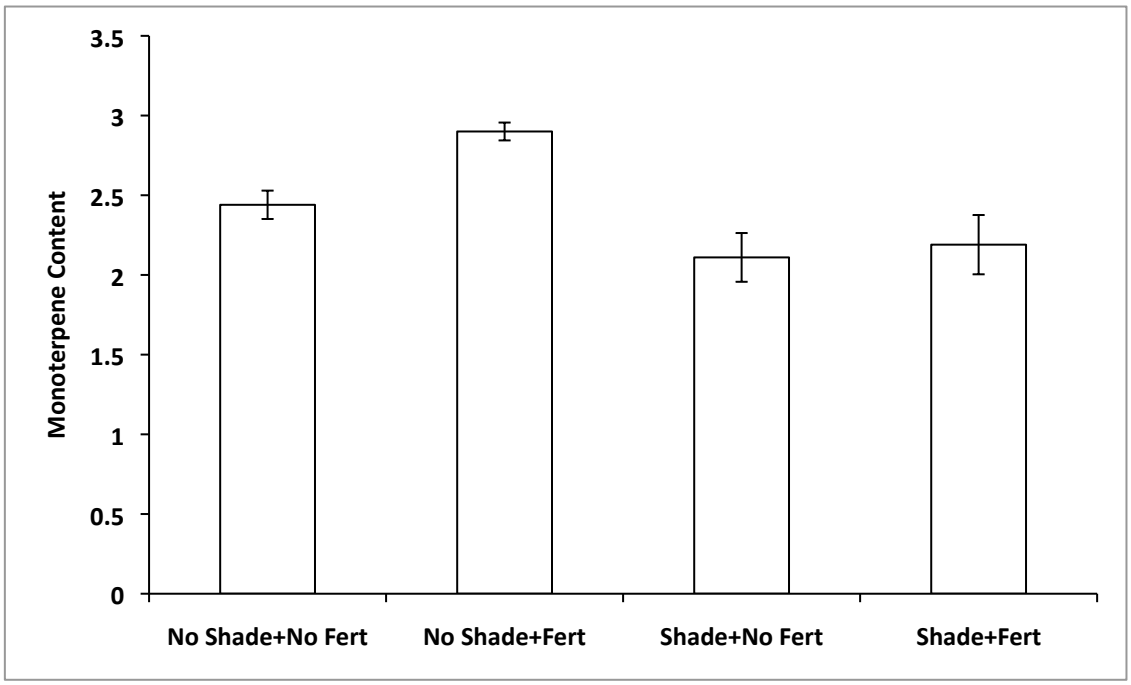

Figure 3.2: Levels of monoterpene content (mg/g of fresh leaf weight) in treatments manipulating nitrogen and light within marsh edge habitat. Error Bars: standard deviations.

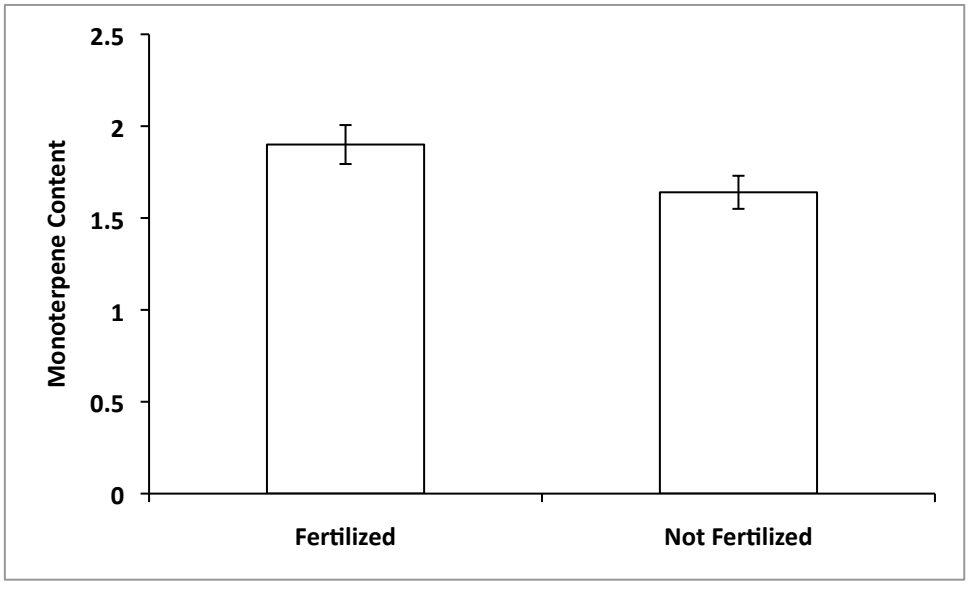

Figure 3.3: Monoterpene content (mg/g of fresh leaf weight) in forest habitat experiments manipulating nitrogen availability; treatments are significantly different. Error Bars: standard deviations.

Table 3.1: Average levels of monoterpene content by levels of light intensity (marsh habitat: shade vs no shade) and nitrogen (fertilizer vs no fertilizer) in the manipulations in the marsh edge and forest habitats. 'SD' is standard deviation. Different superscript letters indicate significant differences from post hoc Tukey HSD test ($p \leq 0.05$).

Light and Nitrogen Intensity	**N**	**Mean**	**SD**
Marsh Edge			
High/Low (no shade, no fertilizer)	5	2.44[a]	0.09
High/High (no shade, fertilizer)	5	2.90[b]	0.06
Low/Low (shade, no fertilizer)	5	2.11[c]	0.15
Low/High (shade, fertilizer)	5	2.20[c]	0.19
Forest			
Low/Low (shade, no fertilizer)	5	1.64[a]	0.09
Low/High (shade, fertilizer)	5	1.90[b]	0.11

3.5 DISCUSSION AND CONCLUSIONS

Nitrogen availability and light intensity are among the abiotic factors that influence monoterpene production in plants. Greenhouse studies examining the effects of nitrogen availability on monoterpene production indicated a positive correlation between high nitrogen concentration and monoterpene production in *Morella cerifera* (See Chapter 2). Results from this field study not only support that of the previous greenhouse study, but also revealed that monoterpene concentration is significantly higher under conditions of high light intensity and high nitrogen availability, than under low light

intensity and high nitrogen availability. Additionally, the observed higher concentration of monoterpenes in the unshaded treatment group within the marsh edge compared to those artificially shaded within the same habitat, as well as the treatment group in the forest interior (native shade habitat) may be also attributed to a phenomenon referred to as the shade-avoidance syndrome (Izaguirre et al., 2006). Plants that naturally thrive under high light conditions have been reported to use far-red radiation (FR) as a major signal in sensing the nearness of potential competitors. Perception of elevated concentration of FR by such plants can trigger a collection of responses (increased stem elongation, production of erect leaves, reduced lateral branching, and production of leaves with larger surface area), which enhances their accessibility to light. Izaguirre et al (2006) reported that perception of FR activated the down-regulation of chemical defenses, and reduced the accumulation of herbivore-induced phenolic compounds in wild tobacco (*Nicotiana longiflora*).

The result from this study is also in alignment with other studies that either evaluated the effects of high light intensity (Burbott and Loomis, 1967, Wang and Lincoln, 2004), or soil nitrogen availability (Kainulainen et al., 2000; Close et al., 2004, Ormeño et al. 2007). More importantly, this study examined the combined effects of soil nitrogen availability and light intensity on monoterpene concentration, and found that both factors have a positive influence on monoterpene production. On the other hand, some studies have also evaluated the role of monoterpene production in the reduction of herbivory in varying plant habitats and concluded that there is a negative correlation between individual plant species monoterpene concentration and level of herbivory (Degenhardt et al. 2003, Wang and Lincoln, 2004). Although results from our previous

greenhouse study showed a positive correlation between nitrogen availability and monoterpene production, which is in alignment with our findings herewithin, however this study suggests that light has greater effect than nitrogen. However, N fertilization nevertheless enhanced monoterpene production. All plants in both marsh edge and forest interior have *Frankia* associated with their roots and that may have played a role in the monoterpene production. In the forest interior habitats, the fertilized group had higher leaf monoterpene concentration compared to the unfertilized group (Figure 3.3), but this was not true of the artificially shaded marsh edge fertilized versus not fertilized (Figure 3.2, Table 3.1). However, within the marsh edge habitat, the unshaded fertilized treatment group had higher leaf monoterpene content than the unshaded unfertilized group. The highest monoterpene production in the marsh edge was seen under high light intensity and high nitrogen soil availability, compared to low light intensity and low nitrogen availability, suggesting a synergistic interaction between the two factors.

It seems clear from this study that plants grown under nitrogen enhancement (through fertilization or biological nitrogen fixation) and sunny environment will be better defended against herbivores due to increase monoterpene concentration. Herbivory will undoubtedly be better defended against under the sunny and high nitrogen environmental conditions. Additionally, since allocation of resources to defense deters growth, such plants will be able to adequately balance growth and defense.

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