To my husband Marcus, who has stood by me and been such a great support through this process. Also, to my sweet Aurelia who has brought great joy to her mommy, and to my little one yet to arrive this year who is along for the ride.
ACKNOWLEDGMENTS

I thank my family who has supported all of my academic efforts, including my parents and in-laws who encouraged me to keep writing, even over holiday visits. I thank Andy Schreffler, who has provided technical laboratory and greenhouse support. Also, thank you to the student workers who provided many hours of tissue sample handling for this body of work: Francisco Sotomayor, Alex Hill, Travis Ewald, and Diana Hart. I must also thank Dr. Sinclair for his personal encouragement when I have become tired of this project and for his comments that kept me thinking.

I thank my committee members for their suggestions and contributions to the writing of this manuscript. Specifically, I also need to thank Cheryl Mackowiak and Ann Blount for sourcing plant materials and field plots for this work. Finally, I need to thank Max Teplitski and the graduate students and post-docs in his laboratory, especially Cory Krediet, Jason Noel and Mengsheng Gao who have tirelessly answered all of my questions and provided demonstrations of the techniques that were necessary for the completion of my analysis.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>10</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>12</td>
</tr>
<tr>
<td>Legumes in Forage Systems</td>
<td>12</td>
</tr>
<tr>
<td>Origin, Cultivars and Uses of Rhizoma peanut</td>
<td>13</td>
</tr>
<tr>
<td>Establishment Concerns</td>
<td>14</td>
</tr>
<tr>
<td>Site Preparation, Seasonality of Planting, and Competition</td>
<td>15</td>
</tr>
<tr>
<td>Rhizome Characteristics for Establishment</td>
<td>16</td>
</tr>
<tr>
<td>Water and Nutrient Demands</td>
<td>16</td>
</tr>
<tr>
<td>Nitrogen Fertilization of Leguminous Crops</td>
<td>17</td>
</tr>
<tr>
<td>Nitrogen Fertilization and Forage Legume Production</td>
<td>18</td>
</tr>
<tr>
<td>Rhizoma peanut Yield Response to N Fertilization</td>
<td>19</td>
</tr>
<tr>
<td>Rhizoma peanut Rhizome N Response to N Fertilization</td>
<td>19</td>
</tr>
<tr>
<td>Legume Transpiration and N₂-Fixation Response to Drying Soil</td>
<td>19</td>
</tr>
<tr>
<td>The Challenge of Inoculating Legume Crops</td>
<td>20</td>
</tr>
<tr>
<td>Specificity</td>
<td>21</td>
</tr>
<tr>
<td>Competitiveness and efficiency</td>
<td>21</td>
</tr>
<tr>
<td>Confirming inoculation</td>
<td>22</td>
</tr>
<tr>
<td>Overview of Chapters</td>
<td>23</td>
</tr>
<tr>
<td>2 RHIZOMA PEANUT RESPONSE TO NITROGEN FERTILIZATION</td>
<td>25</td>
</tr>
<tr>
<td>Introduction</td>
<td>25</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>27</td>
</tr>
<tr>
<td>Growth Response to N Fertilization</td>
<td>27</td>
</tr>
<tr>
<td>Nitrogen Fixation Response to N Fertilization</td>
<td>29</td>
</tr>
<tr>
<td>Field Fertilization Trial</td>
<td>32</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>34</td>
</tr>
<tr>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>Growth Response to N Fertilization</td>
<td>34</td>
</tr>
<tr>
<td>Nitrogen Fixation Response to N Fertilization</td>
<td>36</td>
</tr>
<tr>
<td>Field Fertilization Trial</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>40</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Number of nodules pot(^{-1}) and Nodule mass (mg pot(^{-1})) after 8 wks of N application at dosages of 0, 228 and 580 mg N m(^{-2}) wk(^{-1})</td>
<td>46</td>
</tr>
<tr>
<td>2-2</td>
<td>Number of nodules (pot-1) and nodule mass (mg pot-1) for Experiment 2. Data shown are means ±1 SE. There were no significant differences among the means within each genotype.</td>
<td>49</td>
</tr>
<tr>
<td>2-3</td>
<td>Rhizome mass and rhizome N concentrations (mg g(^{-1}) DW). Data shown are means ± 1 SE separated by harvest month and N fertilization rate (kg N ha(^{-1})).</td>
<td>51</td>
</tr>
<tr>
<td>2-4</td>
<td>Accumulated N in shoot and rhizome tissues (kg N ha(^{-1})). Data shown are means ± SE separated by harvest month and N fertilization rate.</td>
<td>51</td>
</tr>
<tr>
<td>3-1</td>
<td>Concentration of N in shoots 12 wks after inoculation (mg N g(^{-1}) dry weight). The data shown are shoot N for Ecoturf and Florigraze ± 1 SE.</td>
<td>74</td>
</tr>
<tr>
<td>3-2</td>
<td>Shoot and rhizome mass (g pot(^{-1})) from Experiment 2 by core origin. Data shown are means for each genotype ± 1 SE.</td>
<td>74</td>
</tr>
<tr>
<td>3-3</td>
<td>Nodulation by core origin. a.) Number of nodules pot(^{-1}) b.) Nodule mass (mg pot(^{-1})). Data shown for both genotypes are means ± 1 se.</td>
<td>75</td>
</tr>
<tr>
<td>3-4</td>
<td>Comparable strains for phylogenetic analysis.</td>
<td>77</td>
</tr>
<tr>
<td>4-1</td>
<td>Shoot mass harvested at the conclusion of first and second drying treatments in Experiment I and at the conclusion of Experiment II.</td>
<td>96</td>
</tr>
<tr>
<td>4-2</td>
<td>Nodulation data from well-watered (WW) and water-stressed (WS) plants examined in Experiment 2.</td>
<td>96</td>
</tr>
<tr>
<td>B-1</td>
<td>N-free nutrient solution protocol.</td>
<td>107</td>
</tr>
<tr>
<td>C-1</td>
<td>Yeast extract mannitol media.</td>
<td>108</td>
</tr>
<tr>
<td>C-2</td>
<td>PCR Master Mix for Platinum Taq Enzyme and 2.5 mM dNTP protocol.</td>
<td>109</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Shoot yield (g) and greenness measurements after 8 wks of growth under three N-fertilization rates.</td>
<td>45</td>
</tr>
<tr>
<td>2-2</td>
<td>Concentration of N in shoots of rhizoma peanut in response to four N rates (0, 288, and 580 mg N m⁻² wk⁻¹).</td>
<td>46</td>
</tr>
<tr>
<td>2-3</td>
<td>Accumulated N resulting from N fertilization in the nitrogen response and growth response greenhouse experiments.</td>
<td>47</td>
</tr>
<tr>
<td>2-4</td>
<td>Yield resulting from four N fertilizer rates.</td>
<td>47</td>
</tr>
<tr>
<td>2-5</td>
<td>Shoot N concentration (mg N g⁻¹ shoot DW) and acetylene reduction activity.</td>
<td>48</td>
</tr>
<tr>
<td>2-5</td>
<td>Daily rainfall (mm) for Marianna, FL during 2008 as reported by the Florida Agricultural Weather Network.</td>
<td>49</td>
</tr>
<tr>
<td>2-7</td>
<td>Forage mass yield (kg ha⁻¹) by month and 2009 total and crude protein (g kg⁻¹ DW) results from Experiment 3.</td>
<td>50</td>
</tr>
<tr>
<td>3-1</td>
<td>Nodulation by inoculant and genotype.</td>
<td>73</td>
</tr>
<tr>
<td>3-2</td>
<td>Indices of N₂ fixation.</td>
<td>76</td>
</tr>
<tr>
<td>3-3</td>
<td>Phylogenetic relationships between recovered isolates, inoculants, and reference strains.</td>
<td>78</td>
</tr>
<tr>
<td>4-1</td>
<td>Daily normalized transpiration rates (NTR) as a function of the fraction of transpirable soil water (FTSW) present that day from Experiment 1.</td>
<td>92</td>
</tr>
<tr>
<td>4-2</td>
<td>Normalized acetylene reduction activity (ARA).</td>
<td>93</td>
</tr>
<tr>
<td>4-3</td>
<td>Daily normalized transpiration rates (NTR) and normalized acetylene reduction activity (ARA) as functions of the fraction of transpirable soil water (FTSW) present in the pot.</td>
<td>94</td>
</tr>
<tr>
<td>4-4</td>
<td>Relative Water Content (RWC) of leaflets collected across the FTSW gradient. Each point represents a single data point.</td>
<td>95</td>
</tr>
<tr>
<td>A-1</td>
<td>Flow-through pot/chamber for intact acetylene reduction assays.</td>
<td>106</td>
</tr>
</tbody>
</table>
Rhizoma peanut (*Arachis glabrata* Benth.) is an important perennial forage and hay legume in the southeastern United States. The potential nitrogen (N) limitation on rhizoma peanut growth and options for enhancing the symbiotic N$_2$ fixation inputs were explored in the current study.

The potential N deficit for maximum rhizoma peanut production was evaluated in two experiments. For Ecoturf and Florigraze, optimal N accumulation in two greenhouse studies was saturated by the 192 mg m$^{-2}$ wk$^{-1}$ treatment (21 kg ha$^{-1}$). In the field significant differences in yield and N accumulation occurred only at the first harvest date. All three experiments confirm that N$_2$ fixation does not fully provide the N necessary for optimal perennial peanut production.

Inoculant application to increase N$_2$ fixation in rhizome peanut was explored in two experiments. In the greenhouse, four inoculants were applied to intact cores of rhizoma peanut growing in field soil. Yield, nodulation, nitrogenase activity, and N accumulation responses to the inoculants were not significant. Differences in yield and N accumulation were attributable to the core origins. This result raises further questions about interactions between management practices, soil characteristics and N$_2$ fixation.
potential of this crop. Inoculation was further evaluated by DNA analysis. Rhizoma peanut was found to be nodulated with multiple strains indigenous bacteria that were very similar to the inoculants applied. Only three of 12 nodules evaluated contained either of the two inoculants, indicating low inoculant competitiveness.

Drought tolerance may limit rhizome peanut growth and N\textsubscript{2} fixation. Transpiration rates and nitrogenase activity of Florigraze, Ecoturf, and Arbrook rhizoma peanut were evaluated under drying soil conditions. The normalized transpiration rate breakpoints versus fraction of transpirable soil water were 0.32, 0.23, and 0.23, respectively, which indicates that rhizoma peanut is a relatively drought tolerant crop. Normalized nitrogenase activity of Florigraze and Ecoturf did not respond to drying soil, and Arbrook rates were too low to estimate.

Rhizoma peanut was N-limited under both controlled and field conditions. Available inoculants were not able to increase yield or N\textsubscript{2} fixation, and rhizoma peanut is a drought tolerant crop with respect to both transpiration rate and nitrogenase activity.
CHAPTER 1
INTRODUCTION

Legumes in Forage Systems

Grazing lands comprise approximately 25% of the world’s land area. This land area contributes many environmental services besides providing forage intake for livestock. When properly managed, grazing lands provide water management and filtration, nutrient re-uptake from livestock excreta, carbon sequestration, and soil protection. Due to the limited cultivation of forage land soils, carbon storage under pastoral grasslands is greater than other ecosystem soils (Soussana et al., 2004). Incorporation of perennial grasslands into longterm rotation strategies may also increase carbon storage in soil.

In the tropics and subtropics, the addition of legumes to grazing systems supplies dietary protein to livestock in quantities greater than from tropical forage grasses alone. Where legumes are grown in mixture with grasses, biologically-fixed N may be transferred from the legume to the grass. One study estimated that as much as 30% of the N present in the grasses of a mixed sward was derived from to the legume as a result of tissue turnover and redistribution via animal excreta (Vieravargas et al., 1995). Forage and hay production land in Florida of the perennial legume, rhizoma peanut (Arachis glabrata Benth.) was estimated in 1999 to be 8,000 ha (Quesenberry, 1999), and was increased greatly since that time. In Florida, the highly digestible and high protein hay of rhizoma peanut is often used for lactating or growing animals, but the largest market is for horses, sheep and goats.

The nutritive value and digestibility of rhizoma peanut is similar to that of alfalfa. In a study conducted in Central Georgia where alfalfa and rhizoma peanut were grown
alongside one another for three years, the in vitro organic matter disappearance of the two crops was similar on a total herbage basis, and the yields were not different by the third year (Terrill et al., 1996). For this location, production of alfalfa was possible, but this is not the case for locations further south where rhizoma peanut is the predominant perennial forage legume.

**Origin, Cultivars and Uses of Rhizoma peanut**

The center of origin for the *Arachis* genus is South America, specifically Brazil, Bolivia, Paraguay, Argentina, and Uruguay. The two sections from which perennial peanut species have been developed for forage applications are Caulorhizae (*Arachis pintoi*) and Rhizomatosae (*Arachis glabrata*) (Valls et al., 1993). *Arachis pintoi*, which is able to be propagated by seed or vegetatively by stolon pieces, is represented by several cultivars, including ‘Amarillo,’ which is widely used across the tropics and subtropics and, to a limited degree, in the U.S. *Arachis glabrata*, is a rhizomatous species that does not frequently form seeds. Due to low seed production, *A. glabrata* is propagated by rhizome transplantation, which makes genetic improvement through breeding for this crop limited to observing and capturing favorable mutations that occasionally occur in the field. For the purposes of this study, reference to "rhizoma peanut" will specifically mean *A. glabrata*.

The University of Florida has released several cultivars of *A. glabrata*. ‘Florigraze’ (PI 421707) and ‘Arbrook’ (PI 262817) are forage cultivars that have had wide usage since their release (Prine et al., 1986). The most common forage cultivar, ‘Florigraze,’ originated from a seedling that was found growing in Gainesville, FL, between two plots of *A. glabrata* introductions from Brazil (Prine et al., 1981). Ecoturf’ (PI 262840) was originally collected along the Brazil-Paraguay border, and today this genotype is
commonly used as a groundcover plant that requires little maintenance or mowing. There have been other releases of forage-type cultivars, but none have been as successful as Florigraze, to date. A recent release of two new cultivars, UF Tito (PI 262826) and UF Peace (PI 658214) show promise for increased yields, persistence and tolerance to peanut stunt virus (Quesenberry et al., 2010).

Beyond use as a forage and hay crop, rhizoma peanut has been used as a living mulch and an ornamental groundcover. Florigraze has been used as a cover crop in citrus groves, where it effectively suppressed weeds without the use of herbicides (Linares et al., 2008). As the evidence for reduced-tillage systems to conserve soil structure and soil carbon mounts, there may be need to use legumes such as rhizoma peanut as a living mulch. As the evidence for reduced-tillage systems to conserve soil structure and soil carbon mounts, there may be further need to use legumes such as rhizoma peanut as a living mulch. There has been some success with this method of planting into a living mulch with snap bean (*Phaseolus vulgaris*) (de Oliveira et al., 2006).

**Establishment Concerns**

Although rhizoma peanut is a promising perennial legume crop for the southeastern U.S., establishment of the crop is slow relative to other forage crops, and the purchase, transportation and planting of rhizomes are expensive. However, once a field is established, it can last for many years, eliminating the cost of re-planting. Plots of rhizoma peanut near Gainesville, FL, have been in continuous use for more than 50 years. Rhizoma peanut has been widely-adopted in the Southeast as a high-quality hay legume, however, there is much regarding the management and physiology of this crop that remains unknown.
Site Preparation, Seasonality of Planting, and Competition

Slow canopy closure is one of the main issues facing rhizoma peanut producers. Site preparation, planting season, rhizome planting rate, and weed suppression are all management factors that affect establishment rate. In a multi-location study, rhizoma peanut establishment was best achieved when rhizomes were planted in the winter season after the ground had been prepared by plowing (Williams, 1993). In this study, varying degrees of site preparation were performed from no grass sod removal to disking to reduce grass cover to fully plowed soil. Two years following planting, there was no discernable difference between the site preparation treatments, even though emergence and canopy closure occurred more quickly in the plowed subplots.

No-till establishment is also a viable option for rhizoma peanut establishment when mixed stands are acceptable. A study comparing site preparation and machinery available for establishing rhizoma peanut found that commercially available machinery was effective in establishing no-till plots of rhizoma peanut with acceptable canopy closure after 3 years (Williams et al., 2002).

Competition from weeds can delay canopy closure of rhizoma peanut. Both high rhizome planting densities and herbicide usage during establishment can help to control weed encroachment. A study conducted in Gainesville, FL evaluating the use of herbicides during the establishment year of Arbrook and Florigraze planted at different densities to control weed competition showed that higher density plantings and herbicide application to control grassy weeds resulted in the best yields the first year. Control of only broadleaf weeds did not result in yield increase. The optimal rhizome planting rate determined by this study was 2.5 t ha\(^{-1}\) (Canudas et al., 1989). Although
weed suppression with herbicides is a common practice during establishment, there is no known scientific literature available evaluating the efficacy of weed control or potential crop damage for the herbicides labeled for rhizoma peanut.

**Rhizome Characteristics for Establishment**

Successful rhizoma peanut establishment may depend heavily on certain characteristics of the rhizome planting material. The rhizome N and total non-structural carbohydrate (TNC) concentrations have been shown to affect establishment. A study evaluating rhizome planting sources differing in TNC and N concentrations was evaluated for shoot emergence and forage yield (Rice et al., 1996). The greatest shoot emergence and forage yield in this study resulted from rhizome N concentrations of at least 20 g kg^{-1} N. Rhizoma peanut that was not defoliated maintained rhizome N levels within this prescribed range. Saldivar et al. (1992) showed that undefoliated rhizoma peanut rhizome N concentrations followed a positive quadratic pattern over one year with rhizome N concentrations beginning and ending just above 20 g kg^{-1}.

One other species has been shown to have a similar response to rhizome N concentration. In a study evaluating rhizome characteristics and planting techniques, a dune-building seashore grass, *Panicum racemosum*, also displayed a positive correlation between rhizome N concentration and sprout emergence from the rhizomes (Cordazzo and Davy, 1999). It is common practice for a “nursery” crop of rhizoma peanut to remain uncut for at least one year before rhizomes are harvested for transplanting.

**Water and Nutrient Demands**

Water availability is an important factor in establishment that may also have a strong interaction with planting season response. The results of a study comparing
establishment dates and site preparation practices indicated that consistent rainfall (or irrigation) for the first 60 to 90 d after planting was the most influential factor for achieving rapid canopy closure of rhizoma peanut (Williams et al., 1997). Other establishment studies have also pointed to sufficient water availability as a key factor for rapid rhizoma peanut stand establishment (Williams, 1993; Williams et al., 2002).

The nutrient demands of rhizoma peanut for best production are still under investigation. There have been a small number of studies on a limited number of soil types in Georgia, Louisiana, and Florida. Mooso et al. (1995) added varying rates of phosphorus, potassium (K), and a commercial product containing K, magnesium, sulfur, and boron to rhizoma peanut grown on a Louisiana Coastal Plain soil. The study found no yield response differences resulting from the application of any of the nutrients except for K, which increased rhizoma peanut yield at the 75 and 150 kg K ha$^{-1}$ rates versus 0 kg K ha$^{-1}$. For most leguminous crops, it is assumed that N$_2$-fixation rates in the field are sufficient for plant production, especially when additional N fertilization does not have a strong yield response in the field (Venuto et al., 1998).

**Nitrogen Fertilization of Leguminous Crops**

Due to the ability of most commercially cultivated legume crops to fix atmospheric N2 to usable plant forms, it is commonly considered unnecessary to add N fertilizer to these production systems. There have been reports of both synergistic responses to low-rate N fertilization of legumes (Eaglesham et al., 1983) and inhibition of nodule formation that can interfere with crop productivity (Streeter, 1988). Nitrogen fertilization has been shown to improve productivity in legumes in the field when applied under specific conditions and at the appropriate timing in the life cycle of the crop (Hannaway and Shuler, 1993).
Supplemental N has been shown to increase yield of annual peanut, especially under conditions with low soil N and where rhizobia populations may be too low for adequate nodulation to occur. Lanier et al. (2005) found a linear response of annual peanut (*Arachis hypogea* L.) to N fertilizer application, regardless of whether or not they were inoculated.

**Nitrogen Fertilization and Forage Legume Production**

A study of alfalfa (*Medicago sativa* L.) grown in monoculture and fertilized with 0, 40 and 80 kg N ha\(^{-1}\) during a seedling year found that hay production was greatest at the 40 kg N ha\(^{-1}\) fertilization rate (Belanger and Richards, 2000). During the establishment year, N\(_2\)-fixation and soil N reserves were not sufficient for meeting the crop N demand for maximum productivity of alfalfa. When soil N is low (<15 ppm soil NO\(_3\)) or when soil temperatures are low during the early growing season, addition of small amounts of N fertilizer can improve the yield of alfalfa, according to a review by Hannaway and Shuler (1993).

Forage legume crops, particularly perennials, may respond in a more complex manner than annual legume crops to N fertilization. The addition of N fertilizer often can shift the composition of a field toward existing grass competitors, effectively decreasing the productivity of the legume. This effect has been documented in several studies of white clover (*Trifolium repens* L.), where clover coverage has declined in response to N fertilizer (Harris and Clark, 1996; Ledgard et al., 2001). In one study with nine cultivars of white clover, the cultivars were actually ranked according to their “N tolerance,” or ability to persist in a pasture under increasing N fertilization (Ledgard et al., 1996).
Rhizoma peanut Yield Response to N Fertilization

Rhizoma peanut has responded favorably to N fertilization under controlled conditions (Adjei and Prine, 1976; Venuto et al., 1998). In a greenhouse study, Venuto et al. (1998) applied 0, 85 and 170 kg N ha$^{-1}$ to rhizoma peanut and found a linear response in yield to N rate for the second and third of three harvests. However, limited increase in yield due to N fertilization has been observed in the field due to soil water availability and pH interactions (Redfearn et al., 2001).

Rhizoma peanut Rhizome N Response to N Fertilization

The N concentration and total non-structural carbohydrate content of rhizome propagules for efficient establishment of perennia peanut was evaluated by Rice et al. (1996). As previously mentioned, Rice et al. (1996) found that the greatest forage yield and shoot emergence resulted from rhizome N concentrations of at least 20 g kg$^{-1}$ N. Venuto (1998) reports a maximum N concentration of 18.0 g kg-1 in rhizomes of defoliated rhizoma peanut receiving 220 kg N ha$^{-1}$ grown on Louisiana Coastal Plain soils. It is unknown whether acceptable rhizome N concentrations can be achieved in Florida under a defoliated and N-fertilized regime, but the ability to harvest forage mass during a year when rhizomes are being prepared for propagation could result in more profit for farmers.

Legume Transpiration and N$2$-Fixation Response to Drying Soil

Water is frequently the most limiting factor in crop production systems. Water available for transpiration has been shown to be linearly related to crop yield. The ability of a plant to continue transpiring is directly linked to its ability to maintain carbon-fixation (Sinclair et al., 1984).

(Sinclair et al., 1998) and annual peanut (Devi et al., 2009).
Di-nitrogen fixation by legumes can be more sensitive to drought stress than the transpiration rate of the same legume (Sinclair et al., 2000). In soybean, N\textsubscript{2}-fixation rates have been shown to be more sensitive than NTR to soil drying (Sinclair and Muchow, 1986). However, some soybean and annual peanut genetic material has been found for which nitrogenase activity decrease in response to drought was less sensitive than transpiration rate. Eight soybean lines have been identified as being able to continue N\textsubscript{2} fixation well beyond the FTSW of decline in leaf gas exchange (Sinclair et al., 2000). Screening of annual peanut germplasm for drought-sensitivity of N\textsubscript{2}-fixation rates has identified lines that were highly sensitive and some that were highly-insensitive to soil drying (Devi et al., 2009).

The acetylene reduction assay is an N\textsubscript{2} fixation measurement technique with that is well-suited for evaluating the short-term responses of nitrogenase to drought stress. This rapid and repeatable assay has been used repeatedly and non-destructively to monitor the nitrogenase activity of intact legumes in response to a variety of stressors (Cathey et al., 2010; Devi et al., 2009; Sinclair, 2000).

**The Challenge of Inoculating Legume Crops**

Many legumes are able to fix atmospheric N\textsubscript{2} to plant available forms through the activity of bacteria in their nodules. These bacteria, of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and others, are collectively referred to as ‘rhizobia.’ Infection of the legume root occurs through two known mechanisms, and is mediated by a complex series of chemical signals exchanged between the plant and the rhizobium (Rengel, 2002). The first mechanism is by infection thread, whereby a root hair becomes infected by the rhizobia and develops into a tubule that provides a direct pathway for the rhizobia to enter differentiating nodule cells that have begun to develop
adjacent to the root hair (Monahan-Giovanelli et al., 2006). The second mechanism for infection has been observed in many sub-tropical and tropical genera, and it has been confirmed for annual peanut. In this process, the rhizobium (or rhizobia) breaches the root epidermis through a crack, typically near where a lateral or adventitious root is beginning to protrude, creating tiny fissures in the epidermis (Boogerd and van Rossum, 1997). This crack invasion stimulates nodule formation by the plant.

**Specificity**

Legume-symbiont pairings vary from highly-specific to relatively non-specific, commonly referred to as promiscuous nodulation. For example, the signaling relationship between alfalfa and *Sinorhizobium meliloti* has identified two-way chemical communication pathways where not only are signals from the bacteria recognized by the roots, but flavonoids produced by alfalfa roots down-regulate nod genes in the bacteria (Zuanazzi et al., 1998). However, some species of legumes may have a more promiscuous nodulation strategy, especially those growing in their native range where the bacteria and plant species likely have co-evolved (Rodriguez-Echeverria et al., 2003; Van Berkum et al., 1998). The broad host potential of rhizobia and native plants may result from horizontal gene transfer between many strains of rhizobia indigenous to a given ecosystem (Schofield et al., 1987). The host-specificity of rhizoma peanut nodulation is still unknown.

**Competitiveness and efficiency**

Finding potential inoculants for a crop is difficult due to the differences in competitiveness and efficiency of potential candidates. The efficiency of a given rhizobium-legume relationship can vary widely, even among strains that are capable of occupying the nodules. For example, Hungria et al. (2003) found significant increases
in growth and N-accumulation by cultivars of common bean (*Phaseolus vulgaris* L.) nodulated with a highly-efficient *Rhizobium tropici* strain versus other inoculating strains.

Inoculants must also be able to compete with indigenous bacteria present in the soil to which the inoculant is applied. Thies et al. (1991) found that number of nodules occupied by inoculating strains decreased as numbers of indigenous rhizobia populations increased, based on inoculation of seven agronomic crops. Nodulation with indigenous strains of rhizobia may be a key reason why inoculation with specific strains may fail for species such as peanut (Bogino et al., 2006).

**Confirming inoculation**

Once an inoculant has been applied, experimentally, it is important to evaluate whether the strain applied is indeed the strain that is occupying the nodules and producing the observed results. There are many methods for confirming the presence of an inoculant in nodules. Comparison of inoculated plants to an uninoculated control can give an indication of the effectiveness of nodulation with native rhizobia (Purcino et al., 2000). Other methods include measuring the fluorescence of nodules due to strain-specific fluorescent antibodies (Thies et al., 1991) and pre-screening inoculants for antibiotic resistance (Ramirez et al., 1998).

Molecular techniques using DNA sequences for comparison of nodule occupants to inoculant can confirm nodule formation by inoculants as well as give potential insight into the relatedness of any indigenous nodule occupants and the inoculant (Botha et al., 2004). DNA fingerprinting, gene homology, and rDNA intergenic spacer sequencing are commonly used methods for such comparisons (Saeki et al., 2000; Vinuesa et al., 1998). These methods can allow for rapid comparisons once the protocol for analysis is established.
Overview of Chapters

Chapter 2 evaluates the N demand of rhizoma peanut and assesses whether yield can be improved by the addition of N fertilizer. In addition, rhizome N concentration was evaluated in a field study to determine whether N fertilization could increase rhizome N concentrations to levels desireable for propagules in the presence of defoliation. The objectives of chapter 2 were 1) to determine what rate of N fertilization would maximize shoot production of rhizoma peanut, 2) to assess changes in nodulation, nitrogenase activity, shoot N concentrations, and N accumulation in response to N fertilization, and 3) to assess the impact of N fertilization on rhizome N concentrations in a field that was managed for hay production. Three experiments, two greenhouse studies and one field study, were conducted to address these objectives.

Chapter 3 addresses the potential for the addition of inoculants to rhizoma peanut crops. The overall objective of this study was to evaluate the effectiveness of inoculants applied to rhizoma peanut grown in field soil. Subobjective 1 was to evaluate the effects of applying three inoculants to Ecoturf or Florigraze genotypes in terms of shoot mass, nodulation, and shoot N concentration under non-sterile soil conditions. Subobjective 2 was to further evaluate the effect of four inoculants on Ecoturf and Florigraze rhizoma peanut by evaluating N₂-fixation activity of plants growing in three different Florida soils. Subobjective 3 was to evaluate the competitiveness of inoculant strains 8B4 and 8B6 for nodule formation with Florigraze versus the existing strains present in each of three soils.

Four different inoculants were evaluated on cores of rhizoma peanut extracted from established fields. In the plant response experiment, one site was used as a source for the cores, and in the inoculant effectiveness experiment, three sites were
used as sources for cores samples of rhizoma peanut in order to better evaluate the
competitive ability of the inoculants versus ambient soil bacteria conditions. Follow-up
analysis using molecular techniques was conducted in the inoculant effectiveness
experiment to evaluate nodule occupancy of some of the replicates in the study to more
specifically address the competitiveness of the inoculants.

In chapter 4, two experiments were implemented to evaluate the transpiration and
nitrogenase activity response of three cultivars to drying soil stress: Arbrook, Ecoturf,
and Florigraze. The first objective of Experiment I was to determine the transpiration
threshold for three genotypes of rhizoma peanut in response to soil drying and to
determine a second threshold response to a repeat drying event following a recovery
period. The second objective of Experiment I was to evaluate the sensitivity of
nitrogenase activity to drying soil, again for an initial drying period and to a second
stress event following a recovery period. Since the Ecoturf and Florigraze cultivars are
the two most economically-important cultivars of this crop, a second experiment was
implemented to validate their responses to soil drying. The objectives to Experiment II
were to confirm the transpiration responses to soil drying for Ecoturf and Florigraze, to
confirm the nitrogenase activity response to soil drying, to evaluate RWC of rhizoma
peanut leaves as a viable tool for monitoring soil drying, and to evaluate the impact on
short-term drought stress on nodulation of rhizoma peanut.
CHAPTER 2
RHIZOMA PEANUT RESPONSE TO NITROGEN FERTILIZATION

Introduction

Rhizoma peanut (*Arachis glabrata* Benth.) is an important crop in the southern United States for forage production and as an ornamental groundcover. Through symbiosis with soil bacteria, collectively known as “rhizobia,” many leguminous crops can fix their own nitrogen (N). This relationship typically results in high quality forage without the use of N fertilizers. Rhizoma peanut is not ordinarily fertilized with N in mixed grass/peanut hay or grazed production systems since competing grasses, such as bahiagrass (*Paspalum notatum* Flugge) and bermudagrass (*Cynodon dactylon* (L.) Pers.) can accumulate dry matter more quickly, thereby suppressing rhizoma peanut growth (Williams, 1994). In spite of the high quality forage produced by rhizoma peanut without added N, producers are always looking to increase yields.

For most leguminous crops, it is assumed that N$_2$-fixation rates in the field are sufficient for plant production, especially when additional N fertilization does not have a strong yield response in the field (Venuto et al., 1998). However, yellow leaves observed in some rhizoma peanut fields (personal observation) and low productivity on some soils have led researchers and producers to explore various reasons for both this symptom and low productivity. Nitrogen fertilization may be an option for increasing productivity by providing N beyond that supplied by biological N$_2$ fixation. Greenhouse studies have indicated that rhizoma peanut yield may increase with N fertilization under controlled conditions (Adjei and Prine, 1976; Venuto et al., 1998). Previous studies have shown that the limited yield increase in the field from N fertilizer application may
be due to interactions with drought conditions and soil pH (Redfearn et al., 2001; Venuto et al., 1998).

To date, there have been no studies to precisely define the N-limitation of rhizoma peanut under controlled conditions in order to determine a basis for field fertilization practices. Additionally, no other studies have looked specifically at the interaction of nodulation or nitrogenase activity with N fertilization of this crop in order to determine the N rate where nodule formation may be inhibited.

Rhizome N status has been shown to be an important factor in the success of establishing new stands of rhizomatous plants from rhizomes. In a study evaluating rhizome characteristics and planting procedures for reestablishing a dune-building seashore grass, *Panicum racemosum*, Cordazzo and Davy (1999) found a positive correlation between rhizome N concentration and sprout emergence from the rhizomes. A similar result was found for rhizoma peanut in a study conducted by planting rhizomes differing in total non-structural carbohydrates and N concentrations resulting from differing grazing regimes (Rice et al., 1996). The emerging rhizoma peanut was evaluated for shoots per unit area as well as additional characteristics such as canopy coverage and forage yield.

Rice et al. (1996) found that the best forage yield and shoot emergence resulted from rhizome N concentrations of at least 20 to 20.5 g kg\(^{-1}\) N. Saldivar et al. (1992) showed that undefoliated rhizoma peanut, without N fertilization had rhizome N concentrations that followed a positive quadratic pattern over 1 y with rhizome N concentrations beginning and ending just above 20 g kg\(^{-1}\), and the lowest concentration for the year was around 18 g N kg\(^{-1}\). It is unknown whether N fertilizer applications can
maintain rhizome N content over multiple defoliations during a growing season. The ability of a farmer to produce hay on a "nursery" crop of rhizoma peanut during a season when rhizomes are to be harvested might benefit if the sale of the hay was greater than the cost of N fertilizer.

The objectives were 1) to determine what rate of N fertilization would maximize shoot production of rhizoma peanut, 2) to assess changes in nodulation, nitrogenase activity, shoot N concentrations, and N accumulation in response to N fertilization, and 3) to assess the impact of N fertilization on rhizome N concentrations in a field that was managed for hay production. Three experiments were conducted to address these objectives.

**Materials and Methods**

**Growth Response to N Fertilization**

Samples of Florigraze and Ecoturf genotypess of rhizoma peanut were collected on 10 October, 2007 from their respective field plots at the North Florida Research and Education Center (NFREC) near Live Oak, FL using a cup cutter designed for golf greens (10-cm diameter by 20-cm depth). The sample cores were immediately transferred to black plastic tree seedling pots measuring 35-cm tall and tapering from 132 cm² at the mouth of the pot to 56cm² at the base of the pots (Stuewe and Sons, Corvallis, OR). Additional field soil was placed in the bottom of the pots before inserting the core as needed, in order to raise the surface of the core to approximately 2 cm below the pot rim. Forty-five potted cores of each genotype were maintained in a greenhouse at Gainesville, FL with a thermostat setting of 30°C. Daylength was extended beyond ambient to 16 h using incandescent lamps. This extended daylength was shown by (Williams et al., 2008) to be sufficient to sustain rhizoma peanut growth.
The experiment was initiated on 1 February by first harvesting all the leaves and stems from each pot and the shoot materials were dried to constant weight at 60 °C. This harvest allowed an equal starting point for all the plants. On Monday and Thursday of each subsequent week for 8 weeks, beginning 6 March 2008, a nutrient solution was applied to each pot according to a factorial treatment design (genotype x NO₃ dosage x Fe dosage). Each treatment consisted of a dosage combination of KNO₃ and sequestered iron (10% Fe) in addition to an N- and Fe-free nutrient solution. The low nitrate dose was 288 mg N m⁻² wk⁻¹ and the high nitrate dose was 580 mg N m⁻² wk⁻¹. The three Fe fertilization rates were 0, 70 and 140 kg Fe sequestrene ha⁻¹. These amounts of application were equivalent to a total N application during the 8-wk experiment of 0, 23, and 46 kg N ha⁻¹.

The treatments at each application were applied by first adding 120 ml of N- and Fe- depleted but otherwise complete nutrient solution (Table B-1; 100 ml). In addition 20 ml of water or the appropriate solution of KNO₃ plus Fe solution was added to each pot. Plants were lightly watered following application of the nutrients to ensure nutrient movement into the rooting zone. Leaf color was measured weekly using a SPAD chlorophyll meter (SPAD-502, Minolta Co, LTD, Japan) between 6 March and 1 May 2008. Three upper leaflets from different areas of each pot canopy were measured and the average recorded. At the end of 8 wks of treatment, the shoots were harvested at soil level, as at the beginning of the experiment. The belowground tissues were stored at -4°C until the nodules could be collected and counted. Senesced nodules, which appeared concave, were excluded. Shoots, roots + rhizomes, and nodules were dried to constant weight at 60°C and their mass recorded.
Shoot tissues were analyzed for N at the Forage Evaluation Support Laboratory at the University of Florida, Gainesville, FL. Dried shoot tissues were prepared by grinding them in a coffee grinder (Capresso, Inc.) and then further crushing them in a ball mill (SPEX CertiPrep, 8000M Mixer/Mill) so that they passed through a 1-mm sieve. For nitrogen analysis, dry weight fraction of the submitted samples was first determined, and then the samples were digested using a modification of the aluminum block digestion procedure of Gallaher et al. (1975). The sample weight was 0.25 g, and the catalyst used was 1.5 g of 9:1 K2SO4:CuSO4. The digestion was conducted for at least 4h at 375°C using 6 ml of H2SO4 and 2 ml H2O2. Nitrogen in the digestate was determined by semiautomated colorimetry.

Nitrogen accumulation was determined by multiplying the shoot mass harvested from each pot by the dry weight fraction used to determine N concentration. Then, the shoot mass dry weight per pot was multiplied by the fraction of N in the tissue to give the total accumulated N for each pot. This accumulated N value was then divided by the surface area of the pot so that N accumulation was expressed on a land area (g N m⁻²) basis.

**Nitrogen Fixation Response to N Fertilization**

Cores of rhizoma peanut were collected from their respective fields at the North Florida Research and Education Center, Live Oak, FL, on 29 July 2008, as described previously. Twenty cores each of Florigraze and Ecoturf genotypes were placed in approximately 3.6-L pots constructed of PVC pipe (10.5-cm dia. and 35-cm length). A toilet flange was seated at the top of each pot, and the pot was closed at the bottom with a cap into which was threaded a compression fitting to accept rigid plastic tubing (0.64-cm diameter). All of the pot pieces were glued with PVC cement to provide a
water- and air-tight seal. Approximately 2 cm was left above the cores in each pot for watering purposes. The plants were grown under greenhouse conditions in Gainesville, FL with daylength extended to 16h using incandescent lamps and a thermostat setting of 30°C.

The range of N treatments was decreased in this experiment in an attempt to obtain more data in the range of greater sensitivity to N. The four N dosage treatments were 0, 38.5, 96, and 192 mg N m\(^{-2}\) wk\(^{-1}\), supplied as KNO\(_3\). The total N applied during the 11-wk experiment was 0, 4.2, 10.5, and 21.0 kg N ha\(^{-1}\) respectively. Nutrient solution was applied to each of the pots twice weekly on Tuesdays and Fridays. A total of 60 ml of nutrient solution was added at each application: 50 ml of N-free, but otherwise complete nutrient solution (Table B-1) and 10 ml of the appropriate NO\(_3\) solution. The plants were lightly watered after each nutrient solution addition to ensure that the nutrients were distributed into the soil. Shoots were harvested by cutting them at the soil surface the day before treatments were first applied in order to provide consistent initial above ground biomass with each pot.

**Nitrogenase activity measurements.**

Acetylene reduction assays were conducted to assess nitrogenase activity using a non-destructive flow-through system. The lid portion of a clear plastic food storage container (3.8 L; Rubbermaid, Inc.) was attached to the top of each pot flange and sealed with rope caulking. Holes were drilled through the lid and through the flange so that bolts could be inserted through the holes to hold the lids in place with wing nuts, and the center of the lids were removed to match the opening of the mouth of the pot. The clear food storage containers were then inverted over the top of the pots and
attached to the lids to create a sealed chamber. A plastic barbed tubing connector was placed through a hole drilled in the bottom of the food storage container, and this connection was sealed using the rope caulking. Positive flow through the sealed chamber was tested by attaching the hose from an air pump to the base of each of the pots and checking that a bubble would form at the top of the barbed connector at the top of the chamber when wetted.

Once the system was connected to the input lines that flowed through a manifold that created a 1:9 mixture acetylene: air, the venting lines leaving the top of the pot were subsequently attached to a central exhaust line that exited out of the top of the greenhouse. After 15 min to allow for gas equilibrium to establish in the pot-chamber system, duplicate 1-ml samples of the gas mixture were collected from the venting line just above each pot using a syringe. Due to the presence of some background levels of ethylene in the acetylene tank, eight baseline samples were collected randomly from the input lines along the row of pots. Samples were analyzed using gas chromatography to determine the ethylene content (Shimadzu GC-8A). The total exposure time of the plants to for the acetylene assay was a maximum of 20 min, followed by 1 h of airflow through the closed system. After 1 h of airflow, the tops of the pots were removed so that the plants were again exposed to greenhouse ambient conditions.

The plants were assayed weekly over 8 wks, beginning 3 wks after the N treatments were imposed (majority of pots at approximately 75% canopy coverage). An average of four readings (from the venting lines) from each pot was taken at week 4, which was the middle of the experiment when readings were stable and the highest average levels for the eight weeks. The five lowest input readings for the gas input out
of the eight taken per assay were averaged to determine the input blank for the respective days. For each assay, the average input blank was deducted from the average of the two output readings from each pot to estimate acetylene reduction activity (ARA). Due to the variability in ARA data from day to day, the time factor was not assessed when analyzing these readings.

**Harvest.** At the conclusion of the experiment, 11 wks following the original cutting, the plants were harvested. Shoots were harvested at soil level, and the roots and rhizomes were washed free of soil and stored at -4°C until the nodules could be recovered and counted. As previously, only turgid nodules were collected. Shoots, roots + rhizomes, and nodules were dried at 60°C to constant weight and their weights recorded. Shoot tissues were subsequently processed and analyzed for N concentration, and N accumulation was calculated as described previously.

**Field Fertilization Trial**

A field experiment was initiated at the North Florida research and Education Center, Marianna, FL (30.87° N, 85.19° W), which is located on the Atlantic Coastal Plain. The plots were located in a 1.1 ha field that had been established in Florigraze rhizoma peanut in 2000. Prior to the rhizoma peanut planting, the field was used for conventional row crop production. The soil is a Red Bay sandy loam (Fine-loamy, kaolinitic, thermic Rhodic Kandiudult) that is deep and well-drained, with approximately 0.5% soil organic carbon, and a clay content of about 14% (USDA, 2007).

The sampled plots were part of a larger, on-going study that was initiated in 2004 to test the effect of increasing potassium (K) fertilization rates with and without supplemental nitrogen (N). The study used a split-plot design with N treatment defining the main plots and K treatments defining the subplots. Nitrogen (as NH₄-NO₃) was
applied in three applications, at a rate of 0 or 112 kg N ha\(^{-1}\) event\(^{-1}\), and K (as K\(_2\)O) was applied in four applications, at 0, 43.4, 65.1, and 86.7 kg K ha\(^{-1}\) event\(^{-1}\). The first fertilizer application was on 22 May 2009, and the remaining fertilization events occurred on the day following the first and second harvests. Only the 43.4 kg K ha\(^{-1}\) event\(^{-1}\) subplots within the N main plots (0 or 112 kg N ha\(^{-1}\) event\(^{-1}\)) were sampled for results presented here, since it represents typical K application rates for rhizoma peanut hay production.

Plots were clipped (8-cm stubble height) three times during 2009: 2 July, 27 August, and 23 October. A rotary mower with a bag attachment was used to sample a 1.95-m\(^2\) swath from each subplot during each harvest. Following each forage sampling, the entire plot was clipped to a uniform height (8 cm) with a riding mower and bag attachment. Forage was dried at 60°C to constant mass from each sample clipping.

Rhizoma peanut rhizomes were sampled using the same golf cup cutter that was used to harvest cores in Experiments 1 and 2. The soil volume sampled was approximately 10 cm in diameter to a depth of 7.6 cm. The core depth was defined by the depth at which no rhizome pieces were remaining in the bottom of the core once removed from the soil. Two samples of rhizomes were taken from each of the subplots on the same day or the day prior to the shoot harvests, with one additional sample taken on 15 May, the day prior to the first fertilizer application. Rhizomes were frozen at -4°C until they were washed free of soil and small root fragments were removed; then the rhizomes were dried at 60°C to constant mass.

Dried forage samples and rhizomes collected from each plot were ground so that they would pass through a 1-mm sieve using a coffee grinder (Mr. Coffee, Inc.).
samples were then analyzed for N concentration at the Forage Evaluation Support Laboratory using the same procedures as described previously. Nitrogen accumulation was calculated for each subplot on a land area basis by separately multiplying the forage mass and the rhizome mass by the fraction of N in the dry mass.

Data Analysis

The data generated from the two greenhouse experiments were analyzed using the SAS General Linear Model for analysis of variance (ANOVA) with genotype, N treatment (and Fe treatment) and genotype x N treatment as main effects ($\alpha = 0.05$). Student-Newman-Kuels post-tests were used following ANOVA for separation of means, also at the $\alpha = 0.05$ level. Data from the field experiment was analyzed using the Mixed Model Procedure of SAS for repeated measures analysis of variance with forage yield, shoot N concentration and rhizome N concentration as dependent variables. Fertilization rate, harvest date, and their interaction were fixed effects, while replication was a random effect. Separation of means for field experiment data was determined using the “pdiff” statement in the model. Data from all three experiments were plotted and linear regression and student t-test analyses were performed using Prism (Prism 1996).

Results

Growth Response to N Fertilization

Shoot yield was significantly affected by the addition of N ($P < 0.01$), with no significant genotype interaction. The addition of Fe had no significant main effects, and there was no significant interaction between the N and Fe treatments. The Fe treatment did not have a significant effect on any of the additional variables measured in the current study, and the results from the iron treatments were combined.
The data pooled from both genotypes indicates a significant difference in yield between the 0 mg N m\(^{-2}\) wk\(^{-1}\) and 288 mg N m\(^{2}\) wk\(^{-1}\) treatments, but there was no further significant increase between the 288 mg N m\(^{2}\) wk\(^{-1}\) and 580 mg N m\(^{2}\) wk\(^{-1}\) treatments (Fig. 2-1a).

The final readings of leaf color (as measured by SPAD; \(P < 0.01\)) indicated a significant response in chlorophyll content as a result of N addition, but no significant genotype effect. The pooled mean from the 288 mg N m\(^{2}\) wk\(^{-1}\) treatment was significantly higher than the mean of the 580 mg N m\(^{2}\) wk\(^{-1}\) treatment, which was also significantly greater than the mean of the 0 mg N m\(^{2}\) wk\(^{-1}\) treatment (Fig. 2-1b).

Nodulation was suppressed by N fertilization, with a significantly greater number of nodules in the unfertilized pots versus the fertilized pots, but no significant difference in the number of nodules in the pots receiving 580 mg N m\(^{2}\) wk\(^{-1}\) versus the pots receiving 288 mg N m\(^{2}\) wk\(^{-1}\). The Florigraze plants had a greater number of nodules than the Ecoturf (\(P = 0.03\), Table 2-1). Nodule mass was also significantly affected by N fertilization effect, but was not significantly affected by genotype. For the combined data from Ecoturf and Florigraze, the 0 mg N m\(^{-2}\) wk\(^{-1}\) treatment produced greater nodule mass than either the 580 or 228 mg N m\(^{2}\) wk\(^{-1}\) treatments (Table 2-1).

The N concentrations of the rhizoma peanut shoots was also significantly altered through N fertilization. Shoot N concentration (mg N g\(^{-1}\) shoot DW) was significantly increased by N fertilization (\(P < 0.001\)), and although the genotype effect was not significant, the interaction between genotype and N dosage was significant (\(P < 0.01\)). As a result of the significant interaction between the genotype and the N dosage, the means were separated by genotype for further evaluation. Florigraze shoot N
concentrations had a linear response to N dosage ($R^2 = 0.99$), and the mean concentration was significantly different between each N treatment (Fig. 2-2). Ecoturf concentrations also had a linear response to N treatment ($R^2 = 0.91$), and the means of the three treatment responses were significantly different (Fig. 2-2).

Nitrogen accumulation was significantly affected by N fertilization treatments ($P < 0.01$), but there was not a significant genotype effect. The pooled data for the 580 and 228 mg N m$^{-2}$ wk$^{-1}$ treatments was $3.62 \pm 0.19$ and $3.35 \pm 0.16$ g N m$^{-2}$ accumulated, respectively, which was significantly more than the amount of N accumulated by the mg N m$^{-2}$ wk$^{-1}$ treatment ($2.07 \pm 0.17$ g N m$^{-2}$) (Figure 2-3).

**Nitrogen Fixation Response to N Fertilization**

The shoot yield main effect of N fertilization was significant ($P < 0.01$), but genotype effect was not significant (Fig. 2-4). Yield was higher in Florigraze than in Ecoturf, but there were no significant difference between N treatments within either genotype. There was a linear trend in the Florigraze yield with yield increasing with increasing N fertilization, but no significant differences between individual treatments. A second-order polynomial was fitted to the Ecoturf data, and a linear regression was attempted for the Florigraze data, but both regressions resulted in very low $R^2$ values ($R^2 = 0.20$ and $0.28$, respectively) due to the variation among replicates.

Concentration of N in the shoot tissues was not significantly different between genotypes, but it was significantly influenced by N rate ($P = 0.01$), and was also marginally affected by an interaction between the genotype and N rate ($P = 0.06$). Overall, the application of 192 mg N m$^{-2}$ wk$^{-1}$ led to greater tissue N concentrations than did 0 mg N m$^{-2}$ wk$^{-1}$ but these concentrations were not different from the 96 mg N m$^{-2}$ wk$^{-1}$ treatment. Additionally, the 96 mg N m$^{-2}$ wk$^{-1}$ rate did not significantly increase
shoot N concentration versus the application of 0 mg N m\(^{-2}\) wk\(^{-1}\) or 38.5 mg N m\(^{-2}\) wk\(^{-1}\).

Due to the marginally significant interaction between the genotypes and N treatments \((P = 0.06)\), the means were separated by genotype for further analysis. Florigraze means were not significantly different by N treatment. Ecoturf means were different. The 192 mg N m\(^{-2}\) wk\(^{-1}\) treatment was significantly different from the 0 mg N m\(^{-2}\) wk\(^{-1}\) or 38.5 mg N m\(^{-2}\) wk\(^{-1}\) treatments, but was not different from the 96 mg N m\(^{-2}\) wk\(^{-1}\). The 96 mg N m\(^{-2}\) wk\(^{-1}\) treatment was significantly different from the 0 mg N m\(^{-2}\) wk\(^{-1}\) treatment, but not different from the 38.5 mg N m\(^{-2}\) wk\(^{-1}\) treatment. The 38.5 mg N m\(^{-2}\) wk\(^{-1}\) treatment was not significantly different from the 0 mg N m\(^{-2}\) wk\(^{-1}\) treatment (Fig. 2-5).

Nitrogen accumulation was significantly affected by both genotype and N fertilization treatment \((P = 0.04\) for each). Florigraze N accumulation was higher than the Ecoturf accumulated N \((1.50 \pm 0.12 \text{ and } 1.20 \pm 0.10 \text{ g N m}^{-2}, \text{respectively})\). The N accumulation from the 192 mg N m\(^{-2}\) wk\(^{-1}\) treatment was significantly greater than that of the 0 mg N treatment \((1.58 \pm 0.19 \text{ and } 1.00 \pm 0.15 \text{ g m}^{-2}, \text{respectively})\). The N accumulated as a result of the other two intermediate N treatments, 1.34 \pm 0.08 and 1.49 \pm 0.17 g N m\(^{-2}\), respectively \((96 \text{ and } 38.5 \text{ mg N m}^{-2} \text{ wk}^{-1})\) were not significantly different from each other or from the 192 mg N m\(^{-2}\) wk\(^{-1}\) or the 0 mg N m\(^{-2}\) wk\(^{-1}\) treatments (Figure 2-3).

Acetylene reduction activity (ARA) was not significantly different between N treatments for either of the two genotypes studied, due to variation in the data. According to ANOVA results, cultivar effect was not significant. However, there was more nitrogenase activity measured in Florigraze nodules than Ecoturf nodules on an
absolute basis at all but the highest N treatment. ARA also appeared to be suppressed in Florigraze at the two highest N treatments.

Nodule mass means were not significantly different between genotypes or among the four N treatments. Number of nodules per pot was significantly affected by both genotype and N fertilization main effects ($P < 0.01$ and $P < 0.01$, respectively; Table 2-2). The number of nodules declined with increasing N application. Due to the extremely small nodule sizes, many less than 1 mm in diameter, and the tendency of small root fragments to remain with the nodules regardless of careful collection techniques, the nodule mass did not show any clear trend.

**Field Fertilization Trial**

The results from N application in this field experiment were confounded by precipitation events. The rainfall during the 2009 growing season was greatest in May, which accounted for approximately half of the total rainfall for the remaining months. Rainfall was more evenly distributed across the remaining months with an average of 93 mm per month from June through October (Fig. 2-6). Additionally, rain events coincided with each of the three N fertilizer applications. For six days following the 22 May application, a total of 121.2 mm of rain was recorded. The 3 July N application was followed by 64.5 mm of rain within 6 days of the application. In the three days following the 25 October N fertilization, the plots received 21.3 mm of rain. Average temperatures were stable across the season, averaging 25°C across the growing season.

The N fertilization and harvest date main effects were both significant for yield, ($P < 0.01$ and $P < 0.01$, respectively). Yields were significantly greater in July than in August or October, and August yields were significantly greater than October yields.
The increase in yield due to N addition was significant at each harvest date. The yield percent increase resulting from the 112 kg N ha\(^{-1}\) addition was 15.3% in July, 11.2% in August, 11.2% in October, and 13.3% for the entire 2009 season (Fig. 2-7a). Crude protein (based on DM) increased slightly with each harvest (Fig. 2-7b). Both N treatment and harvest date were significant (\(P = 0.01\) and \(P < 0.01\), respectively). Crude protein concentrations were also significantly increased by the fertilizer addition at each of the harvest dates (Fig. 2-7b).

Rhizome mass was not consistently affected by N fertilization or harvest date (Table 2-3). In some months, the fertilized plots had greater rhizome mass than the unfertilized plots, while for other months, the reverse occurred. In unfertilized plots, rhizome mass tended toward a linear rate of decline over the growing season, but due to the variability in the data, a linear regression had a very poor fit \((R^2 = 0.12)\). Rhizome N concentration was consistently higher in plots receiving N fertilizer than those that did not, and rhizome N concentration increased over the course of the season, irrespective of fertilization. The month of harvest \((P < 0.01)\) and fertilization \((P < 0.01)\) main effects were both significant. The N concentration in the rhizomes also increased over the season, with or without fertilizer application, but rhizome N concentration was greater in the fertilized subplots at each of the harvest dates (Table 2-3).

The amount of N accumulated in forage mass declined over successive harvests through the season, from 75 to 42 to 35 kg N ha\(^{-1}\) at the three harvests, despite additional applications of N fertilizer (Table 2-4). The fertilization and harvest date main effects were both significant \((P < 0.01\) and \(P < 0.01)\). N accumulation was significantly different between the 112 and 0 kg N ha\(^{-1}\) event\(^{-1}\) subplots for the July harvest date, but
not for the other two harvests (Table 2-4). For the first harvest, N accumulation increased from 66.5 to 83.4 kg N ha\(^{-1}\) with the addition of 112 kg N ha\(^{-1}\).

Nitrogen accumulation in the rhizomes was significantly affected by fertilizer application \((P < 0.01)\), but was not affected by harvest date. Rhizome N accumulation remained constant over the season, at approximately 0.25 and 0.29 kg N ha\(^{-1}\) in the 0 and 112 kg N ha\(^{-1}\) treatment subplots, respectively. Although the main effect of fertilization was significant, there were no differences between the fertilized and unfertilized subplots for any of the harvest dates (Table 2-4). The total amount of N recovered by rhizoma peanut declined along with yield over the season, in spite of additional N fertilization events.

**Discussion**

The first objective of this study was to determine the N fertilizer addition necessary to maximize rhizoma peanut shoot production. Through two consecutive greenhouse studies, we estimated a saturating response to N addition to be between 10.5 and 23 kg N ha\(^{-1}\) during the duration of the pot experiments. These results differ from the findings of (Venuto et al., 1998) who found a linear response under similar greenhouse conditions for N dosages on an annual basis of 0, 75 and 170 kg ha\(^{-1}\) with no reduction in yield at the highest rate. It is unlikely that other nutrients were limiting in our study, due to the application of N along with a complete nutrient solution.

The combination of fluctuating water availability under rainfed conditions and potential losses due to volatilization or immobilization in the field may make it difficult to see similar results in the field as in the greenhouse. However, in our study applying 336 kg N ha\(^{-1}\) resulted in significantly higher yields than the 0 kg N ha\(^{-1}\) N application treatment in the field (Fig. 2.6). Other studies have failed to show significant yield
improvement in the field with N fertilization. In a similar field study, 0, 110 and 220 kg N ha\(^{-1}\) were applied to plots of Florigraze. These N rates did not produce significant yield improvement, in part, due to too little rainfall (Redfearn et al., 2001). Venuto et al. (1998) also tested 0, 110 and 220 kg N ha\(^{-1}\) on three sites and further concluded that rhizoma peanut must be fixing adequate N to meet its own needs, rendering fertilization at these rates ineffective at increasing yield. These discrepancies could be explained by any number of factors differing between the sites as alluded to by the authors. Variability in water availability, pH or other necessary nutrients make it difficult to see consistent results in field fertilization trials.

The second objective was to evaluate nodulation, nitrogenase activity (measured as ARA), shoot N concentration and N accumulation in response to fertilization. Nitrogen fertilization at the 288 and 580 mg N m\(^{-2}\) treatment levels in the first pot experiment inhibited nodulation, and there was also a noticeable, although not statistically significant, loss of nodules in response to N fertilization above 192 mg N m\(^{-2}\) wk\(^{-1}\) (Table 2-1, Table 2-2). Although higher rates of N fertilizer meets the N needs of the crop, the plant may lose the ability to supply some of its own N through \(N_2\) fixation when nodulation is inhibited. Nodule inhibition may be an additional factor in the limited yield responses observed field studies with high rates of N fertilization (Redfearn et al., 2001; Venuto et al., 1998). A low rate of N fertilizer that did not inhibit nodulation would be ideal so that N may be accumulated by the crop through both fertilizer and biological sources. Under controlled conditions, the nodule numbers were not diminished at the 38.5 mg N m\(^{-2}\) wk\(^{-1}\) or 96 mg N m\(^{-2}\) wk\(^{-1}\) rates (Table 2.2).
Alfalfa yield has been increased under low N fertility conditions with the addition of fertilizer N. Alfalfa grown in monoculture and fertilized with 0, 40 and 80 kg N ha$^{-1}$ produced greater hay production at the 40 kg N ha$^{-1}$ fertilization application during an establishment year (Belanger and Richards, 2000). During the establishment year, N$_2$-fixation and soil N reserves were not sufficient for meeting the crop N demand for maximum productivity of alfalfa. According to a review by Hannaway and Shuler (1993), small amounts of N fertilizer can improve yields of alfalfa when soil temperatures are low and when soil N is low (<15 ppm soil NO$_3$). Field assessment of nodulation at increasing fertilizer rates would be needed to find complementary N rates under rain fed field conditions for rhizoma peanut.

Nitrogenase activity rates in Florigraze tended to be even more sensitive to N fertilization than nodulation at N rates ≥ 10.5 kg N ha$^{-1}$ (Figure 2-4b). This pattern was not apparent in Ecoturf, which showed little ARA response to N addition. It is also interesting to note that for neither genotype was ARA fully suppressed by fertilization, even at 21 kg N ha$^{-1}$, when nodulation was strongly suppressed. Florigraze showed higher ARA overall than did Ecoturf, which may have been related to its ability to produce similar amounts or slightly more dry matter while still maintaining similar N concentration in the shoots (Fig. 2-4; 2-5a).

The concentration of N in forage mass was increased by N fertilizer application (Fig. 2-2; Fig. 2-5a; Fig. 2-7b). In greenhouse studies, rhizoma peanut N concentration increased with fertilizer application in both Florigraze and Ecoturf genotypes with rates up to 580 mg N m$^{-2}$ wk$^{-1}$, which indicates that rhizoma peanut has a large capacity for N uptake. In a similar study where 0, 40 and 80 kg N ha$^{-1}$ was applied to newly-planted
alfalfa, an approximately 13% shoot N concentration increase was observed in one of the two years of the study, while there was no appreciable increase during the second year (Belanger and Richards, 2000). Ecoturf did not respond as strongly to fertilization overall as did Florigraze, indicating that fertilization of Ecoturf in ornamental settings where protein content is not important is unnecessary.

Although ARA data indicates that N₂ fixation was not fully suppressed by N fertilization, it may be more important to evaluate the impact of N fertilization on the N accumulation data, which indicates the ability of the plants to utilize N from either N₂ fixation or from soil/fertilizer sources. Nitrogen accumulation in both greenhouse experiments was enhanced with N fertilization. This offers clear evidence that N₂ fixation activity was inadequate to supply sufficient N for the potential growth of the plants. Under the pot conditions, N accumulation was saturated by the 192 mg N m⁻² wk⁻¹ treatment. Evidence for inadequate N₂ fixation was also obtained in the field where the N accumulated on first harvest date was significantly greater in the subplots receiving 112 kg N ha⁻¹ event⁻¹ than the subplots receiving no N (Table 2-4). Consequently, all three experiments offered evidence that symbiotic N₂ fixation was inadequate to maximize N accumulation and storage in shoots of rhizoma peanut.

Objective III was to evaluate the impact of N fertilization on rhizome N concentrations under field conditions. Even after harvesting the field experiment three times in a season the rhizome N concentrations for the October harvest had achieved levels recommended by Rice et al. (1996) for effective establishment of transplanted rhizomes under rainfed conditions (Table 2-3). Belanger and Richards (2000) also found that alfalfa accumulated N in the taproot under N fertilization. Further analysis of
non-structural carbohydrates is needed to further confirm the capacity of rhizomes harvested after fertilized hay production management for establishment success.

The addition of N fertilizer to rhizoma peanut could be useful for specific management needs. Crude protein of forage mass can be improved by N fertilization, and rhizome N concentrations may reach transplant-acceptable levels even under defoliation pressure with N fertilization. Further study is needed to find the balance between desirable nodulation, CP, and fertilizer application in the field.
Figure 2-1. Shoot yield (g) and greenness measurements after 8 wks of growth under three N-fertilization rates. (A) Shoot yield (g) after 8 wks of growth under three N-fertilization rates (0, 288, and 580 mg N m⁻² wk⁻¹). (B) Greenness as measured by SPAD meter. For both panels, data shown are pooled means from the Florigraze and Ecoturf genotypes, and the error bars represent 1 SE mean. Significance was determined at the α = 0.05 level, and different letters indicate significant differences as determined by a Student-Newman-Kuels post-test.
Table 2-1. Number of nodules pot\(^{-1}\) and Nodule mass (mg pot\(^{-1}\)) after 8 wks of N application at dosages of 0, 228 and 580 mg N m\(^{-2}\) wk\(^{-1}\).

<table>
<thead>
<tr>
<th>N Dosage (mg m(^{-2}) wk(^{-1}))</th>
<th>Number of nodules pot(^{-1})</th>
<th>Nodule mass (mg pot(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Florigraze</td>
<td>Ecoturf</td>
</tr>
<tr>
<td>0</td>
<td>439 ± 51 a</td>
<td>392 ± 83 a</td>
</tr>
<tr>
<td>228</td>
<td>161 ± 25 b</td>
<td>103 ± 29 b</td>
</tr>
<tr>
<td>580</td>
<td>161 ± 34 b</td>
<td>143 ± 41 b</td>
</tr>
</tbody>
</table>

†Letters following the means ± 1 se indicate significant differences among dosage responses within a column at the \(\alpha = 0.05\) level determined by a Student-Newman-Keuls post-test.

Figure 2-2. Concentration of N in shoots of rhizoma peanut in response to four N rates (0, 288, and 580 mg N m\(^{-2}\) wk\(^{-1}\)). Solid squares represent Florigraze means, and the solid circles represent Ecoturf means. The bars represent ± 1 SE of the mean. The solid line represents the linear regression of the Florigraze data, and the dashed line the Ecoturf data. Letters located above (Ecoturf) and below (Florigraze) the symbols represent statistical differences between the respective means. The goodness of fit for the regressions (R\(^2\)) and the means comparisons (t-tests) were tested at the \(\alpha = 0.05\) level.
Figure 2-3. Accumulated N resulting from N fertilization in the nitrogen response and growth response greenhouse experiments. The left panel shows data as a result of 0, 38.5, 96, and 192 mg N m\(^{-2}\) wk\(^{-1}\) treatments, and the right panel shows data from the 0, 288 and 580 mg N m\(^{-2}\) wk\(^{-1}\) treatments. Data shown are means and one SE of the mean. Different lower-case letters represent significantly different means within a panel (α = 0.05).

Figure 2-4. Yield resulting from four N fertilizer rates (0, 38.5, 96, and 192 mg N m\(^{-2}\) wk\(^{-1}\)). The grouped bars represent Ecoturf and Florigraze means, and the error bars represent 1 SE of the mean.
Figure 2-5. Shoot N concentration (mg N g⁻¹ shoot DW) and acetylene reduction activity. (a) Shoot N concentration based on shoot dry weight (mg N g⁻¹ shoot DW). (b) Acetylene reduction activity (ARA) as defined in the methods. Data are shown for panel B as instrument readings to show relative magnitude. For both figures, the legend identifies each bar style to its respective N fertilizer rate. The error bars represent 1 SE of the mean. Different lowercase letters represent significantly different means within a genotype grouping (α = 0.05).
Table 2-2. Number of nodules (pot⁻¹) and nodule mass (mg pot⁻¹) for Experiment 2. Data shown are means ±1 SE. There were no significant differences among the means within each genotype.

<table>
<thead>
<tr>
<th>N dosage (mg N m⁻² wk⁻¹)</th>
<th>Number of nodules pot⁻¹</th>
<th>Nodule mass (mg pot⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Florigraze</td>
<td>Ecoturf</td>
</tr>
<tr>
<td>0</td>
<td>151 ±34</td>
<td>120 ±29</td>
</tr>
<tr>
<td>38.5</td>
<td>106 ±19</td>
<td>106 ±26</td>
</tr>
<tr>
<td>96</td>
<td>70 ±16</td>
<td>53 ±25</td>
</tr>
<tr>
<td>192</td>
<td>87 ±56</td>
<td>100 ±43</td>
</tr>
</tbody>
</table>

Figure 2-5. Daily rainfall (mm) for Marianna, FL during 2008 as reported by the Florida Agricultural Weather Network. The solid line demonstrates daily rainfall patterns for days receiving >1 mm d⁻¹. The arrows indicate days when N fertilizer was applied to the appropriate subplots, and dates given on the x-axis are days when harvesting occurred.
Figure 2-7. Forage mass yield (kg ha\(^{-1}\)) by month and 2009 total and crude protein (g kg\(^{-1}\) DW) results from Experiment 3. a.) Forage mass yield (kg ha\(^{-1}\)) by month and total harvest for 2009. b.) Crude protein based on dry weight (g kg DW\(^{-1}\)). For both panels, the open bars represent the 0 kg N ha\(^{-1}\) application\(^{-1}\) fertilizer treatment and the solid bars represent the 112 kg N ha\(^{-1}\) application\(^{-1}\). Also for both panels, the * and ** symbol indicates a significant difference (\(P < 0.5\) and \(P < 0.01\), respectively) between the means at each harvest date. The error bars represent 1 SE of the mean.
Table 2-3. Rhizome mass and rhizome N concentrations (mg g⁻¹ DW). Data shown are means ± 1 SE separated by harvest month and N fertilization rate (kg N ha⁻¹).

<table>
<thead>
<tr>
<th>Harvest Month</th>
<th>N Fertilizer Rate (kg ha⁻¹)</th>
<th>Rhizome Mass (g)</th>
<th>Rhizome N (mg g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>112</td>
<td>0</td>
</tr>
<tr>
<td>May</td>
<td>21.38 ± 4.54</td>
<td>17.73 ± 3.40</td>
<td>12.9 ± 0.19 a</td>
</tr>
<tr>
<td>July</td>
<td>19.35 ± 5.43</td>
<td>21.25 ± 4.85</td>
<td>13.1 ± 0.19 a</td>
</tr>
<tr>
<td>August</td>
<td>17.20 ± 2.62</td>
<td>14.98 ± 0.25</td>
<td>15.8 ± 0.34 b</td>
</tr>
<tr>
<td>October</td>
<td>14.70 ± 2.24</td>
<td>16.78 ± 2.08</td>
<td>16.8 ± 0.12 b</td>
</tr>
</tbody>
</table>

†Different letters indicate significantly different means within a column.

**Indicates that rhizome N was significantly greater in the 112 kg N ha⁻¹ subplots for all harvest dates based on the SAS mixed procedure pdiff lsmeans comparisons (P < 0.01).

Table 2-4. Accumulated N in shoot and rhizome tissues (kg N ha⁻¹). Data shown are means ± SE separated by harvest month and N fertilization rate.

<table>
<thead>
<tr>
<th>Harvest month</th>
<th>N fertilizer treatment (kg ha⁻¹)</th>
<th>Forage mass accumulated N (kg N ha⁻¹)</th>
<th>Rhizome accumulated N (kg N ha⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>112</td>
<td>0</td>
</tr>
<tr>
<td>May</td>
<td>--</td>
<td>--</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>July</td>
<td>66.47 ± 5.93 a</td>
<td>83.43 ± 9.37 a**</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>August</td>
<td>38.24 ± 3.40 b</td>
<td>45.91 ± 2.59 b</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>October</td>
<td>31.86 ± 1.58 b</td>
<td>37.99 ± 2.24 b</td>
<td>0.23 ± 0.04</td>
</tr>
</tbody>
</table>

†Different letters indicate significantly different means within a column.

††The harvest date main effect was not significant for the rhizome data.

**Indicates that accumulated N was significantly greater in the 112 kg N ha⁻¹ subplots for the given harvest date based on the SAS mixed procedure pdiff lsmeans comparisons (P < 0.01).
CHAPTER 3
NODULATION AND NITROGENASE ACTIVITY RESPONSE OF RHIZOMA PEANUT TO INOCULATION

Introduction

Rhizoma peanut (Arachis glabrata, Benth), is a species of growing utility in the southeastern U.S. This species is important as a forage crop in areas where other forage legumes, such as clover (Trifolium spp.) and alfalfa (Medicago sativa) have limited production opportunity due to climate. The most common cultivar for forage production is Florigraze. In 1999, this cultivar occupied more than 8,000 ha in Florida alone (Quesenberry, 1999), and current land area may be much greater. A second variety, Ecoturf, is commonly used as a low-maintenance ornamental groundcover. Enhancing the productivity and appearance of these cultivars could provide economic benefit for producers. One possibility for enhancing N₂ fixation and shoot mass production of rhizoma peanut is through inoculation. There is currently no inoculant on the market for this crop.

Many legumes are able to fix atmospheric dinitrogen to plant available forms through the activity of bacteria in their nodules, collectively referred to as ‘rhizobia.’ The specificity between a legume and rhizobia bacteria strains range from highly-specific to non-specific, and pairings can have varying degrees of efficiency. There have been specific plant-rhizobia pairings confirmed based on chemical signaling, such as the combination of alfalfa with Sinorhizobium meliloti (Zuanazzi et al., 1998). However, some species of legumes may have a more promiscuous nodulation strategy, especially those growing in their native range where the bacteria and plant species likely have co-evolved (Rodriguez-Echeverria et al., 2003; Van Berkum et al., 1998). The host-
specificity of rhizoma peanut is unknown, as there have been no studies to examine the bacteria-legume relationship of this crop.

The efficiency of a specific combination of rhizobia strains and legume may also vary. Efficiency refers to some comparative measure of a particular rhizobia or suite of rhizobia in combination with a given plant, such as plant/crop yield or nitrogenase activity per unit nodule mass. For example, Fening and Danso (2002) examined the relative efficiency of nodules formed by several strains of indigenous rhizobia with cowpea (*Vigna unguiculata*). Of the 33 plants that formed more than 100 nodules with one of 100 strains applied, only 9 of them produced as much shoot biomass as the uninoculated plants. An inoculant applied to an existing population of soil bacteria must be able to outcompete the existing organisms for proliferation resources as well as outcompete indigenous strains for nodulation. Once inoculation is achieved, that inoculant must also provide a more effective mutualism with the plant than the previous strains. All of these qualifications for an inoculant must be met in order to have successful inoculation in the field.

Molecular techniques may be used to verify strain identity in nodules. The use of a chromosomal metabolic gene as markers allows for examination of strain identity apart from the plasmid, which can move laterally among a population of bacteria (Wernegreen and Riley, 1999). Glutamine synthetase II (*glnII, also called gsII*) is critical for ammonium assimilation, and can serve as a marker for identifying sequence differences among rhizobia strains (Taboada et al., 1996). The ability of an inoculant to nodulate the legume to which it was applied in spite of the presence of
indigenous soil bacteria may be confirmed by the presence or absence of the inoculant in the nodules of legumes inoculated under non-sterile conditions.

The overall objective of this study was to evaluate the effectiveness of inoculants applied to rhizoma peanut grown in field soil. Subobjective 1 was to evaluate the effects of applying three inoculants to Ecoturf or Florigraze genotypes in terms of shoot mass, nodulation, and shoot N accumulation under non-sterile soil conditions. Subobjective 2 was to further evaluate the effect of four inoculants on Ecoturf and Florigraze rhizoma peanut by evaluating N₂-fixation activity of plants growing in three different Florida soils. Subobjective 3 was to evaluate the competitiveness of inoculant strains 8B4 and 8B6 for nodule formation with Florigraze versus the existing strains present in each of three soils.

**Materials and Methods**

**Plant Effects of Inoculants**

**Core collection.** Cores of Florigraze and Ecoturf rhizoma peanut were collected from the North Florida Research and Education Center (30.29°N, 83.02°W), near Live Oak, FL, on 29 July 2008 using a golf cup cutter (10-cm diameter by 20-cm depth) to extract undisturbed cores containing shoots, rhizome, roots, and accompanying soil. Twenty cores each of Florigraze and Ecoturf were placed in approximately 3.6-L pots constructed of PVC pipe (10.5-cm dia. and 35-cm length, Fig. A-1). A toilet flange was seated at the top of each pot and the pot closed at the bottom with a cap into which was threaded a compression fitting to accept rigid plastic tubing. All of the pot pieces were glued with PVC cement to provide a water- and air-tight seal. Approximately 2 cm was left at the top of each pot for watering purposes. The plants were grown under greenhouse conditions in Gainesville, FL with daylength extended to 16h using
incandescent lamps and a thermostat setting of 30°C. On 4 October 2008, 120 ml of an N-free, but otherwise complete nutrient solution was applied to each pot (Table B-1). Cores were watered to avoid water stress for the duration of the experiment.

Inoculation. Five replicates of each perennial peanut genotype received one of four different inoculation treatments, (1) water (no additional inoculant control), (2) 8B4, (3) 8B6, and (4) EL Cowpea. The inoculant strains were received from EMD Crop Bioscience (Brookfield, WI) where they had been stored as potential inoculants. The strains were collected from rhizoma peanut nodules on plants that had recently been imported from their native range in the late 1970s (Ann Blount, University of Florida, personal communication). The liquid cultures were diluted to 1 x 10^8 cells ml⁻¹ with deionized water. Solution bottles, beakers to be used for applying inoculants, and measuring glassware were sanitized by rinsing with 100 percent ethanol prior to use. Cross-contamination was prevented by using separate glassware for measuring each inoculant and by changing gloves between preparations and between applications in the greenhouse. On 22 October 2008, the shoots were harvested from each pot (2-cm stubble height) to provide a consistent starting point for regrowth. Each pot was inoculated with 100 ml of the appropriate 1 x 10^8 cells ml⁻¹ solution, or 100 ml deionized water on 29 October 2008. By applying inoculant to cores of field soil, the inoculants added must compete with the indigenous bacteria present in the soil cores.

Harvest. Entire plants were harvested at the conclusion of the experiment. The shoots were cut at soil level, and the roots + rhizomes were washed free of soil and stored at -4°C until the nodules could be recovered and counted. Only turgid nodules
were collected in order to avoid nodules that had already been senesced at the time of harvest. Shoots, roots + rhizomes, and nodules were dried at 60°C to constant weight.

**Tissue preparation and N analysis.** Shoot tissues from Experiments 1 were analyzed for N concentration at the Forage Evaluation Support Laboratory at the University of Florida, Gainesville, FL. Dried shoot tissues were prepared by grinding them in a coffee grinder (Mr. Coffee) so that they would pass through a 1-mm sieve. For nitrogen analysis, samples were digested using a modification of the aluminum block digestion procedure of Gallaher et al. (Gallaher et al., 1975). The sample weight was 0.25 g, and the catalyst used was 1.5 g of 9:1 K2SO4:CuSO4. The digestion was conducted for at least 4h at 375°C using 6 ml of H2SO4 and 2 ml H2O2. Nitrogen in the digestate was determined by semiautomated colorimetry.

Nitrogen accumulation was determined by multiplying the shoot mass harvested from each pot by the fraction of N in the tissue. This accumulated N was then divided by the surface area of the pot so that N accumulation was expressed on a land area (g m⁻²) basis.

**Inoculant Effectiveness**

**Core collection and herbicide applications prior to collection.** Cores of Florigraze were collected from three different locations in northern, north-central and central Florida as described previously. Twenty cores were collected on 15 May 2009 from the North Florida Research and Education Center at Marianna, Fl (30.650°N, 85.165°W) from soils that are a Red Bay sandy loam (Fine-loamy, kaolinitic, thermic Rhodic Kandiudult, USDA, 2007) that is deep and well-drained, with approximately 0.5% soil organic carbon, and a clay content of about 14 %. The second set of Florigraze cores was collected on 3 June 2009 from a private farm located near
Inverness, FL (28.75°N, 82.35°W), from soils that are deep, excessively-drained sands (hyperthermic coated Typic Quartzipsammets; USDA, 1988). A third set of Florigraze cores was collected on 1 June 2009 from the North Florida Research and Education Center near Live Oak, FL (32.29°N, 83.02°W), where soils are somewhat poorly to moderately well drained surface sands underlain by an eluvial layer followed by deep clay (fine, smectitic, thermic Aquertic Chromic Hapludalfs; USDA 2006).

Each of the three fields from which Florigraze cores were taken had been treated with herbicides prior to core collection. The Marianna field was treated with Plateau at a rate of 284 ml ha\(^{-1}\) (23.6% imazapic; ammonium salt, 3-Pyridinecarboxylic acid, 2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-5-methyl-, monoammonium salt; BASF Corp., Florham Park, NJ) three days prior to core collection. The Inverness field had been treated with Select Max at a rate of 2.3 L ha\(^{-1}\) (12.6% clethodim, (E)-2-[(3-chloro-2-propenyl)oxy]imino)propyl]-5-[(2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one); Valent USA, Walnut Creek, CA) approximately 10 weeks before cores were collected.

The Live Oak Florigraze field had been treated with three herbicide applications prior to collecting the cores. The first application was DualMagnum at a rate of 568 ml ha\(^{-1}\) (83.7% S-metolachlor, Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)\(_2\)-(S), Synenta Crop Protection, Inc., Greensboro, NC) on 2 February 2009. The second herbicide application was Impose at a rate of 284 ml ha\(^{-1}\) (23.3% Ammonium salt of imazapic (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H imidazol-2-yl]-5-methyl-3-pyridinecarboxylicacid); Makhteshim Agan of North America, Raleigh, NC) on 16 April 2009. Finally, Select 2EC was applied 6 May 2009 at
Ecoturf cores were collected as previously described from the North Florida Research and Education Centers near Live Oak, FL and near Quincy, FL (30.55°N, 84.60°W) on 1 June 2009. The soil at the Quincy location was a Dothan loamy fine sand (fine-loamy, siliceous, thermic Plinthic Kandiudult) (Williams et al., 1997). The cores were placed directly into 40 cm-deep PVC pots constructed as previously described. The cores were again grown under greenhouse conditions in Gainesville, FL with 16 h daylength and a thermostat setting of 30°C as described previously.

The Ecoturf fields had also been treated with herbicides prior to the core collection dates. The Quincy field had been sprayed with Select 2EC at a rate of 1.1 L ha⁻¹ seven weeks before the cores were collected. The Live Oak Ecoturf field had been treated with the same herbicide applications previously described for the Florigraze field.

**Inoculation.** The cores of each cultivar were inoculated with one of four different inoculants or deionized water. The same protocol was used as for Experiment 1 with 4 replicates per core origin. The inoculants used were 8B4, 8B6, EL Cowpea, and Organica. The Organica inoculant is a proprietary blend of strains that are being marketed for annual peanut inoculation (Organica Biotech, Inc., Norristown, PA).

**Acetylene reduction assay.** Nitrogenase activity in the nodules was estimated using the acetylene reduction assay. Assays were conducted at approximately one-week intervals between 27 July and 24 September 2009 for Florigraze and between 7 December 2009 and 11 January 2010 for Ecoturf. A non-destructive flow-through
system was used for these assays (Fig. A-1). The PVC pots in which the plants were grown were covered using an inverted 3.8 L, clear plastic food storage container (Rubbermaid, Inc.). The center of the container lid was removed to expose the mouth of the pot, and this lid was then secured to the toilet flange at the top of the pot using bolts and wingnuts. A seal was created between the toilet flange and the lid using rope caulking so that once the clear food storage container was inverted over the top of the pot and attached to its lid, a sealed chamber was created. On the bottom of the food storage container, a hole was drilled and a barbed connector was screwed into place. Once the container was inverted over the pot and sealed, gas lines were attached to the bottom of the pot and to the barbed connector at the top of the container, creating a flow-through chamber (Fig. A-1).

For each daily assay, an acetylene:air mixture (1:9) was flowed through each pot at a rate of 1 L min\(^{-1}\) for 10 minutes, followed by an additional 30 minutes of flow at 0.4 L min\(^{-1}\). Samples of the gas were collected from the input lines to establish the base level of ethylene that is commonly present in the acetylene tank. Gas samples were also collected from the exhaust line above each pot, using a 1-ml syringe, so that the composition of the gas after flowing through each pot could be determined. Gas samples were analyzed using gas chromatography (Shimadzu GC-8A). The pots remained sealed after each assay with air flowing for 1 h to remove any remaining ethylene and acetylene, and then the plastic covers were removed so that the plants were covered for a minimal amount of time each day.

An estimate of acetylene reduction activity (ARA) was calculated for each genotype x soil x inoculant replicate. The daily ARA for each assay was calculated by
subtracting the average of the five lowest input readings from the average of the two output readings from each pot, and then ARA was expressed as μmoles ethylene h⁻¹ m⁻².

**Harvest.** All plants were destructively harvested on 8 October 2009. Shoots were bagged separately to be dried, while roots/rhizomes/nodules were washed free of soil and then refrigerated immediately. Once a subset of nodules were collected for molecular analysis, as described below, the remaining belowground tissues were frozen until nodules could be counted. Shoots and nodules were then dried at 60°C until constant weight and evaluated for dry mass. Dried shoots were prepared for N analysis for N concentration as previously described. Nitrogen accumulation was calculated as described previously.

**Molecular methods.** A subset of Florigraze replicates grown in soils from Live Oak and Marianna and inoculated with the 8B4 and 8B6 strains was selected for analysis of nodule constituents using molecular techniques. Three nodules were harvested from two plants representing each of the core origin x inoculant combinations described above. Within 24 hrs of washing roots free of soil, the nodules were harvested and surface sterilized. Each nodule was transferred to a 1.5 ml sterile Eppendorf microcentrifuge tube and surface sterilized through a series of washes adapted from Vincent (1970). First, 1 ml 95% ethanol was added to the nodule and allowed to soak for 10 s before being removed. Next, 1 ml of a 1% sodium hypochlorite solution was added, and the nodule was exposed for 3 min with occasional agitation. Next, four successive washes with 1 ml sterile water were performed. The water was agitated by pipetting up and down before being removed from the tube.
Surface-sterilized nodules were crushed and plated individually. After the final wash, each nodule was resuspended in 400 µl of sterile deionized water. The nodule solution was then pipetted onto a yeast extract mannitol plate (YEM; Table C-1) and spread with a glass spreader. Plates were allowed to dry and then incubated 48 h at 37°C. The plates were then stored at 4°C.

Isolates from each plated nodule were prepared for DNA sequence comparison by isolating the *glnII* gene. Single colonies were inoculated into 5 ml YEM broth (Table C-1) and then grown, with shaking at 220 rpm, for 48 h at 30°C. Genomic DNA was extracted from the fresh cultures using the GenElute Bacterial Genomic DNA Kit, following the manufacturer's instructions, but with final elution by DNA-free water (Sigma-Aldrich, St. Louis, MO). Next, the *glnII* gene was amplified by the polymerase chain reaction (PCR) using GlnIIIF (YAAGCTCGAGTACATYTGGCT-3') and GlnIIR (TGCATGCCSGAGCGCTCCA-3') primers, and Platinum TAQ polymerase (Invitrogen, Carlsbad, CA) in a reaction volume of 25 µl (see Table C-2 for concentrations). The reaction conditions were denaturing at 95°C for 4 min, 35 cycles of 94°C for 70 s, 58°C for 40 s, and 72°C for 130 s, followed by 72°C for 70 s for final extension. The products were gel-purified using a GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK).

The *glnII* gene was then amplified through cloning for sequence analysis. Purified PCR products were then cloned into pCR2.1 vector using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) using 4 µl of the purified PCR product and omitting the water from the reaction volume. The resulting constructs were then transformed into chemically competent *E. coli* DH5α competent cells (Inoue et al., 1990).
Transformations were carried out as follows. The competent cells were thawed on ice for 10 min, and then 4 µl of each construct was added, followed by 30 min incubation on ice. The cells with the plasmid DNA were then heat-shocked by placing them into a water bath at 42°C for 30 s. The tubes were cooled on ice for 2 min before 1 ml NZY+ recovery broth (Table C-3) was added to each tube, followed by incubation at 37°C for 1 hr (Fisher Scientific, Pittsburgh, PA; Table C-2). The cell suspension was then centrifuged to pellet the cells for 30 s at 13,000 rpm. The supernatant was then removed down to approximately 50 µl.

The E. coli vector cells were then screened for gene insertion into the plasmid. The pelleted cells were resuspended by pipetting up and down into the remaining supernatant and then plated onto LB 1.5% agar plates with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 40 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) added. The plates were incubated overnight at 37°C and then screened to discern colonies with the plasmid insert (white) from those without (blue).

Seven transformed (white) colonies per nodule were then evaluated by colony PCR followed by gel electrophoresis to confirm the insertion of the glnII PCR product into the pCR2.1 vector. Each selected colony was streaked onto a “patch” plate (LB 1.5% agar with 50µg Kanamycin ml⁻¹) to isolate the colony. The PCR reaction was performed using M13F (GTAAAACGACGGCCAG’3) and M13R (CAGGAAACACGCTATGAC’3) primers, and the temperature sequence was 95°C for 10 min to denature, followed by 35 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, then 72°C for 10 min for final extension.
From the seven reactions per transformation, one was selected for plasmid isolation that displayed a tight, single band at 800 bp. This colony was then grown overnight in 5 ml LB broth plus Kanamycin (50 µg ml⁻¹) with shaking at 220 rpm. The broth was then divided into two aliquots and centrifuged at 13,000 rpm for 3 min to pellet the cells. The supernatant was then removed, and the cells were washed twice with DNA-free water, combining the two aliquots into a single tube with the second wash. Plasmids were then extracted from the pelleted cells using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The final elution was done with 50 µl DNA-free water. Plasmid DNA was submitted for sequencing at ICBR, University of Florida with M-13 forward primers.

**Sequence analysis.** An error rate due to laboratory error was determined for the pCR2.1 vector DNA sequences by aligning a 70bp section of the DNA occurring before the insertion point of the PCR fragment. The alignment was done using ClustalW analysis in MacVector (8.0). Paired comparisons between the nodule isolate sequences and the four selected inoculant strain sequences were performed using CustalW sequence alignment to determine whether the number of nucleotide differences between each isolate and the applied inoculant was within the error tolerance range. Those isolate sequences with a number of differences within the tolerance range were considered to be the same as the inoculating strain, indicating successful nodulation with that inoculant.

Phylogenetic analysis of the nodule isolate sequences, inoculant sequences, and reference sequences was performed using MacVector (Table 3-4). Organisms with comparable glnII sequences to the inoculants and select isolates were located using
BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), and these glnII sequences were added to the phylogenetic analysis. For additional reference, Methylobacterium extorquens and Agrobacterium radiobacter glnII sequences were also included.

Data analysis

Data from the plant effects experiment were analyzed according to a completely randomized design where inoculation treatments were randomly assigned to replicates of each genotype. Ecoturf and Florigraze genotypes were randomized and were evaluated separately. For the inoculant effectiveness experiment, the two genotypes were also analyzed separately, since they were grown at different times. The design for the inoculant effectiveness experiment was a split-plot design where core origin defined the plot and inoculant defined the sub-plots. Statistically significant effects were determined by the appropriate one- or two-way analysis of variance (ANOVA) for Experiments 1 and 2, respectively, using the GLM procedure of SAS (SAS 9.2). Significant main effects were further analyzed by the Student-Newman-Kuels post-test for separation of means. All tests were conducted at the $\alpha = 0.05$ level.

Results

Plant Effects of Inoculants

Shoot mass. Neither Ecoturf or Florigraze shoot mass (7.74 ± 0.36 and 10.72 ± 0.65 g pot$^{-1}$, respectively) was significantly influenced by inoculant application.

Nodulation. Nodule counts pot$^{-1}$ were significantly affected by inoculation in Ecoturf ($P = 0.05$). For Ecoturf replicates, nodule counts resulting from application of the 8B4 inoculant and the water control were not different from nodule counts resulting from EL Cowpea, but were greater than the counts resulting from application of 8B6
Inoculation was not a significant main effect for Florigraze nodule count data. The effect of inoculation on Ecoturf nodule mass was not significant. The effect of inoculation on Florigraze nodule mass was significant with a P-value of 0.05. Within the Florigraze replicates, the EL Cowpea inoculant resulted in greater nodule mass than the control, but was not different than the 8B4 or 8B6 treatments. The resultant nodule masses from application of 8B4 and 8B6 were not different from the uninoculated control (Fig. 3-1b).

**Shoot N concentration.** Ecoturf shoot N was significantly affected by inoculation ($P < 0.01$). Application of the 8B4 inoculant to Ecoturf resulted in higher shoot N concentrations than did any of the three other inoculant treatments (Table 3-1). N concentrations in Florigraze shoot dry matter (mg N g$^{-1}$ DM) were not significantly affected by inoculation (Table 3-1).

**Inoculant Effectiveness**

**Survivorship of cores.** Of the 20 Ecoturf cores collected from Quincy, nine had not recovered from the removal of shoots shortly after their collection by experiment initiation, which resulted in nine missing replicates from this soil origin. As a result, the number of replicates per inoculant was reduced to two each for 8B4, EL Cowpea, 8B6 and the uninoculated control, while Organica was added to three replicates.

**Shoot mass and greenness.** Ecoturf shoot mass was significantly affected by core origin ($P = 0.01$), but the inoculant effect was not significant. The cores collected from Quincy yielded 47% higher shoot mass than those collected from Live Oak (Table 3-2). There were no significant main effects for greenness as measured by SPAD (data not shown).
Shoot mass of Florigraze was not significantly affected by inoculant. However, there were significant differences in shoot mass due to the core origin effect ($P < 0.01$). The cores from Marianna had higher shoot mass yield overall than those from either Inverness or Live Oak (Table 3-2). The core origin effect was also significant for leaf greenness in Florigraze, as measured by SPAD ($P < 0.01$), but the inoculant effect was not significant. Again, cores collected from Inverness had higher green color readings ($33.97 \pm 0.65$) than those collected from either Live Oak or Marianna ($29.35 \pm 0.70$, and $30.81 \pm 0.71$, respectively).

**Nodulation.** Ecoturf nodule counts (nodules pot$^{-1}$) were significantly affected by core origin, but the inoculant effect was not significant. The mean nodule counts from Quincy were more than double those in cores from Live Oak (Table 3-3), and the difference in nodule mass (mg pot$^{-1}$) was marginally significant ($P = 0.07$; Table 3-3).

Nodule numbers (nodules pot$^{-1}$) for Florigraze were not significantly affected by inoculant as a main ANOVA effect. However, the core origin effect was significant ($P = 0.02$). Cores collected from Marianna and Live Oak had significantly more nodules than those collected from Inverness, but the difference between the nodule counts from the Marianna and Live Oak material was not significantly different (Table 3-3). There were no significant main effects of inoculant or origin with regard to nodule mass of Florigraze (Table 3-3).

**N concentration.** Nitrogen concentration of Florigraze shoots ranged from 19.46 to 28.36 mg N g$^{-1}$, and was neither core origin or inoculant main effects were significant. There were no significant core origin or inoculant main effects for Florigraze shoot N
concentrations. Florigraze shoot N concentrations ranged from 19.5 to 28.4 mg N g\(^{-1}\), and

**ARA and N accumulation.** Due to the highly-variable nature of the normalized ARA data for Florigraze, there were no significant differences detectable by ANOVA. The data indicated a trend toward greater nitrogenase activity in the cores collected from Inverness, regardless of inoculant. (Fig. 3-2a).

For both Ecoturf and Florigraze, N accumulation was not significantly affected by inoculant, but was significantly affected by core origin \((P < 0.01\) for each). Ecoturf accumulated significantly more N in cores collected from Quincy than from Live Oak. Florigraze N accumulation was significantly greater in shoots grown from the Marianna and Inverness cores than from the Live Oak cores (Figure 3-2b).

**Competitiveness.** Three sequences received were omitted from further analysis due to abnormalities that indicated contamination or error in the sequencing analysis (see Appendix D for sequences). The sequencing error rate as determined by leading sequence comparisons was 1.3%, or 17 errors in 1260 aligned nucleotides. An additional 1.7% error rate for PCR mismatches was added to the sequencing error rate for a threshold of 3% error allowed between sequences that are the same. Paired sequence alignments between 8B4 and the 8B4 inoculation treatment isolates indicated that only one of the six isolates examined contained the recovered inoculant. The nodule containing 8B4 was from a plant grown in soil from Marianna, and only one of the two nodules analyzed from that plant contained the inoculant strain. Sequence alignments of isolates from the 8B6 inoculation treatment indicated that two nodules had recovered the inoculant. The two nodules containing 8B6 were harvested from two
different plants grown on Marianna soil. The first of the nodules recovering the inoculant was one of two analyzed from the first plant, and the second was the sole sequence analyzed from the second plant's nodules.

**Phylogenetic analysis.** Phylogenetic analysis indicates that all of the isolates and the two inoculants except for two outliers were closely related, based on the single gene analysis. The three isolates that were identified as positive recovery of the inoculants, Marianna 1-1 (8B4), Marianna 3-3 (8B6), and Marianna 1-1 (8B6), were all very closely-related to each other and to the 8B4 inoculant. Isolates Marianna 2-2 and Live Oak 1-3 were the most different from the other sequence strains. Marianna 2-2 showed homology to *Bradyrhizobium canariense* and *B. japonicum* CCBAU, and Live Oak showed closer homology to *R. tropici* strains than to the other recovered strains.

**Discussion**

Subobjective 1 was to analyze the affect of inoculation on shoot mass, nodulation, and shoot N concentrations of Ecoturf and Florigraze under non-sterile soil conditions.

Nodulation, shoot mass and N concentrations in shoots were not consistently improved by inoculation. Although Ecoturf had one marginal significant difference in nodule mass resulting from inoculation (Figure 3-1b), Florigraze had no significant increase in any of the three measured variables based on inoculation. The lack of plant response in two experiments to these measures of inoculant effectiveness indicate that none of the inoculants tested should be considered for further investigation.

The range of N concentration in these two experiments, 19.5 to 28.4 mg N g dry matter⁻¹ is similar to but lower than other reported values. Other reports from field studies give ranges of 32 to 40 mg g⁻¹ (Saldivar et al., 1992) and 29 to 35 mg g⁻¹ (Butler et al., 2007). Although daylength was extended in the greenhouse, it is possible that
growing these plants during the winter season may have diminished to some extent both the yield and the N concentration in the shoots in these greenhouse experiments.

Although the inoculant effect was not significant for the plant variables measured, there were differences based on core origin for shoot mass and nodulation for both genotypes. There were no differences in rhizome mass between core origins at the end of the experiment, which indicates that a consistent amount of plant material was collected from each site (Table 3-2). Plant performance differences based on core origin could be accounted for by differences from year-to-year in management practices. The fields were sprayed with herbicide during the growing season prior to the core collection dates, and the influence of these particular herbicides on soil microbial populations or even on rhizome vigor is unknown. Other factors such as the consistency of soil moisture over the season prior to core collection could have had an affect on the indigenous soil bacteria populations. Inoculation efficiency, and nodulation, generally, can be strongly influenced by the presence of indigenous rhizobia (Thies et al., 1991), and nitrogen availability (Abaidoo et al., 1990), regardless of other environmental factors. It is worth noting that the cores taken from fields with more clay content, the Marianna and Quincy, had consistently greater shoot mass and nodulation than the sandier sites, which could indicate an influence of a whole host of soil variables from pH to micronutrient nutrition as reasons why these sites had more productive rhizoma peanut in this study.

Since there is no prior knowledge of the bacterial inhabitants of rhizoma peanut it could be that host specificity (Zuanazzi et al., 1998) also may have also hindered the effectiveness of one or more of the inoculants, especially the EL Cowpea and Organica
inoculants, which contained a mixture of bacteria. A study comparing *Arachis pintoi* plants inoculated with 230 strains of *Bradyrhizobium* strains versus an N-fertilized standard for yield and shoot N resulted in only three strains that the authors deemed worthy of subsequent field testing (Purcino et al., 2000). This prior study indicates that *A. pintoi*, and perhaps other perennial *Arachis* species, such as *A. glabrata* may have some nodulation specificity. Due to the complexity of interactions between environmental conditions, prior field management and soil characteristics, it is difficult to determine which of these barriers to inoculation may be preventing better and more consistent inoculation rhizoma peanut.

Subobjective 2 was to further evaluate the effect of four inoculants on rhizoma peanut Ecoturf and Florigraze by evaluating N$_2$-fixation activity of plants growing in three different Florida soils. An average per pot ethylene production estimate for this study was 0.177 μmoles ethylene h$^{-1}$ pot$^{-1}$, which was comparable to the range of values for well-watered plants measured in Chapter 4 of this study (0.01 to 1.56 μ moles ethylene hr$^{-1}$ pot$^{-1}$). These values are still lower than those reported by other authors for annual peanut (*A. hypogea*), which range from 46.7 to 89.9 μmoles ethylene h-1 plant-1 or up to as much as 200 μmoles ethylene h$^{-1}$ plant$^{-1}$ (Arrendell et al., 1989; Sung and Sun, 1990). The ARA values for Florigraze were highly variable, which prevented statistical differences among treatments or soil types from being determined.

The pattern of relative differences in N$_2$ fixation based core origin indicated by the ARA data is corroborated by the N accumulation data. The inoculants had no influence on the N accumulation of either genotype. Since there was no N applied to these sandy soils during the experiment, N accumulated would have come almost entirely from N$_2$
fixation. There was 43% more N accumulated by the Florigraze plants originating from Inverness than from Live Oak, and more 68% more N accumulated by the Marianna cores versus the Live Oak cores. Ecoturf accumulated N data also reinforced the core difference pattern indicated by the ARA data. Based on this evidence, it is apparent that N₂ fixed by rhizoma peanut may be highly variable across the landscape in FL, and that the amount of N fixed has not been influenced by inoculation thus far, regardless of the soil origin.

Subobjective 3 was to evaluate the competitiveness of inoculant strains 8B4 and 8B6 for nodule formation with Florigraze versus the existing strains present in each of three soils. The inoculants applied in this study were not very competitive with the closely related strains that were already present in the soil bacteria population. The 8B6 strain may be slightly more competitive than the 8B4 strain, since it was recovered from nodules of two replicate plants rather than only one nodule, as was 8B4. The strong similarity between the majority of the isolates and inoculants may indicate that there is a highly-competitive environment in the rhizosphere of rhizoma peanut among very similar organisms competing for nodule occupancy. El-Akhal et al. (2008) also found very a large group of closely-related phylogenetic groups when analyzing indigenous populations of Bradyrhizobium strains in Mediterranean soils. It is not uncommon for several different rhizobia strains or even different groups to associate with a single legume species (Van Berkum et al., 1998). Rhizoma peanut, which had nodules from the same plants with different strains in each of them, appears to be one of those species that can nodulate with many closely-related strains with little difference in efficiency between the strains.
The high degree of similarity between the strains isolated from the nodules of plants originating in Marianna and Live Oak indicate that the rhizobia populations in these two locations are highly similar. The lack of difference in the rhizobia population between these two locations further indicates that the differences in yield lies outside of nodulation efficiency issues, but may instead be related to other soil or locale factors.

Conclusions

Inoculation was not effective for consistently increasing shoot mass or nodulation of rhizoma peanut in Florida soils. Rather, the two single-strain inoculants applied in this study, 8B4 and 8B6, were highly similar to strains that were indigenous to the fields from which the cores were sampled. Although the inoculants were able to induce nodulation, they were not competitive enough to displace the indigenous strains. These results indicate that finding an effective inoculant for rhizoma peanut, which appears to be a non-selective nodulator, could be quite difficult. Yields of other crop species that nodulate with many species of rhizobia are often not improved by nodulation, or demonstrate mixed results in response to nodulation, such as annual peanut (Chen et al., 2003; El-Akhal et al., 2008) and common bean (Graham, 1981).
Figure 3-1. Nodulation by inoculant and genotype. a.) Nodules pot$^{-1}$. Data shown are mean numbers of nodules collected from each potted core separated by genotype and inoculation treatment. b.) Nodule mass pot$^{-1}$ (mg). Data shown are the mean nodule mass pot$^{-1}$ separated by genotype and inoculation treatment. The error bars represent 1 SE of the mean. Lowercase letters represent significant differences between the treatment means within each genotype grouping as determined by a Student-Newman-Kuels post-test ($\alpha = 0.05$).
Table 3-1. Concentration of N in shoots 12 wks after inoculation (mg N g\(^{-1}\) dry weight). The data shown are shoot N for Ecoturf and Florigraze ± 1 SE.

<table>
<thead>
<tr>
<th>Inoculant</th>
<th>Ecoturf</th>
<th>Florigraze</th>
</tr>
</thead>
<tbody>
<tr>
<td>8B4</td>
<td>24.10 ± 4.45 a</td>
<td>23.76 ± 7.23</td>
</tr>
<tr>
<td>8B6</td>
<td>20.80 ± 8.00 b</td>
<td>22.79 ± 2.30</td>
</tr>
<tr>
<td>EL Cowpea</td>
<td>20.42 ± 7.00 b</td>
<td>21.81 ± 8.67</td>
</tr>
<tr>
<td>Water (uninoculated)</td>
<td>19.87 ± 7.57 b</td>
<td>22.13 ± 3.81</td>
</tr>
</tbody>
</table>

†Different letters represent significantly different means within a cultivar, as determined by a Student-Newman-Kuels post-test (α = 0.05).

Table 3-2. Shoot and rhizome mass (g pot\(^{-1}\)) from Experiment 2 by core origin. Data shown are means for each genotype ± 1 SE.

<table>
<thead>
<tr>
<th>Core Origin</th>
<th>Shoot mass (g pot(^{-1}))</th>
<th>Rhizome mass (g pot(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ecoturf</td>
<td>Florigraze</td>
</tr>
<tr>
<td>Inverness</td>
<td>--</td>
<td>182 ± 31 b</td>
</tr>
<tr>
<td>Marianna</td>
<td>--</td>
<td>6.60 ± 0.49 a</td>
</tr>
<tr>
<td>Live Oak</td>
<td>3.50 ± 0.31 b</td>
<td>4.27 ± 0.38 b</td>
</tr>
<tr>
<td>Quincy</td>
<td>5.13 ± 0.57 a</td>
<td>--</td>
</tr>
</tbody>
</table>

†Different letters indicate significantly different means within a genotype, as determined by an SNK post-test (α = 0.05).
Table 3-3. Nodulation by core origin.  a.) Number of nodules pot-1 b.) Nodule mass (mg pot-1).  Data shown for both genotypes are means ± 1 se.

<table>
<thead>
<tr>
<th>Core Origin</th>
<th>Nodules pot⁻¹</th>
<th>Nodule mass (mg pot⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ecoturf</td>
<td>Florigraze</td>
</tr>
<tr>
<td></td>
<td>Ecoturf</td>
<td>Florigraze</td>
</tr>
<tr>
<td>Inverness</td>
<td>--</td>
<td>130 ± 15 b</td>
</tr>
<tr>
<td>Marianna</td>
<td>--</td>
<td>248 ± 22 a</td>
</tr>
<tr>
<td>Live Oak</td>
<td>84 ± 18 b</td>
<td>196 ± 28 a</td>
</tr>
<tr>
<td>Quincy</td>
<td>182 ± 31 a</td>
<td>--</td>
</tr>
</tbody>
</table>

†Different letters indicate significantly different means within a cultivar, as determined by an SNK post-test (α = 0.05).
Figure 3-2. Indices of N₂ fixation: Ethylene produced (μmoles ethylene h⁻¹ m⁻²) and N accumulated in rhizoma peanut shoots. a.) Ethylene production (μmoles ethylene h⁻¹ m⁻²). b.) Accumulated N per unit land area (g N m⁻²). All panels show means by core origin as inoculation main effects were not significant. Different letters indicate significantly different means within a cultivar, as determined by an SNK post-test (α = 0.05).
Table 3-4. Comparable strains for phylogenetic analysis. Information shown is the species or strain name, the abbreviation used in the phylogenetic tree, and the BLAST accession number. The inoculant or core origin x inoculation treatment headings indicate the *glnI1* sequence input to find the comparable sequence. All sequences used from the strains shown had ≥ 98% identity with the search sequence.

<table>
<thead>
<tr>
<th>Species or Strain</th>
<th>Abbreviation</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>8B4 inoculant</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bradyrhizobium canariense</em> bv. genistearum strain BCO-1</td>
<td>B_canariense</td>
<td>AY599110</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em> strain CCBAU 43129</td>
<td>B_japonicum_CCBAU</td>
<td>GU433499</td>
</tr>
<tr>
<td><em>Mesorhizobium</em> sp. WSM3865</td>
<td>Meso_WSM3865</td>
<td>FJ827056</td>
</tr>
<tr>
<td>8B6 Inoculant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncultured <em>Bradyrhizobium</em> sp. clone DDE3</td>
<td>Brady_cloneDDE3</td>
<td>FJ812717</td>
</tr>
<tr>
<td>Live Oak soil x 8B4 Isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobium sp. CCBAU 53004-5</td>
<td>Rhizo_CCBAU_53004_g</td>
<td>GU433507</td>
</tr>
<tr>
<td>Rhizobium tropici strain CCBAU 83795</td>
<td>R_tropici</td>
<td>EU513320</td>
</tr>
<tr>
<td>Rhizobium tropici strain SCAU15</td>
<td>R_tropici_SCAU15</td>
<td>FJ799724</td>
</tr>
<tr>
<td>Marianna x 8B4 Isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradyrhizobium sp. SEMIA 6144</td>
<td>Brady_SEMIA6144</td>
<td>EU196051</td>
</tr>
<tr>
<td>Bradyrhizobium sp. SEMIA 6077</td>
<td>Brady_SEMIA6077</td>
<td>FJ391043</td>
</tr>
</tbody>
</table>
Figure 3-3. Phylogenetic relationships between recovered isolates, inoculants, and reference strains (Table 3-4). Isolates are identified by the location where the core originated followed by two numbers indicating the plant replicate and nodule number from which it was recovered.
CHAPTER 4
TRANSPERSION AND NITROGENASE ACTIVITY OF RHIZOMA PEANUT IN RESPONSE TO DRYING SOIL

Introduction

Soil dehydration is a serious concern for crop production around the world. Although dry matter production is directly related to water availability, there is genetic variability in transpiration response of individual crops to reduced available soil water (Lecoeur and Sinclair, 1996). Symbiotic N₂ fixation by legumes has also been shown to be particularly sensitive to drying soil apart from photosynthetic inputs to the nodules (Marino et al., 2007).

Water available to plants is a difficult parameter to measure. A volumetric measure of the water content of the soil is one of the simplest methods, and a method that has been shown to be a good predictor of physiological response in plants. Transpiration rates of plants are commonly unaffected until soil drying reaches 0.25 of the transpirable soil water, with some genetic variability above and below this point (Sinclair et al., 1998). Another approach focused on the plants is to measure the relative water content (RWC) of leaves (Lazcano-Ferrat and Lovatt, 1999). RWC could be used to predict the transpiration responses of crops in the field if these measurements were calibrated with volumetric water content of the soil. Evaluating the relative nitrogenase activity of plants at a known RWC could also be useful in predicting the N-status of a crop.

Di-nitrogen fixation by legumes is often more sensitive to drought stress than is the transpiration rate of the legume (Sinclair et al., 2000). However, there are some cases in which the response of nitrogenase activity to drought is disproportionate to the transpiration rate, even seemingly de-coupled, such as in some genotypes of annual
peanut (Devi et al., 2009). The acetylene reduction assay, when used in conjunction with a flow-through system, is a quick and repeatable method of measuring nitrogenase activity in nodules of intact legumes. This method is useful for comparing relative rates of nitrogenase activity over a period of time without disturbing the plant (Cathey et al., 2010).

Rhizoma peanut (*Arachis glabrata*), is an important high-value forage crop grown across the southeastern United States that is subject to seasonal drought in the sandy soils of Florida. Although seasonal drought is a problem for Florida crops, there have been no studies evaluating the physiological response of rhizoma peanut to soil drying stress.

In the current study, two experiments were implemented to evaluate the transpiration and nitrogenase activity response of three cultivars to drying soil stress: Arbrook, Ecoturf, and Florigraze. The first objective was to determine the transpiration breakpoint for three genotypes of rhizoma peanut in response to an initial period of soil drying and to a second drying event following a recovery period. The second objective was to evaluate the sensitivity of nitrogenase activity to drying soil, again for an initial drying period and to a second stress event following a recovery period. Since the Ecoturf and Florigraze cultivars are the two most economically-important cultivars of this crop, a second experiment was implemented to validate their responses to soil drying. A third objective was to evaluate RWC of rhizoma peanut leaves as a viable tool for monitoring soil drying, and to evaluate the impact on short-term drought stress on nodulation of rhizoma peanut.
Materials and Methods

Experiment I

Samples of Florigraze, Ecoturf, and Arbrook cultivars of rhizoma peanut were collected from their respective field plots on 9 December 2006 at the North Florida Research and Education Center, Live Oak, FL, as 10-cm diameter by 20-cm deep cores containing shoot, rhizome, roots and the accompanying soil using a golf cup cutter (source). The cores were inserted on-site into pots constructed of PVC plumbing supplies consisting of a toilet flange at the top of the pot attached to a 35-cm length section of 10.5-cm diameter pipe and closed at the bottom with a cap into which was threaded a compression fitting for rigid plastic tubing. All of the pot pieces were glued with PVC bonding glue to provide a water- and airtight seal, and the pot volume was approximately 2.6 L. Additional field soil was added to the bottom of each pot in order to raise the top of each core to approximately 3 cm below the top of the pots.

A 2-cm thick layer of pea gravel (approximately 2- to 3-cm diameter) was applied to the soil surface of each pot to reduce evaporative loss. One pot filled with field soil and covered with gravel was included in each experiment to verify minimal evaporative loss. Potted cores were grown in a glasshouse where the temperature was maintained between 25 and 35° C and the relative humidity during the day was approximately 40 percent. An N-free, but otherwise complete nutrient solution was added to each pot in 200 ml increments prior to the drought stress treatment, 9 and 14 March 2007, and to the Arbrook pots on 8 and 9 May 2007.

The drying of the Ecoturf and Florigraze cultivars was initiated on 15 March 2007. Arbrook plants began drying on 10 May 2007. The night before the start of the drying experiment, each plant was watered until water dripped freely from the bottom of the pot.
with 200 ml of the nutrient solution, followed by water. The next morning, each pot was weighed to determine its fully-watered mass. This fully-wetted mass minus 200 g was used as the well-watered target for daily re-watering of plants. Pots were weighed daily at approximately 16:00 to determine daily transpiration loss from each pot. Water was added to maintain the control plants at the well-watered status, and drying plants were permitted to lose up to 70 g of water per day. Water loss in excess of 70 g in any pot on any day was added to that pot.

The water status of the drying plants was monitored daily by calculating the ratio of the daily transpiration loss of each drying plant to the mean daily transpiration loss of the well-watered plants. Variation in potential transpiration among days due to differing environmental conditions in the greenhouse was accounted for in the calculation of this ratio. To allow ready comparison of response among plants within each genotype, the transpiration rate was normalized for each pot so that its values were centered on 1.0 during the initial well-watered phase. This was done by calculating for each plant the average transpiration ratio for the first four days of the experiment (before the total water loss had reached 200 g). Normalized transpiration rate (NTR) was computed on each day for each pot by dividing the daily transpiration ratio by the average initial transpiration of that plant.

Plants were allowed to dry until their NTR was approximately 0.1, at which time that plant was watered until dripping and the shoots were harvested to the soil surface. Shoots were harvested completely to provide an accurate evaluation of the shoot biomass pot⁻¹ as well as to provide a standard starting point for evaluating re-growth in each pot. This re-watering marked the beginning of the recovery period. After being
watered to dripping, the plants were watered to avoid drought until the drying sequence was repeated, as previously described, beginning 16 August 2007.

Total transpirable soil water (TTSW) was determined for each pot by taking the difference between the drained weight of each pot and the weight when the NTR reached 0.1. The daily value for the fraction of transpirable soil water (FTSW) is defined as the ratio between the amount of transpirable water left in the pot and TTSW.

Nitrogenase activity in the roots systems of the drying and well-watered plants was monitored using the acetylene reduction assay. The PVC pots in which the plants were grown were covered using an inverted plastic food storage container. The center of the container lid was removed to expose the mouth of the pot, and this lid was then secured to the toilet flange at the top of the pot using bolts and wingnuts. A seal was created between the toilet flange and the lid using rope caulking so that once the clear food storage container was inverted over the top of the pot and attached to its lid, a sealed chamber was created. On the bottom of the food storage container, a hole was drilled and a barbed connector was screwed into place. Once the container was inverted over the pot and sealed, gas lines were attached to the bottom of the pot and to the barbed connector at the top of the container, creating a flow-through chamber (Fig. A-1).

For each daily assay, a 1:9 acetylene/air mixture was flowed through this chamber at a rate of 1 L min⁻¹. After 15 min to establish equilibrium, samples of the gas were collected from the input lines to establish the base level of ethylene that is commonly present in the acetylene tank, and from the exhaust line above each pot so that the composition of the gas after flowing through each pot could be determined. A
pot filled only with soil was also assayed along with the pots containing plants. Samples were analyzed using gas chromatography (Shimadzu GC-8A). The pots remained sealed with air flowing for one hour after each assay to remove any remaining acetylene, and then the plastic covers were removed so that the plants were covered for a minimal amount of time each day.

Once the samples were analyzed, the samples taken from the output lines from the soil-only pots had lower ethylene values than the input from the acetylene tank. Due to the apparent reduction in ethylene content due to passing through soil, these soil-only pot output values were used as the ethylene baseline that was subtracted from each of the output readings from each of the potted cores. The adjusted data were normalized in the same manner as the transpiration data to arrive at a normalized ethylene production (NEP) value for each day.

At the conclusion of the experiment, shoots, rhizomes and roots were harvested, dried to constant weight at 60°C, and weighed.

**Experiment II**

The pots were watered to dripping on 13 August 2008 to initiate the gradual drying experiment. Soil drying and daily ethylene production rates were measured as described for Experiment I as plants were allowed to dry to 0.10 FTSW. The relative water content (RWC) of a single leaflet per pot was determined on respective days when NTR was approximately 1, 0.8, 0.6, 0.4, and 0.2, respectively. Leaflets were removed following the acetylene reduction assay, and immediately weighed and then floated on water for 24 hours at which time a fully-hydrated weight was measured after blotting the leaflet to remove surface moisture. Finally, each leaflet was dried to constant mass at 60°C to determine dry weight. RWC was calculated as:
\[ RWC = \frac{\text{fresh weight} - \text{dry weight}}{\text{fully-hydrated weight} - \text{dry weight}} \] (1)

Once a drying core had reached the endpoint of 0.1 FTSW, it was watered again until water dripped freely from the bottom of the pot. All plants were harvested on 8 and 9 September 2008. Leaf area was measured (LI-3100, Li-Cor Corp., Lincoln, NE) by removing leaflets from the shoots, and then shoots were dried to constant weight at 60°C. Roots and rhizomes were washed free of soil and then frozen until nodules could be removed and counted. Once nodules were collected, the roots, rhizomes and nodules were dried and weighed in the same manner as the shoots.

**Data Analysis**

The statistical design for Experiments 1 and 2 was a completely randomized block design with 6 well-watered controls, 2 soil-only controls, and 12 drying plants per block, where block was defined by genotype. Data was plotted and analyzed using Prism (Prism, 1996). For both Experiments 1 and 2, breakpoint analysis was applied to the NTR and ARA data for each genotype, where S1 and S2 are the slopes of the two line segments, respectively, and S2 was manually set to 0 so that the breakpoint would represent deviation from a mean NTR value of 1. The second line was constrained to intersect with the first line segment at the breakpoint. The two line segment slopes were compared to determine whether or not they differed significantly (\( \alpha = 0.05 \)), to confirm that the data should be represented by two segments rather than a linear single regression.

Mass results from shoots and nodulation means from well-watered and drought-stressed plants were tested for analyzed using two-way analysis of variance and one-tailed, unpaired Student’s t-tests (\( \alpha = 0.05 \)) to separate means.
Results

Experiment 1

All three genotypes in the current study had NTR thresholds between 0.17 and 0.32 FTSW. The Ecoturf expressed no reduction in normalized (daily) transpiration rate until 0.23 FTSW. The NTR threshold for Florigraze was 0.32, and for Arbrook it was 0.23 (Fig. 4-1). In the repeated drydown of each genotype following a recovery period, the NTR breakpoint was lower for the same plants: 0.17, 0.19, and 0.18 for Ecoturf, Florigraze and Arbrook, respectively.

Rhizoma peanut exhibited low and highly-variable nitrogenase activity, as measured by ARA, across the range of FTSW with no apparent response to drying soil (Figure 4-2). The NEP of the drying plants ranged from 0 to 1.25 as compared to the 1.0 that represents the well-watered control, and these readings remained scattered across the entire range of FTSW. There was no difference in ARA response to soil drying between genotypes. Absolute ethylene production rates in the well-watered plants, expressed as \( \mu \text{moles ethylene h}^{-1} \text{plant}^{-1} \), ranged from 0.02 to 2.03 for Ecoturf, and from <0.01 to 1.56 for Florigraze. Following the recovery period, the ethylene production in the well-watered plants of Ecoturf and Florigraze was further reduced: 0.01 to 0.29 and 0.01 to 0.11 \( \mu \text{moles ethylene h}^{-1} \text{plant}^{-1} \), respectively. The ethylene production of Arbrook nodules was indistinguishable from the background levels of ethylene present in the acetylene tank.

There was a significant overall treatment effect of water stress on the yield of the first cutting in Experiment I \( (P < 0.001) \), and the cultivar effect was significant \( (P < 0.001) \) with Ecoturf yield greater than that of Florigraze, but not different from that of
Arbrook. Ecoturf yield from this first cutting was reduced by 24% by water stress, and this reduction was significant \((P = 0.02)\). Florigraze yield was only reduced by water stress by 16%, and this reduction was not significant. Arbrook yield was reduced by a significant 33% by water stress \((P < 0.01; \text{Table } 4-1)\). The analysis of variance of shoot yield from the second cutting revealed no significant effect of drought stress or cultivar, but only a significant water stress \(\times\) cultivar interaction \((P = 0.028)\). Yields of Florigraze and Ecoturf following the second harvest were not significantly different. However, Arbrook yield was significantly reduced by drought, a 22% reduction \((P < 0.01)\).

**Experiment II**

The NTR breakpoints for the second year experiment were similar for Ecoturf and Florigraze genotypes at 0.20 and 0.22 FTSW, respectively (Figure 4-3). Normalized nitrogenase activity, as measured by ARA, did not respond to drying soil. The normalized ARA data indicates a fairly constant rate of nitrogenase activity across the entire FTSW range (Figure 4-3). The absolute ethylene production rates for the second year ranged from \(<0.01\) to 1.26 and 0.03 to 1.14 \(\mu\)moles ethylene h\(^{-1}\) plant\(^{-1}\) for Ecoturf and Florigraze well-watered plants, respectively.

The relative water content (RWC) of the leaflets measured from both rhizoma peanut genotypes (data pooled) in this experiment decreased sharply following a threshold FTSW value of 0.15 (Figure 4-4). There was not enough data collected for a single genotype for the data to be analyzed by individual genotype, so the breakpoint was determined using pooled data.

There was no significant overall treatment effect of water stress on the shoot yields in 2008. However, the cultivar effect was significant \((P = 0.014)\), with Florigraze
having higher yield than Ecoturf. Comparisons between yields of well-watered and water-stressed cores showed no significant differences between treatments for Ecoturf or Florigraze. Analysis of the nodulation data, nodule counts (number pot\(^{-1}\)) as well as nodule mass (mg pot\(^{-1}\)), indicates that the watering stress effect was not significant for either measure of nodulation. However, the cultivar effect was significant for both nodule counts and nodule mass (\(P < 0.001\) and \(P < 0.001\)), with Ecoturf having greater nodulation than Florigraze.

**Discussion**

**Transpiration Response**

Experiment I found the NTR threshold range of rhizoma peanut to be 0.17 to 0.32 for three genotypes, which indicates that this crop is relatively tolerant to drying soil for maintaining transpiration. Experiment II further confirms the NTR threshold values for Ecoturf and Florigraze to be approximately 0.2. Similar studies conducted on field pea (*Pisum sativum* L.), annual peanut (*Arachis hypogea* L.), and soybean (*Glycine max* L. Merr.) report NTR threshold values of 0.40, 0.40, and 0.29, respectively (Lecour and Sinclair, 1996; Sinclair et al., 1998; and Sinclair et al., 1995). The three cultivars used in Experiment I also show limited variability for this trait, since the range of threshold values is relatively narrow in comparison to other crops whose germplasm has been more widely examined. A test of 17 genotypes of annual peanut resulted in a continuum of threshold values ranging from 0.28 to 0.59 (Devi et al., 2009).

In Experiment I, there was approximately a 30 and 60% reduction in the breakpoint that resulted from the second drying event versus the first drying event for the Ecoturf and Florigraze cultivars, respectively. The reason for this interesting
adaptive response is unknown, since the roots of the cores should have had adequate
time to reach the bottom of the pots by the start of the first drying sequence.

RWC

The limited data collected in Experiment II to evaluate RWC in relation to FTSW
indicate a similar transpiration threshold of 0.2. However, due to the rapid decline of the
RWC of the leaflets in a single day, many FTSW points were missed. If this method
were to be used in the future, leaflets would need to be collected more than once per
day during the drying period. Although removal of leaflets could potentially change the
soil drying rate due to declining leaf area in the canopy, this issue could be ameliorated
by removing approximately the same leaf area from both the control and drying plants in
order to prevent discrepancies. It would also be interesting to evaluate RWC of the
well-watered control plants versus the drying plants to see at what FTSW available point
differences between the two treatments become apparent.

Nodulation and Nitrogen Fixation

Nodulation was not significantly affected by the drought imposed in this
experiment. The number of nodules and nodule mass were significantly different
between cultivars ($P < 0.001$, and $P < 0.001$, respectively; Table 2).

Normalized ethylene production rates were insensitive to drought stress in both
experiments in this study. In soybean, $\text{N}_2$-fixation rates have been shown to be more
sensitive than NTR to soil drying (Muchow and Sinclair, 1986). In similar studies,
genotypes of annual peanut and soybean have been identified where the nitrogen
fixation rates were both sensitive and insensitive to soil drying. Screening of annual
peanut germplasm for less drought-sensitive $\text{N}_2$-fixation characteristics has revealed
some lines that were both highly sensitive and some that were highly-insensitive to soil drying (Devi et al., 2009).

Due to the very low ethylene production by rhizoma peanut, it is possible that the methods used were not sensitive enough to detect differences in the NTR response to soil drying. The values reported in the current study, ranging from <0.01 to 1.56 for well-watered plants, are very low compared to some other reported values for annual peanut on intact nodules. Arrendell et al. (1989) reported ethylene production values from Spanish-type crosses where ARA was used as a screening tool for breeding at a range of 46.7 to 89.9 µmoles ethylene h⁻¹ plant⁻¹. In another study, even higher rates were reported for both Virginia-type and Spanish-type plants that were evaluated at various growth stages. The maximum ARA rates, during seed-fill, were at approximately 200 and 90 µmoles ethylene h⁻¹ plant⁻¹, respectively (Sung and Sun, 1990).

**Shoot Yield**

Yield reductions due to short-term water stress were significant for yields of the first and second harvests of Arbrook and for Ecoturf cores in Experiment I. However, there were no other instances of significant yield reductions in Florigraze or any other instances of yield reduction of Ecoturf out of the two experiments. These preliminary results on small samples indicate that drought may have a more pronounced impact on the yield of the Arbrook cultivar than the two more common cultivars, Florigraze and Ecoturf.

**Conclusions**

Rhizoma peanut has a relatively low transpiration threshold response to short-term, gradual soil drying and no apparent threshold for reduction in NEP rates. In
addition, reduction in N$_2$-fixation rate of rhizoma peanut in response to soil drying is apparently de-coupled from its transpiration response to the same stress. The low FTSW threshold response to soil drying in addition to this de-coupling of N$_2$-fixation rate from temporary drought stress is a favorable response for a slowly-growing forage legume like rhizoma peanut. Yield was not consistently reduced by short-term water stress in Ecoturf or Florigraze. These characteristics indicate that rhizoma peanut may be able to maintain to low soil water contents its photosynthesis rate as well as its relatively higher nitrogen status versus the grasses with which it must compete.
Figure 4-1. Daily normalized transpiration rates (NTR) as a function of the fraction of transpirable soil water (FTSW) present that day from Experiment 1. The circles, squares and triangles represent individual measurements of the Ecoturf, Florigraze, and Arbrook genotypes, respectively. Plots A, B, and C show data from the first dehydration period, and plots C, D, and E show data from the dehydration period following recovery. S1 is the slope of the portion of the curve from zero up to the breakpoint.
Figure 4-2. Normalized acetylene reduction activity (ARA). The circles and squares each represent the response of an individual Ecoturf and Florigraze potted core sample, respectively, to drying soil.
Figure 4-3. Daily normalized transpiration rates (NTR) and normalized acetylene reduction activity (ARA) as functions of the fraction of transpirable soil water (FTSW) present in the pot. The circles and squares represent individual measurements of the ecoturf and florigraze genotypes, respectively from 2008. $S_1$ is the slope of the portion of the curve from zero up to the breakpoint.
Figure 4-4. Relative Water Content (RWC) of leaflets collected across the FTSW gradient. Each point represents a single data point. The data from the Ecoturf cultivar are represented by closed circles, and Florigraze data is represented by the closed squares. $S_1$ is the slope of the portion of the curve from zero up to the breakpoint.
Table 4-1. Shoot mass harvested at the conclusion of first and second drying treatments in Experiment I and at the conclusion of Experiment II. Data shown are mean shoot yields per pot (g) ± 1 SE for the well-watered (WW) and water-stressed (DS) plants.

<table>
<thead>
<tr>
<th></th>
<th>Ecoturf</th>
<th>Florigraze</th>
<th>Arbrook</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WW</td>
<td>WS</td>
<td>WW</td>
</tr>
<tr>
<td><strong>First harvest</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.00 ± 1.16*</td>
<td>8.33 ± 0.64</td>
<td>5.58 ± 1.15</td>
</tr>
<tr>
<td><strong>Final harvest</strong></td>
<td>9.56 ± 1.28</td>
<td>8.68 ± 1.18</td>
<td>5.58 ± 9.10</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td>8.17 ± 1.25</td>
<td>7.02 ± 0.56</td>
<td>11.07 ± 1.84</td>
</tr>
</tbody>
</table>

†* Indicates a significant difference between the WW and WS treatments for the given harvest at $\alpha = 0.05$, and **indicates that this difference is significant at $\alpha = 0.01$.

Table 4-2. Nodulation data from well-watered (WW) and water-stressed (WS) plants examined in Experiment 2. Values shown are the number of nodules per pot and mass of nodules per pot (g) ± 1 SE. There were no significant differences between WW and WS means within a cultivar, however, the overall cultivar effect was significant between Ecoturf and Florigraze for both nodules per pot and mass ($\alpha = 0.01$).

<table>
<thead>
<tr>
<th></th>
<th>Ecoturf</th>
<th>Florigraze</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WW</td>
<td>WS</td>
</tr>
<tr>
<td>Nodules pot$^{-1}$</td>
<td>600.83 ± 104.69</td>
<td>476.64 ± 54.05</td>
</tr>
<tr>
<td>Nodule mass (g)</td>
<td>0.199 ± 0.026</td>
<td>0.145 ± 0.015</td>
</tr>
</tbody>
</table>
Rhizoma peanut is a crop of growing economic importance in the southeastern United States. More knowledge about the physiology and genetic potential of this crop is needed for it to be produced at maximum efficiency.

**Objectives**

The overall objectives of the current study were to evaluate the potential for N deficiency in rhizoma peanut that may exist in spite of symbiotic N\(_2\) fixation and to monitor the effects of N supplementation on yield, nodulation, nitrogenase activity, and rhizome characteristics, (2) to evaluate the potential for yield improvement and increased N\(_2\) fixation in rhizoma peanut with the addition of an inoculant and to determine the competitiveness of potential inoculants in field soil, and (3) to determine the transpiration and nitrogenase activity responses of rhizoma peanut genotypes to soil drying. Greenhouse experiments utilizing a flow-through system for administering the acetylene reduction assay and a field study were used to evaluate these objectives.

The genotypes evaluated in this study were Florigraze, Ecoturf, and to a limited degree, Arbrook. Florigraze and Ecoturf were the focus of the work, because they are the most economically important cultivar of rhizoma peanut for forage production and the germplasm available for groundcover uses, respectively.

**Findings**

**Response to N Fertilization**

Rhizoma peanut yield can be improved by the addition of N fertilizer under controlled conditions. Although this finding has been previously reported, (Adjei and Prine, 1976; Venuto et al., 1998), the specific level at which N fertilization ceased to
increase yield or where it inhibited nodulation had not been previously reported. Shoot yield for both cultivars increased linearly with increasing N rate up to 96 mg N m\(^{-2}\) wk\(^{-1}\) (approximately 10.5 kg N ha\(^{-1}\) over the duration of the experiment). It appears that 96 to 192 mg N m\(^{-2}\) wk\(^{-1}\) (10.5 to 21.0 kg N ha\(^{-1}\)) may be approximately the optimum range for nitrogen fertilization under controlled growing conditions (Chapter 2). These results are much lower than those reported by Venuto et al. (1998), who found a linear response under controlled conditions for N dosages up to 170 kg ha\(^{-1}\). There were several differences between these two experiments that could have accounted for some of the yield difference. In the Venuto et al. experiment, the N fertilizer treatment was applied in a single dosage, and the plants were grown for 3 months with three harvests. In the current study, the N treatment was added incrementally over the 11 weeks, which was a shorter time period, and the plants may have produced less shoot material without cuttings to stimulate additional growth. Plants using N as it was added in small increments may perhaps use less N than those to which the N was added in a single dose.

Under field conditions, rhizoma peanut yield increased significantly to N fertilization (336 kg ha\(^{-1}\) y\(^{-1}\)) for the 2009 season (Chapter 2). The increase in rhizoma peanut yield may have been more dramatic had significant rainfall events not have fallen so close to the application dates, possibly removing much of the available N. Rainfall is an important factor in rhizoma peanut (and any crop) production, in spite of N fertilization (Venuto et al., 1998). With rainfall events as large as occurred following the May and July applications, much of the N fertilizer applied was likely lost as leachate in the current study. In a similar field study, the addition of 0, 110 and 220 kg N ha\(^{-1}\) did
not produce significant yield improvement, in part, due to too little rainfall (Redfearn et al., 2001). The interaction of weather with fertilization makes improving yield of rhizoma peanut with N addition a very difficult task. It would be interesting to see if a more consistent response to N fertilization would occur under irrigated field conditions.

The final rhizome N concentration (g kg⁻¹) for the final harvest in the field study was 20 g N kg⁻¹ DW, which meets the recommended concentration for propagule rhizomes (Rice et al., 1996). However, the rhizome mass declined over the season with or without fertilization (Table 2-3), which indicates that the interaction of total non-structural carbohydrates (TNC) with N concentration that Rice reported needs to be further investigated under N fertilization before the rhizomes should be used for propagation. If the TNC is adequate as well, under N fertilization, it may be possible to harvest a crop in the same year that a field is being harvested for propagules, which could be an income boost to farmers.

More importantly, however, is the N accumulation findings, which indicate that N₂ fixation both under controlled and field conditions may be inadequate for optimal growth. Nitrogen accumulation in both greenhouse experiments was enhanced with N fertilization. Under greenhouse conditions, N accumulation was saturated by the 21 kg ha⁻¹ (192 mg N m⁻² wk⁻¹) treatment. Further evidence of inadequate N₂ fixation was also obtained in the field were the N accumulation at the first harvest date was increased by a statistically significant 25% in the subplots receiving 112 kg N ha⁻¹ application⁻¹ than the subplots receiving no N (P < 0.01; Table 2-4). Consequently, all three experiments offered evidence that symbiotic N₂ fixation was inadequate to maximize N accumulation and storage in shoots of rhizoma peanut.
Response to Inoculation

Inoculation did not have a significant affect on shoot yield of rhizoma peanut for either genotype (Chapter 3). There were mixed responses in nodulation, but no clear cases where the addition of inoculant resulted in significantly more nodules than the uninoculated control. Acetylene reduction activity levels were low enough that any impact of inoculation fell within the range of variation, and was not detectable.

The results of the second portion of the inoculation study, although conducted under greenhouse conditions with small cores, indicate that rhizoma peanut production varies significantly based on soil type, regardless of inoculation (Chapter 3). The Marianna and Quincy soils supported greater yield (Florigraze and Ecoturf, respectively) than did the sandier soils found at Live Oak and Inverness (Table 3-2). There was not a consistent inoculant x soil type interaction to indicate that a particular inoculant would be more competitive in any of the different bacteria communities that were present in the field soils.

Differences in N$_2$ fixation based core origin indicated by the acetylene reduction assay (ARA) data were corroborated by the N accumulation data. The inoculants had no significant influence on the N accumulation of either genotype. The amount of accumulated N for both the Florigraze and Ecoturf genotypes reinforced the relationship between N$_2$ fixation and core origin indicated by the ARA data. There was 43% more N accumulated by the Florigraze plants originating from Inverness than from Live Oak, and more 68% more N accumulated by the Marianna cores versus the Live Oak cores. This N accumulation difference is in spite of slightly higher (not significant) yield from the Live Oak cores versus the Inverness cores. This initial, albeit limited evidence indicates that N$_2$ fixed by rhizoma peanut may be highly variable across the landscape.
in FL, regardless of yield differences. The influence of soil type, pH, nutrient availability, and herbicide interaction with soil microbial populations should be further explored in order to determine what allows one site to be better for N\textsubscript{2} fixation than others.

Chapter 3 also evaluated the competitiveness of two inoculating strains for nodule formation with Florigraze versus indigenous strains in the field soils. Interestingly, rhizoma peanut plants developed nodules with various strains in each of them, with little apparent difference in preference between the strains (Fig. 3-3). It is not uncommon for several different rhizobia strains or even different groups to associate with a single legume species (Van Berkum et al., 1998).

The inoculants applied in this study were not very competitive with the closely related strains that were already present in the soil bacteria population. The high degree of similarity between the strains isolated from the nodules of plants originating in Marianna and Live Oak indicate that the rhizobia populations in these two locations are highly similar. The lack of difference in the rhizobia population between these two locations further indicates that the differences in yield lies outside of nodulation efficiency issues, but may instead be related to other soil or locale factors, as previously mentioned.

**Response to drying soil stress**

Rhizoma peanut is a relatively drought tolerant crop. The transpiration threshold responses found in response to drying soil indicate that rhizoma peanut responds similarly to the more tolerant lines of annual peanut to soil drying (Chapter 4). There were slight differences between the three cultivars evaluated, which indicates that there may be some genetic variability for this trait in the forage *Arachis* spp. germplasm. The results also indicate that the low nitrogenase activity of rhizoma peanut nodules is not
highly-sensitive to drought stress, but rather regulation of N\textsubscript{2} fixation seems to be decoupled from the transpiration response of the plant. However, there did appear to be some technical difficulties in the use of the ARA system with rhizoma peanut, as made apparent by the low ethylene production rates found that conflicted with the accumulated N data. Further development and testing will explore a different approach system that works well for flowing gases non-destructively through the root systems of multi-stem plants with consistent results.

**Suggestions for Future Work**

Since rhizoma peanut is expanding in its use as a widely-used and profitable hay crop and forage in the Southeast, further exploration into its physiological and specifically N\textsubscript{2}-fixation potential is needed. Although the slow establishment rate of rhizoma peanut may continue to be a problem for producers, if the N accumulation of the crop could be enhanced, both the yield and quality of the hay could be increased.

This study of N-fertilized rhizoma peanut yield, along with previous field studies, have concluded that rainfall inconsistencies have a strong interaction with N fertilizer application, and that this interaction makes it difficult to see dramatic yield differences in response to N application in the field (Redfearn et al., 2001; Venuto et al., 1998). A field study where water is not limiting could be useful for evaluating the genetic potential of perennial peanut in response to N fertilization under water non-limiting field conditions. Although the initial work in this study indicate that rhizoma peanut N\textsubscript{2} fixation is not readily suppressed by drought stress, a field study to confirm these findings and to explore differences in N accumulation and yield potential under field would be an excellent addition to the body of knowledge available for this crop.
Improvement of N₂-fixation potential of rhizoma peanut may be the most economical and environmentally amiable way of increasing forage yield. In addition, the expanded use of Ecoturf as a drought resistant and N self-sufficient perennial ornamental groundcover is especially attractive for areas of the Southeast, especially South Florida, that are developing highly-restrictive nutrient input laws for urban areas. For agricultural production, the combination of improved genetics, such as the new cultivars UF Tito and UF Peace, which have good persistence and are tolerance to peanut stunt virus, with an efficient inoculant could be an ideal situation for maximizing productivity of rhizome peanut (Quesenberry et al., 2010). Realizing this ideal is many years ahead, but there are some steps that could be taken in the near future in order to make progress towards finding a marketable and effective inoculant, if future research documents that inoculation is truly deficient.

Examining the current rhizobia-plant interactions with rhizoma peanut is an important first step for improving N₂ fixation in the field. A field survey to collect and culture nodules from field sites around Florida could be the start of many studies. The cultures could then be examined to find consistent strains that nodulate in multiple locations that have consistently high yields in addition to exploring the genetic diversity and relationships of bacteria that are currently occupying the nodules. An estimate of rhizoma peanut nodulation specificity, where nodule occupant phylogenetic relationships and other characteristics are evaluated, could help researchers to better understand why or why not nodulation with novel inoculants may work in the future. This initial survey could be conducted rapidly and could perhaps be tied-in for funding
purposes with other studies evaluating the rhizobia or general microbial diversity of Florida soils.

The interaction between chemical applications such as herbicides and soil fumigants and bacteria populations in treated rhizoma peanut fields is another area that needs further explanation. In this study, material from Live Oak, to which many different herbicides in multiple applications had been applied, had the lowest N accumulation in the inoculation study, but not the lowest yield (Table 3-2; Fig. 3-2). This relationship may indicate a potential reduction in rhizobia populations as a result of herbicide application. Although the current study only hints at this possibility, further work could evaluate the relationship between specific herbicides and rhizobia populations as well as potential crop damage, including possible rhizome stunting that may occur with different rates of herbicide application.

Sterile rhizoma peanut material maintained under aseptic conditions could be used to further address questions raised by this study about the efficiency of the currently-nodulating rhizobia population/strains. Plants grown under aseptic conditions could then be introduced to a variety of inoculants, and the ability to nodulate and efficiency of the individual strains based on growth and N concentration of tissues could be evaluated on a large scale with many replicates grown in small pots (methods similar to Purcino et al. 2000). Once promising inoculants could be identified, it would then be important to understand their competitiveness with the indigenous populations of rhizobia (Bradyrhizobia) in FL using an extension of the methods used in Chapter 3 of this study, followed by field testing.
Inoculant collection from the native range of perennial Arachis species may be an important next step. Some sampling of the native populations of *A. glabrata* and closely-related species has been done in Argentina in some ongoing work by Dr. Monica Collavino (Agricultural Science Faculty, Universidad Nacional del Nordeste, Corrientes, Argentina). Bacteria collected from thriving populations in the native range of rhizoma peanut are good candidates for future inoculant screening experiments in FL as discussed above. Diversity in the inoculant pool is needed in order to improve the chances of finding a more effective inoculating strain.
Figure A-1. Flow-through pot/chamber for intact acetylene reduction assays.
### APPENDIX B

NUTRIENT SOLUTION

Table B-1. N-free nutrient solution protocol.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock Solution (g L(^{-1}))</th>
<th>Stock Solution Added to Tank (ml 19L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>27.22</td>
<td>95</td>
</tr>
</tbody>
</table>
| MgSO\(_4\)\(
7H\_2\)O\) | 98.59                         | 95                                            |
| KCl                 | 37.20                         | 190                                           |
| CaCl\(_2\)          | 73.50                         | 266                                           |
| Iron Sequestrene    |                               | 95                                            |
| Micronutrient Solution |                             | 95                                            |

†Deionized water added to bring the solution up to volume.
‡Pre-fill tank approximately half-full before adding nutrient stocks to prevent precipitate formation.

#### Micronutrient Stock Solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock Solution (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_3)BO(_3)</td>
<td>0.570</td>
</tr>
<tr>
<td>MnCl(_2)(\cdot4)H(_2)O)</td>
<td>0.360</td>
</tr>
<tr>
<td>ZnSO(_4)(\cdot7)H(_2)O)</td>
<td>0.040</td>
</tr>
<tr>
<td>CuSO(_4)(\cdot5)H(_2)O)</td>
<td>0.016</td>
</tr>
<tr>
<td>H(_2)MoO(_4)(\cdot4)H(_2)O)</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Table C-1. Yeast extract mannitol media.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g L-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol (Sigma 66F-006)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Yeast Extract (Fisher BP 1422-500)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>K2HPO4</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>MgSO4·7 H2O</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
| Deionized H2O           | Bring to 1 L volume | }

†For liquid culture, omit agar.
Table C-2. PCR Master Mix for Platinum Taq Enzyme and 2.5 mM dNTP protocol.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Per Rxn (µl)</th>
<th>3 rxns (µl)</th>
<th>8rxns (µl)</th>
<th>24 rxns (µl)</th>
<th>30 rxns (µl)</th>
<th>Concentration in Master Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>18</td>
<td>54</td>
<td>144</td>
<td>432</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Standard 10x Buffer -MgCl₂ (50mM stock; Invitrogen)</td>
<td>2.5</td>
<td>7.5</td>
<td>20</td>
<td>65</td>
<td>75</td>
<td>200 mM</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1</td>
<td>3</td>
<td>8</td>
<td>24</td>
<td>30</td>
<td>3.5 mM</td>
</tr>
<tr>
<td>2.5 mM dNTPs</td>
<td>2</td>
<td>6</td>
<td>16</td>
<td>52</td>
<td>60</td>
<td>200 µM</td>
</tr>
<tr>
<td>Forward Primer (50 µg ml⁻¹ stock)</td>
<td>0.25</td>
<td>0.75</td>
<td>2</td>
<td>6.5</td>
<td>7.5</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Reverse Primer (50 µg ml⁻¹ stock)</td>
<td>0.25</td>
<td>0.75</td>
<td>2</td>
<td>6.5</td>
<td>7.5</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Platinum Taq Enzyme</td>
<td>0.2</td>
<td>0.6</td>
<td>1.6</td>
<td>4.8</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**dNTP Mixture Protocol**

1) dNTPs (4 total) come in 100mM concentrations from Fischer Scientific.
2) Take 25 µl of each type of dNTP and mix together in an Eppendorf tube (100 µl total volume).
3) Perform a 1:10 dilution by adding 900 µl dH2O to the tube for a total volume of 1000 µl.
4) Store 200 µl aliquots at -20°C.

Table C-3. NZY+ Recovery Media for transformation mixture.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZY media (Fischer Scientific)</td>
<td>45 ml</td>
</tr>
<tr>
<td>1 M MgCl</td>
<td>0.47 ml</td>
</tr>
<tr>
<td>20% Glucose solution</td>
<td>0.9 ml</td>
</tr>
</tbody>
</table>
APPENDIX D
NODULE ISOLATE NUCLEOTIDE SEQUENCES OF THE GLNII GENE

8B4 Inoculant
GAATTCCGATGTCGATGCCGTACTTTCTCGGTCAGGCGCAGCATCAGGTAGCGGGCC
ATCCACATTTGTCAGCGCGCCCTTGGAGCCCTTGCGGAAGATCTGGCAATTCCC
CTGGCCCTTCGCGACTTCCGAGGTGCCTGGTTGATGCCGGCGAG
GCAGAGTGCAGCTCCTCCTGGCACAGATCTTGCGGGCGACGTCGCCGACGTTCG
GAAAGCCGACGCCGCGCCGACGCCGCGTCAGGCGGAA

8B6 Inoculant
GAATTCCGCCCTTTGCATGCCGGAGCCGTTCCAGTCGGTGTCGCCGAGCGCTTGCC
AGTGGAATTCGATGTCGATGCCGTACTTCTCGGATGGCAGTGACATCAGATAGCG
GGCCATCCAGCTCGAGGGATGGTCGCTATTGCCCTTGGAGCCCTTGCGGAAGATCTGGCAATTCC
CTGGCCCTTGGCCACTTCAGCGTTGATGCCGTTCGTGGTTGATGCCGGCTTCAGG
CAGAGGTCGAGGTGCTCTTCAACGATCTCGAGCGACATCGCCGACGTTCTTGAG
CCGACGCCGCGCCGACGCCGCGTCAGGCGGAA

Live Oak replicate 1, nodule 1
GAATTCGATGTCGATGCCGTACTTTCTCGCAGACGCAGCAGCAGGTAGCGTGCC
ATCCAGATCTGGCGCTCTTGGACCGCTTGGCAGATCAGATGACTTCTCTCC
CTGGCCCTTCGCGACTTCCGAGGTGCCTGGTTGATGCCGGCGAG
CAGAGGTCGAGGTGCTCTTCAACGATCTCGAGCGACATCGCCGACGTTCTTGAG
CCGACGCCGCGCCGACGCCGCGTCAGGCGGAA

Live Oak replicate 1, nodule 2
GAAGGTAGCGATGAGGTGAAGAAAGTCGTTTCTTTCGCAAAGAGGATTGGCTTGAAGATGACTTCTCTCC
CTGGCCCTTCGCGACTTCCGAGGTGCCTGGTTGATGCCGGCGAG
CAGAGGTCGAGGTGCTCTTCAACGATCTCGAGCGACATCGCCGACGTTCTTGAG
CCGACGCCGCGCCGACGCCGCGTCAGGCGGAA

Live Oak replicate 1, nodule 2
GAAGGTAGCGATGAGGTGAAGAAAGTCGTTTCTTTCGCAAAGAGGATTGGCTTGAAGATGACTTCTCTCC
CTGGCCCTTCGCGACTTCCGAGGTGCCTGGTTGATGCCGGCGAG
CAGAGGTCGAGGTGCTCTTCAACGATCTCGAGCGACATCGCCGACGTTCTTGAG
CCGACGCCGCGCCGACGCCGCGTCAGGCGGAA
CATTCCTGCAACAAATCCTGACTGTCCTGCTGAATATGATGTGGTAGCTACGTTG
AACCTGAACGGGGACTATGTTTCCGATGCACTTGCTGCACAGGTTGGCGGCATCG
GCATTGCACCGGGGCACAAACATCAACTATGTGAACCGCTCGCGCTATTTTCGAAGC
AACACATGGAACCGGCTCCCGGCATGCAAGGCGGAA

Live Oak replicate 1, nodule 3
GAATTCGCCCTTTGCTATGCGGAGCCGTTCCAGTCGCTGCGACCGGGCTTGC
AGTGGAACTCGATGCTGATGCCTACTTTCTCGGACCAGCGCAACATCGGTAGCG
GGCCATCCACATTTGGAGCCTTCTTGGAGACCCCTTGGCAAGATCTGAAATT
CCCACTGGCCTTTCGCACTTCTCAGCTGATGGGCTGCTGTGAGCCTGACCAGC
AGGGCAAGGTCGAGATCTCCTCTCGAGCTCGTCGGTACCGTGTCGGCGGGTT
CGAGAAGCCGACGCGGCTGAGTGACAGCTCCTGAGGCTGAGGCTGAGGCTG
GGCCTTCATGACCGGAGCATCGGATTCCCGCTGCCGCTGACACAGGCAGG

Live Oak replicate 2, nodule 1
GAATTCGCCCTTTGCTATGCGGAGCCGTTCCAGTCGCTGCGACCGGGCTTGC
AATGGAACTCGATGCTGATGCCTACTTTCTCGGACCAGCGCAACATCGGTAGCG
TGCCATCCAGATCTCGGTGGGCGCTTCTTGGAGACCCCTTGGCAAGATCTGAAATT
TCCAAGGCTTCTTGGACCCTCAGCTGATGGGCTGCTGTGAGCCTGACCAGC
CGGGAAAGCGACGCGGCTGAGTGACAGCTCCTGAGGCTGAGGCTGAGGCTG
GGCCTTCATGACCGGAGCATCGGATTCCCGCTGCCGCTGACACAGGCAGG

Live Oak replicate 3, nodule 1
GAATTCGCCCTTTGCTATGCGGAGCCGTTCCAGTCGCTGCGACCGGGCTTGC
AGTGGAACTCGATGCTGATGCCTACTTTCTCGGACCAGCGCAACATCGGTAGCG
TGCCATCCAGATCTCGGTGGGCGCTTCTTGGAGACCCCTTGGCAAGATCTGAAATT
CCCACTGGCCTTTCGCACTTCTCAGCTGATGGGCTGCTGTGAGCCTGACCAGC
CGGGAAAGCGACGCGGCTGAGTGACAGCTCCTGAGGCTGAGGCTGAGGCTG
GGCCTTCATGACCGGAGCATCGGATTCCCGCTGCCGCTGACACAGGCAGG

Live Oak replicate 3, nodule 2
GAATTCGCCCTTTGCTATGCGGAGCCGTTCCAGTCGCTGCGACCGGGCTTGC
AGTGGAACTCGATGCTGATGCCTACTTTCTCGGACCAGCGCAACATCGGTAGCG

111
TGCCATCCAGATCTGGTCGGCTGCCTTCTTGGAGCCCTTGCCGAAAATCTGGAATT
CCCACTGGCCCTTGGCCACCTTACGCTTTGATGCTCTTCCTGTGGATATGCCCGCTTC
GAGGCAGAGGTGAGTGCTTCTCAAGCTCTCTGAGCGACATCGCCGACGGTT
CTTGTAGCGACGCCCGTGGTAGTACGGGCCCCTGCGGAGCCCGGTAGCCGAGATC
CGGGAACCGGAGCCAGCGCCCGGCCTTACCCTGAGAAAGATCTGGCAATT
CCCACTGGCCCTTGGCCACCTTACGCTTTGATGCTCTTCCTGTGGATATGCCCGCTTC
GAGGCAGAGGTGAGTGCTTCTCAAGCTCTCTGAGCGACATCGCCGACGGTT
Marianna replicate 1, nodule 1
GAATTCCGCCTTTGATGCGGAGGATTCGCCCTTTGCATGCCGGAGCCGTTCCAGTCGGTGTCGCCGAGCGGCTTGG
AGTGGAATTCGATGTCGATGCCGTACTTCTCGGCCAGGCGCAGCATCAGGTAGCGGGC
CATCCACATCTGGTCCACGCGGCTTCTTGGAGCCCTTTGCCGAGATCTGGGAATTCC
CCCACTGGCCCTTGGCCACCTTACGCTTTGATGCTCTTCCTGTGGATATGCCCGCTTC
GAGGCAGAGGTGAGTGCTTCTCAAGCTCTCTGAGCGACATCGCCGACGGTT
Marianna replicate 1, nodule 2
GAACCTCGATGTCCGATGCCTCTTCGTCGGTTCGAGGCGACGATCAGGTAGCGGGAC
CATCCACATCTGGTCCACGCGGCTTCTTGGAGCCCTTTGCCGAGATCTGGGAATTCC
CCCACTGGCCCTTGGCCACCTTACGCTTTGATGCTCTTCCTGTGGATATGCCCGCTTC
GAGGCAGAGGTGAGTGCTTCTCAAGCTCTCTGAGCGACATCGCCGACGGTT
Marianna replicate 2, nodule 1
GAAGATCTGGGAATTCCCACTGGCCCTTGGCCACTTTGCCGAGCCCTGGGAGCACTTGGG
TTGATGCGGAGCAGGAGGTTCGGAGATGTCTCTGAGCTCTTGGAGCCCGCTTGG
TTGATGCGGAGCAGGAGGTTCGGAGATGTCTCTGAGCTCTTGGAGCCCGCTTGG
Marianna replicate 2, nodule 2
GAAGATCTGGGAATTCCCACTGGCCCTTGGCCACTTTGCCGAGCCCTGGGAGCACTTGGG
TTGATGCGGAGCAGGAGGTTCGGAGATGTCTCTGAGCTCTTGGAGCCCGCTTGG
TTGATGCGGAGCAGGAGGTTCGGAGATGTCTCTGAGCTCTTGGAGCCCGCTTGG
CAAGTTCCGAGTCGGCGCTATATCCGTCAAGCCAAATGTACTCGAGCTTTGAAGGGC
GAA

Marianna replicate 2, nodule 2
GAAGGCCACAGCTCGATTGCGTGAAGCAGCGTTCGCGGTTTTCCCGGATGCC
GCCGCAACCAGTGCCGCTGATGTGCGAAGGCAGCAGCGGACATCGCAAGCAG
ACCCCGCATCCGTCAAAACGCGCCACCACATTCTCGACGATTTCCCGGCGCTGT
TCGCGCTTCGAGCAGAAACTCTTCTTCTACAAGAACCGCCGCCGCTGGGCTTCCC
GACCTCGGGCGACGCTGAGCGATCGTCGGCCTGGTCTTCCCTC
GAACGTCGGCGACGTCGCCCCCAAGATCGTCGAGGTAGCGCATCTCGACCTC
CGCGGGCCGCATCAACACGGAGGGTCATCAGGAAGGGCCAGTTGGAGC
GGAATTCAGATCTTCCGACAAGGGGCTTCAAAGACAGCCGGGTACGAAGATGGATG
GCCGCTACCTGAGTGCCTGAGCTACCCGAGAAGACATCGGACATCGAAG

Marianna replicate 3, nodule 1
GAATTCCGCTTCTTGATGCCGAGCCGCTTCCAGTGCCTGAGCAGCCCGCTTGC
AGTGAAATTCGATGCGATGCGCGACTTCTCGGTCAGGCCGACGATCAGGTAGCG
GGCCATCACAATTTCTCAGGGCTTCTTTGAGGGCCAGTCTGGAATT
CCCACTGGCGCTCTCGACGCTGGTGTTGATGCCGGCCGCC
GAGGAGGAGTGCAGATGCTCTCAGCCGCTGTGATGCTGGATGCCGGCCG
GGATGCTGATGCTGACTTCTCGGTCAGGCGCAGCATCAGGTCG
GGCCATCCACATTTCGTCAGCGGCTTCTTGGAGCCCTTGCCGAAGATCTGGAATT
CCCACTGGCGCTTCTGAGCTACCTCAGCCGAGGGTGTAGTACGGACCTGCGGCGCC
GGATACCCGAGGTCGAGATGCTCCTCGACGATCTTGCGGGCGACGTCGCCGCTCTCGA
GAAGGGAAGCCGGACGCGGAGCCCGCTGTCTTGGGTAAAGGTTCTGCGAAGCC
GAACCAGCGCCGCTTGCAGAGTGGTGCCGGCGCCGAGTTGGGAACGGATGGCG
GGTCTTGCACATCCGGCCATCATGACTTCTCGCAGACACGGACAGCCATTGGTCGCG
GCGGATCCGGAGGACACCCCGCACCAGGCTTCCAGCAGCTGAGCTGGGCTT

Marianna replicate 3, nodule 2
GAATTCCGCTTCTTGATGCCGAGCCGCTTCCAGTGCCTGAGCAGCCCGCTTGC
AGTGAAATTCGATGCGATGCGCGACTTCTCGGTCAGGCCGACGATCAGGTAGCG
GGCCATCACAATTTCTCAGGGCTTCTTTGAGGGCCAGTCTGGAATT
CCCACTGGCGCTCTCGACGCTGGTGTTGATGCCGGCCG
GAGGAGGAGTGCAGATGCTCTCAGCCGCTGTGATGCTGGATGCCGGCCG
GGATGCTGATGCTGACTTCTCGGTCAGGCGCAGCATCAGGTCG
GGCCATCCACATTTCGTCAGCGGCTTCTTGGAGCCCTTGCCGAAGATCTGGAATT
CCCACTGGCGCTTCTGAGCTACCTCAGCCGAGGGTGTAGTACGGACCTGCGGCGCC
GGATACCCGAGGTCGAGATGCTCCTCGACGATCTTGCGGGCGACGTCGCCGCTCTCGA
GAAGGGAAGCCGGACGCGGAGCCCGCTGTCTTGGGTAAAGGTTCTGCGAAGCC
GAACCAGCGCCGCTTGCAGAGTGGTGCCGGCGCCGAGTTGGGAACGGATGGCG
GGTCTTGCACATCCGGCCATCATGACTTCTCGCAGACACGGACAGCCATTGGTCGCG
GCGGATCCGGAGGACACCCCGCACCAGGCTTCCAGCAGCTGAGCTGGGCTT

Marianna replicate 3, nodule 3
GAATTCCGATGCGATGCGCCGCTTCTCCGTCAGGCGCAGCAGCACGTGAAAGCCG
ATCCACACCTCGCTCAGGCCCTTGTGAGGGCCCTTTGCCGAAGATCTGGAATTCCCA
CTGCCCTTCCGCACTCTCCGGCTGTTGATGCGCCTCTCGGTTGATGCCGGGCCG
GACAGAGTGCGAGATCGATCTCTCGGCGGAGCGTCCGGACGCTGGTCG
GAAGGCGAGCGGCGGCTGAGTGACGGGACCTCAGCGCCGCGGATAACCCCGAGGTCG
GAAGGCGAGCGGCGGCTGAGTGACGGGACCTCAGCGCCGCGGATAACCCCGAGGTCG
CAGGCGCCGGAATCGTCAGGATGGTGGCGCGCTTGTTGGACGGATGCAGGCTCCAGCAGGACATGCGGGGTC
TTGCCATCGGGCATCATGACTTCGCACATCACCAGCAGCGCCATTGCTGGCGGGCCGG
CATCGGGAACACCACGCACCAGGCTTCAGCAGCAGCAGGAGCTGTGGCCTTCGG
CCTGCTGTTGGAGAGGAGCCATCGAAGCCCAACAGAGCGGAAGCTGCTGAGCCTGCG
GGAACGACGCAGA
APPENDIX E
PCR METHODS USED TO COMPARE INOCULANTS WITH NODULE ISOLATES

Background Information

Isolates were collected from each of the replicates in the first inoculation trial as well as each of the inoculant x core origin treatments as described for a limited number of replicates in Chapter 3. With the intent of analyzing all of the replicates, we sought to find a quick PCR-based method of comparing the isolates to the inoculants applied to the appropriate replicate to confirm inoculation. However, the two methods used in this testing phase did not work, and the ultimate methodology used for comparison was sequencing the \textit{glnII} gene from a limited number of replicates, as explained in detail in Chapter 3.

Methods

RAPD Approach

**First run.** Random Amplification of Polymorphic DNA was the first method used to compare nodule isolate DNA to inoculant DNA. A colony PCR technique was used to amplify the DNA. The PCR reaction was performed in a 25 µl volume (Table C-2) using Platinum Taq polymerase. For this experiment, isolates from the first inoculation trial were used. The DNA template was supplied to each of the reactions as intact bacteria. A pipette tip was touched to a single colony on the corresponding replicate plate and then inserted into the reaction tube and flushed twice to deposit the bacteria. The PCR cycle was 10 min at 94°C, then 40 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, followed by 10 min at 72°C for final extension. The primers used were two nonsense primers that have been successfully used in previous studies for producing
distinct DNA fragment fingerprint patterns from rhizobia, ARP 7 (GTACGTGGCG) and CRL 7 (GCCCGCCGCC) (Mathis and McMillin, 1996; Sato et al., 1999).

Following PCR amplification, the DNA was separated using gel electrophoresis. Dyed PCR products, 18 µl product mixed with 2 µl dye, were placed into corresponding wells in 0.9% agarose + EtBr gel. The gel was run for 25 min at 110 V and then evaluated under UV light.

**Second run.** For the second run, the DNA was extracted using a phenol extraction technique:

1. Place one loop full of bacteria from the appropriate plate into 1 ml water, and centrifuge for 30 s at 16 rcf (max speed).
2. Remove the supernatant and add ___ mg glass beads and 1 volume of pH 8 phenol. (1 vol = add solution so that it is 1:1 with volume already present in tube.)
3. Vortex for a few seconds to shear the cells, then Centrifuge 5 min at max speed.
4. Transfer aqueous phase to a clean tube and add 1 volume of phenol:chloroform:isoamyl alcohol mixture (25:25:1 and pH 8). Invert to mix and centrifuge 5 min at max speed.
5. Add 1 volume chloroform:isoamyl alcohol (24:1), and invert to mix. Centrifuge 5 min at max speed.

- **Spooling the DNA**

6. Add 34 µl (proportionate to the solution volume – use 0.3 volume) of 3.0M Na-acetate, pH 5.2 and 350 µl isopropanol. Invert the tube until the DNA is visible; it will appear cloudy.
7. If DNA does not appear immediately, place in -80°C freezer for 10 min.
8. This step can be stored overnight at -20°C.
9. Centrifuge solution for 5 min at max speed to pellet DNA. Remove supernatant as completely as possible. Dry DNA in clean hood until no liquid remains. Resuspend the DNA in 100 µl water.

- **Ethanol Extraction**
10. Add 0.1 volume (~10 µl) of 3 M Na-acetate and 3 volumes (300 µl), ice cold from the -20°C freezer, absolute EtOH.

11. Freeze at -80°C for 30 min.

12. Spin at max speed for 10 min.

13. Pour off supernatant onto a paper towel. Be careful not to lose the DNA pellet onto the towel.

14. Wash twice with 70% 1 ml EtOH, freezing for 30 min at -80°C after each wash.

15. Dry DNA in hood after the second wash.

16. Resuspend DNA with 50 µl water and store in -20°C.

DNA was visualized using electrophoresis to confirm the presence of extracted DNA in the stored solutions.

A temperature gradient PCR reaction with serial dilution was performed in 25 µl volume (Table C-2) with primer concentration increased to 0.625 µl each primer and 1 µl template DNA. The dilution scheme was to add 1 µl template to the first well, followed by transferring 1 µl of the reaction mixture to the next row. Two rows of each of two templates (8B4 and isolate number 8-1) were prepared and placed in the thermocycler which was programmed with the same temperature cycle as for first run with the addition of a 2°C gradient from front to back of the cycler.

The PCR products were separated in electrophoresis gel with 10 µl of product run for 30 min at 140 V. The gel was then viewed under ultraviolet light.

**RFLP Approach**

**DNA Extraction.** The isolates from the first inoculation trial were used for the DNA extraction experiment. However, isolates and inoculants from the second inoculation trial were used as well as isolate DNA from the first inoculation trial for the digestion experiment. A lysozyme extraction technique was used for isolating the
template DNA. A fresh culture of bacteria was achieved by placing 3 ml inoculant
solution or a scraping of bacteria from an isolate plate in 30 ml of liquid YEM media
(Table C-1) and growing for approximately 48 hr at 30°C, with shaking at 220 rpm. Two
liquid culture preparations were made per plate or inoculant culture.

Lysozyme DNA Extraction

1. Centrifuge the culture at 3,000 to 5,000 x gravity for 10 min to pelletize the
bacteria; discard the supernatant. Resuspend in 5 ml of SET Buffer (20 mM
TrisHCl, pH 7.5; 75 mM NaCl; 25 mM EDTA).

SET Buffer (30 ml stock)
- 6 ml 100 mM TRIS-HCl, pH 7.5
- 450 µl 5 M NaCl (292.2 g L⁻¹)
- 7.5 ml EDTA
- Bring to 30 ml volume with water.

2. Add lysozyme to a final concentration of 1 mg ml⁻¹ (125 µl). Make the
lysozyme dilution fresh, if possible. Lysozyme stock is 45 mg ml⁻¹, need 5 mg
sample⁻¹.

3. Incubate at 55°C for 2 h, inverting every 15 min.

4. Using a chloroform resistant tube, add 1 volume chloroform:isoamyl alcohol
(24:1) solution to the DNA solution.

5. Add 1/3 volume 5M NaCl and mix by gentle inversion (~1.67 ml).

6. Incubate at room temperature for 30 min with frequent gentle inversion to mix.

7. Centrifuge at 5,000 x gravity for 10 min.

Spooling of DNA

8. Transfer upper phase to a new tube and add 1 volume isopropanol. Gently
invert to mix.

9. Use a glass rod or pipette tip to spool the DNA.

10. Wash spooled DNA with 70% EtOH by placing spooled DNA into a tube filled
with EtOH.

11. Centrifuge EtOH and DNA at max speed for 10 min.
12. Remove the majority of the supernatant and dry DNA in the hood, but be sure not to over dry the DNA.

13. Resuspend the DNA in 500 µl DNA water. (Yields ~1 to 5 mg DNA per 30 ml culture.)

DNA was visualized on electrophoresis gel by running 8 µl DNA solution + 1 µl water + 1 µl dye for 20 min at 100 V and then observing under ultraviolet light.

**Amplification and isolation of 16S ribosomal DNA.** Two primer sets were tested for the PCR reaction. Primers 42 and 43 made up the first set (42: 5’-AGAGTTTGATCCTGGCTCAG-3’ and 43: 5’-TACCCTTGTTACGACTTCA-3’), and primers 44 and 45 made up the second set (44: 5’-AGAGTTTGATCCTGGCTCAG-3’ and 45: 5’-AAGGAGGTGATCCAGCCGCA-3’). The following extracts were used, which correspond to the replicate pot number followed by the number of the nodule plated (such that isolates with the same first number are from the same plant): 17-1, 31-2, 32-1, 38-1, 38-2, and the inoculants 8B4 and 8B6. Each isolate or inoculant template was tested with both of the primer sets.

The PCR reaction was performed in 25 µl volume (Table C-2), and the temperature sequence was 95°C for 5 min to warm up followed by 35 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 2 min. The final extension was 10 min at 72°C, followed by storage of the products at 4°C.

PCR products were isolated using electrophoresis by running 18 µl product + 2 µl dye for 25 min at 140 V on a large gel. Since the brightest and most discrete bands were observed in the products from the products produced by the 42 -43 primer pair, these primers selected for further analysis.

For the restriction digestion, DNA was extracted from the following isolates (from inoculation experiment 2, described in Chapter 3): 12-2, 15-1, 56-3, 13-3. Inoculants
EL Cowpea and Organica were also cultured and had DNA extracted. The DNA extraction was done using the Sigma GenElute Kit and procedure described in Chapter 3. The 16S rDNA fragments were amplified using primers 42 and 43 and the temperature sequence previously described. 18 µl PCR product + 2 µl dye were run for 25 min at 140 on a 9% agarose gel. Once the 1800 bp band was observed, it was excised from the gel using a razor blade. The gel pieces were then purified using the GFX PCR DNA and Gel Band Purification Kit described in Chapter 3. Stored PCR products from previous amplification (Primers 42 and 43) of 8B4, 8B6, 17-1, 31-2, 32-1, and 38-1 were also purified for digestion. The combination of these particular isolates allowed for each of the inoculant treatments to be tested in the digestion experiment along with all four of the inoculants that were ultimately used in the two inoculation trials.

**Restriction digestion.** Ultimately, 11 DNA fragments were digested: 8B4, 32-1, 12-2, 8B6, 31-3, 15-1, EL Cowpea, 38-1, Organica, 56-3, 17-1. A digestion replicate with no DNA added was also prepared as a method blank. Two restriction enzymes were evaluated, HPAII, and SAU3AI. The reaction solution was

- 5 µl DNA
- 3 µl Buffer (HPA II = Buffer 1; SAU 3AI = Buffer 2)
- 6 µl BSA stock for 100 µg µl⁻¹ final concentration (stock = 500 µg ml⁻¹)
- 1 µl Hpa II or SAU 3AI
- 15 µl DNA water, for a total volume of 30 µl.

The reaction was incubated at 37°C for a total of 2 hr, followed by stopping the reaction by placing the tube in a heating block that was 65°C for 20 min. After 1 hr and then after 2 hr, 8 µl +1 µl water + 1 µl dye was loaded into a 0.9% agarose gel and run for 20 min at 110 V.
The restriction digestion reaction was repeated again exactly as before in order to test other gel concentrations. The 1 hr digestion product, 10 µl total dyed sample, was run on 3% agarose gel with 4 µl EtBr added for 3 h at 80 V. The 2 hr digestion product, 10 µl total dyed sample, was also run on the denser gel for approximately 2 h at 80 V, removing the gel to the light table to observe the progress of the fragments every 0.5 h.

Results and Discussion

RAPD Approach

First run. No DNA was visible in the gel. Some of the tubes were not capped tightly enough, and the reaction volume had evaporated during the temperature cycles. The colony approach to providing DNA to the reaction was not effective. The next reaction will be done beginning to extracted genomic DNA.

Second run. No PCR products were visible. The DNA visualization resulted in two strong bands and two non-visible bands. This result suggests that the technique needs improving for better quality extracted DNA. The two extracts showing strong bands were used for the temperature gradient PCR experiment. Since no DNA was visible in the PCR products, it seems that perhaps the primers are not finding sequences to anneal to in the templates that are being used. Since the sequences of the inoculants and the isolates are not known, it is difficult to find a RAPD primer that will produce consistent results without considerable developmental work that would be outside the scope of this project.

RFLP Approach

DNA extraction. The lysozyme method of DNA extraction resulted in a strong visual yield of DNA. This method was a viable solution to doing multiple DNA
ex extractions. However, due to the potentially very large number of samples to be
extracted, the DNA elution kit was eventually used, as described in Chapter 3.

**16S rDNA amplification.** The PCR reactions resulted in consistent bands of
approximately 1800 bp fragments of DNA for both of the primer sets and for nearly all of
the isolates tested. The primers and reaction cycle tested are usable for producing
clear fragments that can be used for restriction fragment digestion.

**Restriction digestion.** Each of the gels run after the restriction digestion reaction
had gone for 1 hr and 2 hr, respectively, smeared and showed no distinct banding
pattern. A denser agarose gel may needed in order to slow down the movement of the
potentially very short fragments through the gel.

After the digestion was repeated, the 1 hr products from this reaction run on the
3% agarose gel yielded no visible DNA fragments. It is most likely that the products ran
off the end of the gel in such a long period of time. The 2 hr products were prevented
form running off of the gel by frequently observing their progress, however, the visible
DNA was very faint and smeared after the 2 hr reaction time for both restriction
enzymes. The restriction digestion did not yield a distinct banding pattern that was
usable for comparing the DNA of the nodule isolates with the inoculants.


Prism. 1996. GraphPad Prism, GraphPad Software, Inc., San Diego, CA.


BIOGRAPHICAL SKETCH

Sarah Elizabeth (Wright) Cathey received a bachelor’s degree in biology from Lipscomb University, Nashville, TN, in 2001, graduating with honors designation. As an undergraduate student, she worked as an intern with the TN Department of Environmental Conservation tracking endangered native plants in the office and in the field. She completed a senior honors thesis entitled “An Eradication Study of Vinca minor” for which she conducted field research in an urban landscape.

Cathey completed her Masters’ degree at the University of Florida in 2005. Her thesis was entitled “Growth and N₂-Fixation of Legumes Native to the Longleaf Pine-Wiregrass Ecosystem.” The project was undertaken as part of a cooperative agreement between the University of Florida and the Joseph W. Jones Ecological Research Center, Newton, GA. Her work with perennial legumes gave her particular insight for undertaking this dissertation project.

While working toward the completion of this dissertation and doctoral degree, Cathey gave birth to a little girl and is expecting her second child later this year. Shortly after graduation, she will begin a postdoctoral position with the Department of Environmental Horticulture at the University of Florida to study transpiration response of turfgrasses to minimal irrigation. Cathey intends to pursue a professorship in the future in order to follow her strong desire to teach at the undergraduate and graduate levels.