GROWTH AND N_2-FIXATION OF LEGUMES NATIVE TO THE LONGLEAF-WIREGRASS ECOSYSTEM

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2005

This thesis is dedicated to my husband, partner and most devoted supporter, Marcus.

ACKNOWLEDGMENTS

Financial support for this research was provided through the cooperative graduate research agreement between the Joseph W. Jones Ecological Research Center and the UF College of Natural Resources and Environment. Technical, sample processing and field support were provided by the staff of the Jones Center, especially Scott Taylor, Mary Cobb, and Sarah Becker. A special thankyou goes to Dr. Kay Kirkman of the Jones Center who provided all of the nursery plants for my experiments, and to Dr. Ken Quesenberry who provided additional seeds.

I would like to thank my committee for their advice and revisions regarding my research and the writing of this thesis. Special thanks go to Tom Sinclair and Lindsay Boring for their assistance in revisions and for personal support. I would like to thank my parents for their moral support, as well. I especially need to thank Susan Sorrell, technician at the USDA ARS lab at the University of Florida, for her tireless efforts in data collection and sample preparation, and for sharing her expertise in spectrophotometry analysis.

Finally, I would like to thank my husband, Marcus, for his tireless dedication to my graduate studies and attendant technical support and for his willingness to move all the way to central Florida from Tennessee in order for me to pursue this degree.

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Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

GROWTH AND N_2-FIXATION OF LEGUMES NATIVE TO THE LONGLEAF-WIREGRASS ECOSYSTEM

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December 2005

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The longleaf pine- (*Pinus palustris* Mill.) wiregrass (*Aristida stricta*, Michx.) savanna ecosystem once dominated the southern coastal plain of the United States, but presently less than 1.5 million of the historic 37.2 million ha remain intact. Restoration plantings reclaiming more than 283,000 ha of former agricultural fields, pulpwood plantations, and other fire-suppressed lands are being established in the Southeast. Groundcover reestablishment of native legumes and grasses is the key to restoring soil nitrogen levels, wildlife habitat and continuous fuels for frequent prescribed burning in young longleaf pine plantings. More information is needed about the growth and N_2-fixation rates of native legumes under shade in order for informed selections to be made for groundcover restoration plantings.

A potted plant study was used to assess growth and N_2-fixation responses of 10 species under two light regimes. Total biomass accumulation and root-to-shoot ratios were used to examine growth responses to shading, and several corroborative techniques

including analysis of nodule biomass, $\delta^{15}N$ natural abundance, percent N derived from the atmosphere, transport product analysis, and the acetylene reduction assay were used to assess potential N_2-fixing capabilities. Overall, N_2-fixation rates increased throughout the season. Relative N_2-fixation rates, as assessed by the above approaches, indicated that *Mimosa quadrivalvis*, *Crotalaria rotundifolia* and *Centrosema virginianum* were quickly developing species and effective N_2-fixers, and that *Lespedeza hirta* and *Orbexillum lupinellus* showed lower N_2-fixation rates in a one-year study. Shade did not have a significant effect on N_2-fixation in this controlled study. The acetylene reduction assay is best used as a check for nitrogenase activity and for following seasonal patterns, but is not as useful for a quantitative estimate of N_2-fixed. The nitrogen transport product analysis may have limited usefulness for these species due to extremely low nitrate levels, but should also be tested as a field technique. The $\delta^{15}N$ natural abundance method was a useful technique for estimating N_2-fixation inputs over time.

A garden plot study, situated in a 14 year-old longleaf pine plantation on an old agricultural site, was used to assess growth and N_2-fixation of eight species of native legumes under three levels of canopy openness. Growth and N_2-fixation declined rapidly between approximately 60 and 80 percent canopy openness, indicating that legumes native to the longleaf pine-wiregrass ecosystem have a limited degree of shade tolerance. Root-to-shoot ratios also indicated that belowground growth was dominant among plants growing under shaded conditions. Groundcover restoration plantings involving native legumes will be most effective when conducted after an initial thinning after about 15-20 years of tree growth

CHAPTER 1
INTRODUCTION

The longleaf pine- (*Pinus palustris* Mill.) wiregrass (*Aristida stricta*, Michx.) savanna ecosystem dominated the coastal plain region of the southeastern United States in the pre-European era, covering as much as 37.2 million ha, but less than 1.2 million ha remain intact. Private hunting lands and planted stands constitute most of the longleaf pine coverage that still exists in the U.S. (Landers et al., 1995). Over the past 100 years, land managers have used frequent prescribed burning to maintain the longleaf pine-wiregrass ecosystem for wildlife habitat (Boring et al., 2004).

Although frequent fire disturbance is necessary to maintaining the structure of the longleaf-wiregrass ecosystem by suppressing the oak midstory, some have hypothesized that nutrient losses due to the consumption of litter and volatilization may cause a continual decline of overall N in the system (Carter and Foster, 2004). Nitrogen and phosphorus are potentially co-limiting to net primary productivity in these woodlands, secondary only to water limitations (Hendricks et al., 2002). Recent studies have shown that periodic mineralization of phosphorus, which occurs when large amounts of accumulated needle litter is burned, may serve an important role in the nutrition, growth and reproduction of phosphorus-demanding legumes, leading to the eventual replacement of nitrogen lost during burning (Boring et al., 2004).

Native herbaceous legumes constitute more than 10 percent of the vascular plants in frequently-burned longleaf pine savannas (Hainds et al., 1999). Due to their high numbers and density as well as their quick regeneration following fire, the potential for

significant input of biologically-fixed nitrogen is great. Life history adaptations such as high belowground biomass allocation, opportunistic flowering and N_2-fixation (in legumes) are important to the success of species native to frequently burned ecosystems (Knapp et al., 1998; Morgan, 1999; Hiers et al., 2000; Jacobs and Schloeder, 2002).

Legumes have been considered to be a significant component of the N-cycles of many ecosystems, but the actual quantification of N from biological N_2-fixation is difficult to estimate. Using a series of field-conducted population surveys and acetylene reduction assays (and corroborative $\delta^{15}N$ data), Hendricks and Boring (1999) conservatively estimated legume nitrogen inputs from biological fixation in the longleaf-wiregrass system to range from 7 to 9 kg N ha^{-1} yr^{-1}. Other studies have used this technique to estimate potential contributions of N_2 fixed by legumes in other fire affected ecosystems including prairies (Becker and Crockett, 1976). However, N-input estimates made from the acetylene reduction assay are limited by the ability to recover nodules in the field. Recent studies seeking to quantify biological N_2-fixation in natural ecosystems and agroforestry systems often rely more heavily on isotopic methods, such as ^{15}N enrichment or the natural abundance of ^{15}N (Peoples et al., 1996; Hendricks and Boring, 1999; Medina and Izaguirre, 2004). A comparison of studies using these diverse tools, and an understanding of the limitations inherent in each of the assessment techniques reinforce the need to use corroborative methods when assessing N_2-fixation in legumes.

Because of the high energy requirements necessary to maintain nitrogen fixation, legumes outside of the tropics have generally been assumed to be shade intolerant (Sprent, 1999). Sprent (1973) found that *Lupinus arboreus* plants grown under shade conditions produced less nodule biomass and subsequently lower nitrogenase activity

(acetylene reduction). Although the longleaf-wiregrass ecosystem has a relatively open canopy compared to other forest systems, the numerous legumes in this ecosystem must be able to tolerate a light environment that is variable but averages approximately 40 percent of full sunlight (Battaglia et al., 2003; Pecot et al., 2005). Light levels in young, planted longleaf pine stands may be substantially higher than the native woodland, but canopy closure occurs only a few years after planting. Thus some of the more shade tolerant native legume species may be more satisfactorily adapted for restoration plantings.

Reforestation initiatives on public and private lands since 1998 have resulted in the planting of approximately 700,000 acres of former agricultural, pulpwood plantation and fire suppressed land back into longleaf pine stands through the USDA Conservation Reserve Program (CRP) and other independent landowner efforts. Due to the long rotation period typical of longleaf pine stands they have the potential to generate income for private landowners over many years through timber revenue, hunting leases and government subsidies paid by the CRP (Landers et al., 1995). Groundcover restoration in young longleaf pine stands planted on depleted, former agricultural soil is important for rebuilding soil organic matter and N (Markewitz et al., 2002), for providing wildlife food and cover (Stoddard, 1931), and for enhancing pyrrhic fuel continuity necessary to reintroduce frequent prescribed fires (Mulligan and Kirkman, 2002). Reintroduction of native legumes for groundcover restoration rather than exotic or agricultural species should prevent problems of invasiveness with non-native legume species in the past, such as serecia lespedeza (*Lespedeza cuneata*) and *L. bicolor* (Miller, 2003), and agronomic species' lack of adaptability to woodland environments.

The overall objectives of this study were (1) to explore the impact of various degrees of shading on relative growth and N_2-fixation rates of legume species native to longleaf pine-wiregrass savannas, (2) to make initial observations of phenological development and nodule morphology for each species, and (3) to examine the effectiveness of corroborative methods for assessing N_2-fixation. These objectives have not been previously addressed for most of these species. Controlled potted studies and a common garden experiment were used to assess species responses to shading under potted and field growing conditions.

Chapter 2 examines the affects of shade on growth of nine species of native legumes and provides descriptions of phenological development and nodule morphology after one growing season in a pot study. Descriptive information regarding the species in this study, *Chamaecrista nictitans* (L.) Moench, *Centrosema virginianum* (L.) Benth., *Clitoria mariana* L., *Crotalaria rotundifolia* J.F. Gmel., *Lespedeza hirta* (L.) Hornem., *Mimosa quadrivalvis* (L.), *Orbexillum lupinellus* (Michx.) Isley, *Rhynchosia reniformis* D.C., and *Tephrosia virginiana* (L.) Pers, is very limited. Phenological development data is helpful for better understanding the life history of a species, especially in regard to fire adaptation. Nodule morphology can be an important taxonomic tool for the *Leguminoseae* (Sprent 2002), and relative nodule biomass is often indicative of relative N_2-fixation capacity. In addition, biomass accumulation and allocation (root-to-shoot ratios) patterns are indicative of shade tolerance and environmental adaptability.

Chapter 3 describes a companion study to Chapter 2 that examines the N_2-fixation patterns and capabilities of the same nine species of native legumes during a growing season, using five corroborative methods of assessment: nodule biomass, the acetylene

reduction assay, nitrogen transport and storage product analysis, $\delta^{15}N$ natural abundance, and total N content. Each of these methods has been independently used to assess relative N_2-fixation rates among species in both controlled and field studies, and to determine the affect of shading on N_2-fixation rates. However, this study is the first instance in which the nitrogen transport product analysis has been used to assess these species. In this study, the relative effectiveness of each corroborative assessment was determined for use in a small, controlled study.

Chapter 4 further examines the growth and N_2-fixation capabilities of eight species of native legumes planted under three canopy opening conditions in a 14 year-old longleaf pine plantation. Because of their dominant growth form and importance for wildlife cover and food, more semi-woody species were included in this field study than in the potted studies. The species examined were *Centrosema virginianum*, *Desmodium ciliare* (Muhl. ex Willd.) DC., *Lespedeza angustifolia* (Pursh.) Ell., *Lespedeza hirta*, *Mimosa quadrivalvis*, *Orbexillum lupinellus*, *Pediomelum canescens* (Michx.) Rydb., and *Tephrosia virginiana*. As in Chapter 2, biomass accumulation and root-to-shoot ratios were used to assess growth responses to shade in each of the species. The $\delta^{15}N$ natural abundance method was used to compare relative N_2-fixation rates among species and across light environments.

Together, these studies should provide a greater understanding of the growth habits, morphology, phenological development, N_2-fixation capabilities and shade tolerance characteristics of several species of native legumes. This information can be applied to further studies of these and similar species and could be used to make preliminary

decisions about which species should be used for groundcover restoration plantings in longleaf pine stands.

CHAPTER 2
GROWTH AND PHENOLOGY OF NATIVE LEGUMES IN TWO LIGHT ENVIRONMENTS

Introduction

N_2-fixing legumes are generally considered to be shade intolerant species -due to the high energetic cost of nodule production, maintenance, and N fixation (Vitousek et al., 2002). Among temperate ecosystems, native legumes are most diverse and abundant in grasslands (Becker and Crockett, 1976) and savanna ecosystems (Hainds et al.,1999). The variably-shaded and frequently burned environment of the longleaf pine- (*Pinus palustris* Mill.) wiregrass (*Aristida stricta*, Michx.) ecosystem supports over forty species of native herbaceous legumes. These legumes constitute more than 10 percent of the vascular species in these pine savannas and occur in high densities across a great range of site conditions (Hainds et al., 1999). They demonstrate fire tolerance, adaptability to infertile and droughty soils, values for wildlife food resources, and may fix varying amounts of N (Hainds et al., 1999; Hendricks and Boring, 1999; Hiers et al., 2003).

Hiers et al. (2000) showed that flowering of native legumes in this system is tied to occurrence of fire, but responses to a specific seasonal burn varied for *Tephrosia virginiana* (L., Pers.), *Centrosema virginianum* (L., Benth.) and *Rhyncosia reniformis* (D.C.). These legumes are found ubiquitously across the longleaf-wiregrass savanna landscape with only the most extreme deep sands or seasonally-inundated lowlands having a lowered abundance and diversity of species (Hainds et al., 1999). In addition to surviving droughty conditions and frequent fire, legumes are able to overcome the problem of very low-

fertility soils through N_2-fixation (Hiers et al., 2003). However, shade tolerance of these species has not been explored. Scientific knowledge of the biology of N_2-fixing species is limited to a few ecosystems, and environmental tolerances have not been explored for most of these species (Vitousek et al., 2002).

Since 1998, reforestation initiatives on public and private lands in the southeastern U.S. have resulted in the planting of approximately 283,000 ha of former agricultural fields, harvested pulpwood plantations and otherwise fire suppressed land back into longleaf pine stands, with 48,000 ha of marginal coastal plain farmland in Georgia alone under the USDA Conservation Reserve Program (Coffey and Kirkman, 2004). Many of these sites are characterized by coarse sandy soils that are prone to drought and may be highly depleted in C and N from prior agricultural production (Markewitz et al., 2002). Groundcover restoration was proposed to be vital in the recovery of soil organic matter and N-availability. N_2-fixing legumes could be especially valuable. Although longleaf pine overstory has been successfully established, there is a great need to better determine the compatibility of groundcover species to a range of canopy light conditions so that recommendations may be made to integrate suitable species into habitat restoration projects.

This potted-plant study was designed to observe the effects of two light conditions on growth responses of ten species of legumes native to the longleaf-wiregrass ecosystem over the course of a single growing season. The specific objectives of the research reported here were: (1) to document the influence of shading on growth habits and biomass accumulation; and (2) to make initial observations of phenological development and nodule morphology for each species. Various observations of root, shoot and nodule

growth were all used as indicators of growth response to light. A companion study also measured N_2-fixation responses of these species and results are reported in Chapter 3.

Methods and Materials

Planting

Young plants of ten species of native legumes (Table 2-1) were grown outdoors in Gainesville, Florida (82° 20' W, 29° 38' N) between April and November of 2004. One-half of the plants from each species was grown in the sun and one-half under a shade cloth that excluded approximately one-half ambient light. Difference in photosynthetically active radiation (PAR) between light treatments was determined using a Li-Cor Quantum Sensor, LI-185A. Measurements were taken on a clear day, 19 March 2004, at approximately 13:00 Eastern Standard Time. Three readings each were taken under the shade cloth and outside adjacent to the potted plants. PAR in the shaded area (753 ± 82 μmol $s^{-1}m^{-2}$) was 56 percent of full sun (1340 ± 51 μmol $s^{-1}m^{-2}$).

The seedlings were initially propagated by Dr. L. Katherine Kirkman at the Joseph W. Jones Ecological Research Center (JWJERC) from seeds collected from throughout the native woodland on the 12,500 ha Ichauway reserve, located in Baker County, Georgia, USA (31°19'N and 80°20'W). Seeds were scarified by physical abrasion and then germinated in a soil mix consisting of 8 parts Fafard 3B soil mix, 2 parts peat (sphagnum), 2 parts sand, and 1 part perlite, contained in plug flats. The seeds were sown June/July 2003 and kept in a greenhouse over the winter.

Table 2-1. Description of Native Legumes used in study. Nomenclature follows Wunderlin and Hansen (2003). Descriptions adapted from Isley (1990).

Species	Code	Common Name	Subfamily	Category	Growth Habit	Mature Size
Centrosema virginianum (L.) Benth.	CEVI	Spurred Butterfly Pea	Papilionoideae	Vining/Spreading	Perennial	Stems 1-1.5m
Clitoria mariana (L.)	CLMA	Butterfly Pea	Papilionoideae	Vining/Spreading	Perennial	Stems 30-100cm
Chamaecrista nictitans (L.) Moench	CANI	Sensitive Pea (Partridge Pea)	Caesalpiniodeae	Erect herb	Annual	Plant 15-60cm
Crotalaria rotundifolia J.F. Gmel.	CRRO	Rabbitbells	Papilionoideae	Vining/Spreading	Perennial; prostrate	Stems 1-7cm
Lespedeza hirta (L.) Hornem.	LEHI	Hairy Lespedeza	Papilionoideae	Erect herb	Perennial; semi-woody	Plant 0.8-1.5m
Mimosa quadrivalvis (L.)	MIQU	Sensitive Briar	Mimosoideae	Vining/Spreading	Perennial; trailing, thorned stems	Stems 1-2m
Orbexillum lupinellus (Michx.) Isley	ORLU	Piedmont Leatherroot	Papilionoideae	Erect herb	Perennial; rhizomatous	Stems 20-60cm
Pediomelum canescens (Michx.) Rydb.	--	Buckroot	Papilionoideae	Erect herb	Perennial; diffusely branched to bushy	Plant up to 1m
Rhynchosia reniformis D.C.	RHRE	Dollarleaf	Papilionoideae	Erect herb	Perennial; rhizomatous	Plant 7-15cm
Tephrosia virginiana (L.) Pers.	TEVI	Goats Rue	Papilionoideae	Erect herb	Perennial; brancing from a central point	Plant 30-60cm

Table 2-2. Fruit and nodule descriptions. Nodule sizes indicate an average, and are represented as follows: --, not nodulated; +, <1mm; ++, 1-2mm; +++, <2 to ≤6mm; ++++, >6mm to 10mm. The number of plants examined to estimate nodule size is also given.

		Nodules		
Species	Fruit	Shape	Size: Sun	Size: Shade
Centrosema virginianum	Legume; Linear, 7-12cm x 3-4 mm, dehiscent	Spherical	+++ (n=2)	++ (n=5)
Clitoria mariana	Legume; Oblong, 3-5 cm x 5-7 mm, seeds sticky	Spherical	+++ (n=2)	+++ (n=5)
Chamaecrista nictitans	Legume; Oblong, flat 2-4 cm x 4-5 cm	Spherical	-- (n=4)	++++ (n=3)
Crotalaria rotundifolia	Legume; Ellipsoid, inflated, 1.5-2.5 cm x 7-12 mm	Coralloid	++++ (n=8)	+++ (n=8)
Lespedeza hirta	Legume; 5-8 mm long	Spherical	+++ (n=7)	++++ (n=6)
Mimosa quadrivalvis	Legume; Oblong to linear, 3-5cm long, prickled	Coralloid	++++ (n=4)	++++ (n=2)
Orbexillum lupinellus	Legume; Obliquely transverse-ridged	Spherical	+++ (n=3)	++ (n=2)
Pediomelum canescens	Legume; 8-11mm long	Spherical	n/a	n/a
Rhynchosia reniformis	Legume; Oblong or elliptically-oblong, 1.2-1.8 cm x 6-7 mm	Spherical	-- (n=1)	++ (n=2)
Tephrosia virginiana	Legume; Oblong, flat, 3-5 cm x 4 mm	Elongated	++++ (n=4)	++++ (n=7)

The small, 7 month-old seedlings from the JWJERC were transferred into oneo f the following: clear acrylic rooting tubes (90cm long x 3.5cm diameter), cylindrical polyvinyl chloride (PVC) pots, 35cm x 10.5cm diameter, or black plastic tree seedling pots, 35cm tall x 80cm^2 (Stuewe and Sons, Corvallis, OR). PVC pots were also used to assess N_2-fixation using the acetylene reduction assay (Chapter 3). A total of eight rooting tubes, six PVC pots, and 14 black plastic pots were planted for each species in the experiment. One half of each set of pots was grown under the shade treatment. For this experiment, plants in the PVC and black plastic pots were measured and harvested as a single group since the volume was approximately the same. Transplanting was completed on 11 February 2004 (Day 42). Dates are represented in figures and tables as numbered days beginning with 1 January 2004 as Day 1.

Seeds for the annual *Chamaecrista nictitans* (CANI) were obtained from Dr. Ken Quesenberry (Agronomy Department, University of Florida). The seeds were collected from along the roadside in Gainesville, FL. Seeds for (CANI) were scarified with sand paper and then germinated on moist filter paper. Emerged CANI seedlings were planted directly into PVC and black plastic pots on 21 April (Day 122).

Plants were inoculated by introducing native soil to each pot. Two topsoils (0-20cm) were collected at the Jones Center, from a fine-loamy, kaolinitic, thermic Typic Kandiudult (Orangeburg Series), and from a loamy, kaolinitic, thermic Arenic Kandiudult (Wagram Series). Collections were taken from areas with thriving and diverse legume populations. Soils were transported to Gainesville, FL where they were stored in a cool, dark room and covered with plastic to maintain moisture. A mixture of equal parts of each native soil type was used as the inoculation source. The pots had been

previously filled with commercial topsoil (Walmart Corp.) to within 6 cm from the top, then 2 cm of native soil was added to the surface of all pots. Finally, the plant was placed in the pot, and additional topsoil was used to cover the roots as needed. For the rooting tubes, the 2 cm of native soil was added below where the seedling was pressed into the top of the tube. Seedlings in the rooting tubes were inserted so that there was approximately 2-3 cm in the tube above the soil to facilitate watering.

Water was applied by drip-irrigation every 12 hours (5:00 and 17:00 EST) using a battery-operated timer (Rainbird), but was adjusted as needed throughout the experiment to prevent excessive watering during periods of heavy rainfall.

Aphids were detected on CRRO, beginning around 3 April (Day 94), but the plants were not detrimentally affected by the infestation. *Pediomelum canescens* began to yellow and to develop brown leaf spots as early as 30 June (Day 182), followed by rapid leaf loss and, consequently, this species was omitted from our results.

Gainesville experienced hurricane activity around 14 August (Day 227) and 3 September (Day 247; Figure 2-1). Some plants experienced leaf loss due to wind, and LEHI, which was moved indoors, experienced some water stress. However, the reason for loss of leaves at harvest was difficult to distinguish, because senescence had begun by that time for most species.

Measurements

Height measurements were taken weekly of plants in both pot types and the rooting tubes. Height was determined by measuring the distance from the soil surface to the highest point on the plant. The height of vining plants such as MIQU, CEVI, and CLMA was considered to be the length of the longest stem. Each of these vines was measured until the date when the branches became too tangled for a measurement to be plausible.

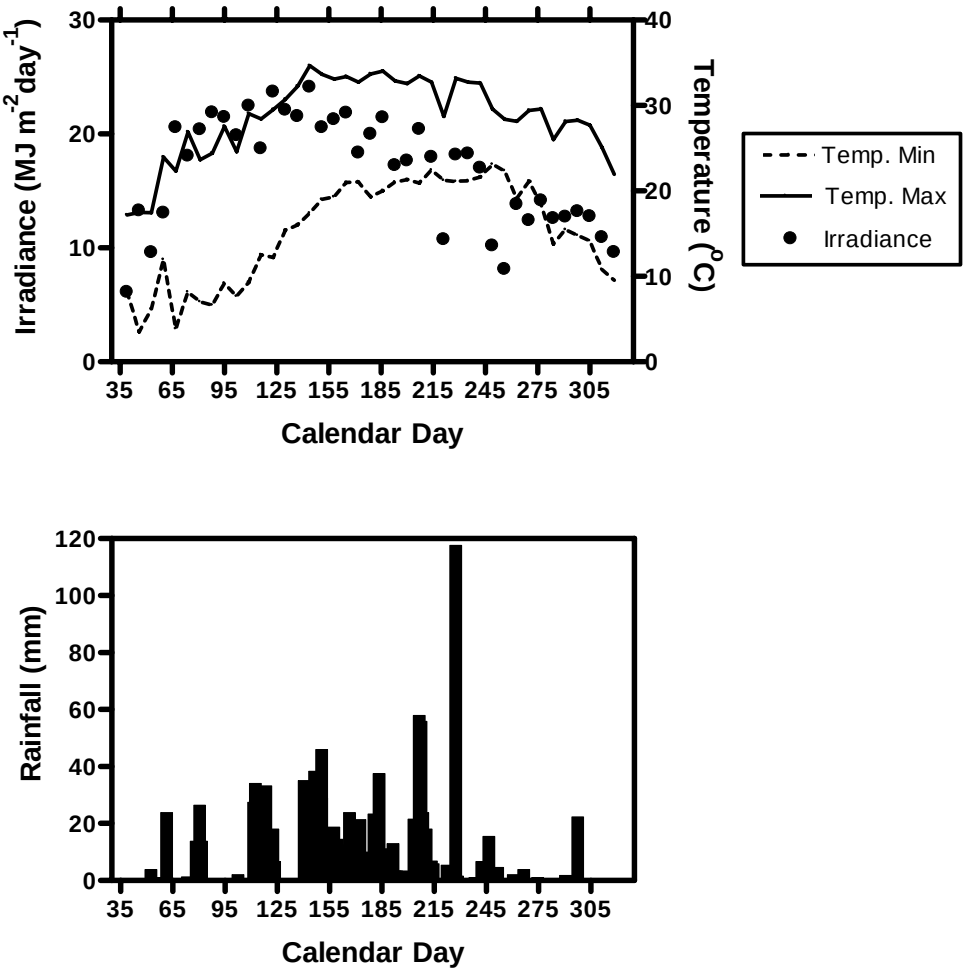


Figure 2-1. Weather for Gainesville, FL, 8 February to 20 November 2004. Temperature and irradiance values are weekly averages. Rainfall values are daily totals. Data from FAWN (2005).

Root elongation was measured along the side of the clear acrylic tubes from the top of the tube to the tip of the longest visible root using a measuring tape. Each tube was placed in a white PVC sleeve. Root depth measurements and notations regarding nodule presence were taken twice weekly until the roots reached the bottom of the tube or until there was no increase in rooting depth for at least three readings.

Leaf addition was measured by counting the number of leaflets on each plant on a weekly basis. This method was continued throughout the experiment for CLMA, LEHI, RHRE, and TEVI. However, due to the large number of leaflets or the indistinguishable nature of the leaflets, CEVI, MIQU, CRRO and ORLU leaf addition was instead determined by measuring the width of the plant. The width of CEVI and MIQU was determined to be the sum of the lengths of the two longest stems. Widths of CRRO and ORLU were determined as the width of the plant at the widest point, leaf tip to leaf tip. Width measurements were taken using a measuring tape or ruler.

The presence of flowers and fruits was noted along with the height measures. A phenological phase was considered to be initiated when half of the specimens for each species had expressed the particular characteristic such as presence of flowers or fruit, or the absence of flowers while fruit remained.

At the conclusion of the experiment, all plants were destructively harvested. Aboveground material was collected from specimens grown in root tubes. Both above- and belowground biomass was collected from all specimens grown in pots. Roots were washed free of soil and nodules were collected. Nodules were counted and then individually measured by laying each one alongside a millimeter ruler. The diameter of spherical nodules and the longest dimension of elongated nodules was measured in order

to estimate an average nodule size for each plant. All tissues were dried to constant weight at 80^{o}C.

Data and Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with species and light environment as main effects. If differences existed ($p<0.05$), Duncan's multiple comparison post-test was used to determine which means differed significantly. The GLM procedure performed in the Statistical Analysis System (SAS, 2003) was used for ANOVA and post-tests. Patterns of stem elongation were analyzed using a non-linear regression model (third-order polynomial), followed by an analysis of slope using the derivative (dh/dt). The slope of the curve at selected points was calculated and compared using ANOVA to test for species and treatment effects. Student's T test ($\alpha=0.05$) was used to test for significant differences in plant height, number of leaves and plant width response to light treatments within a species. Non-linear regression and t-tests were performed using Prism (Prism, 1996).

Results

Survivorship

Out of the 20 individual plants of each species that were planted in the black plastic and PVC pots, an average of 7 survived. LEHI had the highest survival with 13 remaining, and ORLU and RHRE had the lowest survivorship with only 4 and 3 plants remaining, respectively. The cause of mortality for most of these young seedlings is unknown. Most of the species were still dormant when transplanted, and many of the individuals never reemerged. Some of the MIQU plants were lost due to desiccation during May during a dry and windy period when conductance and evaporative demand must have exceeded the watering rate for this large species.

Survivorship in the rooting tubes was also low, with only an average of 3 plants surviving out of the 8 transplanted per species. Lack of reemergence, inadequate space for watering in the top of the tubes and soil compaction (leading to inundation of roots) were the reasons for the small success of these individuals. Despite low survivorship overall, 6 of the 10 species generally had enough survivorship to allow for treatment analysis, excluding *Pediomelum canescens*, MIQU, ORLU and RHRE.

Morphology

Although they are quite different in structure and life history, the ten species in this experiment can be categorized into two general morphological types, a vining/spreading herb or an erect herb (Table 2-1). The vining/spreading species, including CEVI, CLMA, CRRO and MIQU, although quite different from each other, all tend to be either prostrate or to climb over adjacent plants. In contrast, the erect herbs are generally upright, but LEHI or ORLU may droop over other plants or onto the ground. CANI, RHRE and TEVI branch from a central stem or root crown, and the branching stems may be nearly horizontal to the ground.

The legume fruits of these species ranged in size from 1.2 to 12 cm long (Table 2-2). CEVI, CLMA, CRRO, and RHRE pods are dehiscent, but the dense, bract-like, tightly-fitting pods of LEHI are persistent (Kirkman et al., 2004). Dispersion strategies represented besides dehiscing include pods that stick to clothing or fur, such as LEHI, individual seeds that are coated with an adhesive material, such as CLMA, and pods that simply fall to the ground, such as MIQU and TEVI. CRRO legumes are inflated, and the seeds will rattle inside of the pod when dry.

Inoculation with a mixture of native soils appeared to be effective due to nodule development in all species. Nodule morphology differs among species, as well. CEVI,

CLMA, CANI, LEHI, ORLU and RHRE all have similarly shaped, spherical nodules. These nodules vary from 1mm to nearly 10mm in diameter (Table 2-2). CRRO and MIQU have coralloid nodules from >2mm to <10mm in length. Coralloid nodules have a central branching point from which they randomly bifurcate, with occasional twice-bifurcation. The shape of this type of nodule is highly irregular, and a definitive size is difficult to estimate. TEVI nodules are elongate, cylindrical and often bifurcated. The length of TEVI nodules average >6mm to 10mm, although largest nodules are >10-mm long.

Phenology

Shade-grown plants did not demonstrate significant phenological delay in comparison to plants grown in full sun. However, weekly observations of phenological change did not allow high resolution for determination of treatment effects on flowering and fruit initiation. Data for the two light treatments within a species were combined, since there were no significant differences. Dates given are the days on which one half of the specimens for each species had begun to express the given phenological change, such as flowering.

MIQU was the first of the study species to flower (28 May, Day 149; Figure 2-2) and to produce fruit (30 June, Day 182), followed by CRRO, CLMA, CEVI, and LEHI, respectively. Flowering and fruiting continued throughout the season for MIQU, CRRO and CEVI. The lack of a specific time for flowering and fruiting to occur indicates that these species may be opportunistic in their fruit production. Both flowers and fruits were present on MIQU, CRRO and CEVI from the first fruiting until the end of the experiment. In contrast, CLMA, which stopped flowering around 14 September (Day 258), had a much more determinant flowering and fruiting pattern. Flowers and fruits

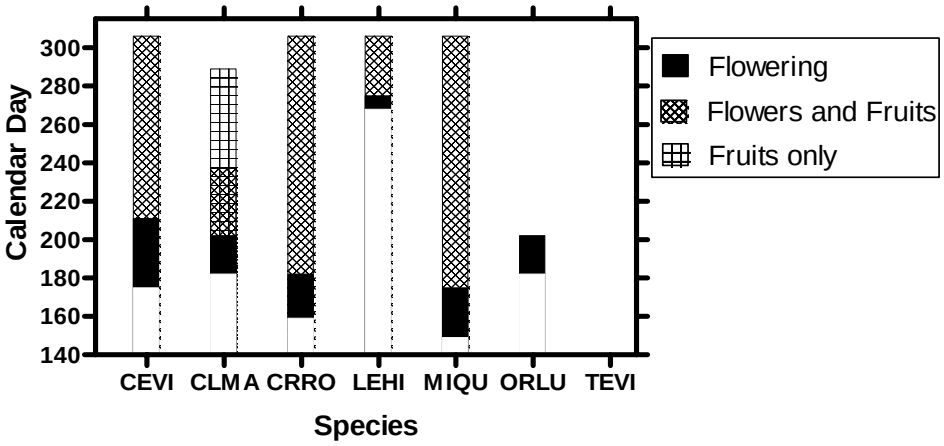


Figure 2-2. Phenological change by species. Bars represent the time at which over one-half of specimens for a species had reached the prescribed phenological phase, such as the onset of flowering. TEVI did not flower during the experiment.

were only concurrently present for approximately 30 days on CLMA before fruit production ceased and the plant returned to a vegetative state.

All species examined in this study were late spring and summer-flowering and fruiting except for LEHI, which is considered to be fall flowering and did not begin to flower until around 24 September (Day 268). TEVI did not flower during this experiment. ORLU flowered for the shortest duration of any of the species in this study, only 20 days. Although ORLU flowered, fruits were neither detected nor collected.

Plant Responses to Light Environment

Overall, the height patterns of all species showed rapid increase in stem elongation toward the beginning of the season, with the exception of CRRO, and LEHI. Growth curves were fitted to a third order polynomial (Figure 2-3; Table 2-3), but CEVI and MIQU had a poor fit for this equation (r^2<0.300) in a particular treatment, making them difficult to analyze. Stem elongation rates for all species were greatest around 14 May (Day 135), followed by decline as the season progressed, with the exception of LEHI, which had its strongest elongation rate during the middle portion of the season, between 29 June and 19 August (Day 181 and 232) (Table 2-4). CANI, CRRO and LEHI underwent a slight decline by the final measurement before harvest, 22 October (Day 296).

Stem elongation rates of CRRO, LEHI, ORLU and TEVI diverged according to treatment on 19 August and 22 October (Day 232 and 296 (Table 2-4). Height was also statistically different (unpaired t test, $p< 0.05$) at dates within the range that slope diverged for CLMA, CRRO, and LEHI (Figure 2-3). For CLMA, stem elongation rates in the shade were approximately twice that of sun-grown plants across all calculated

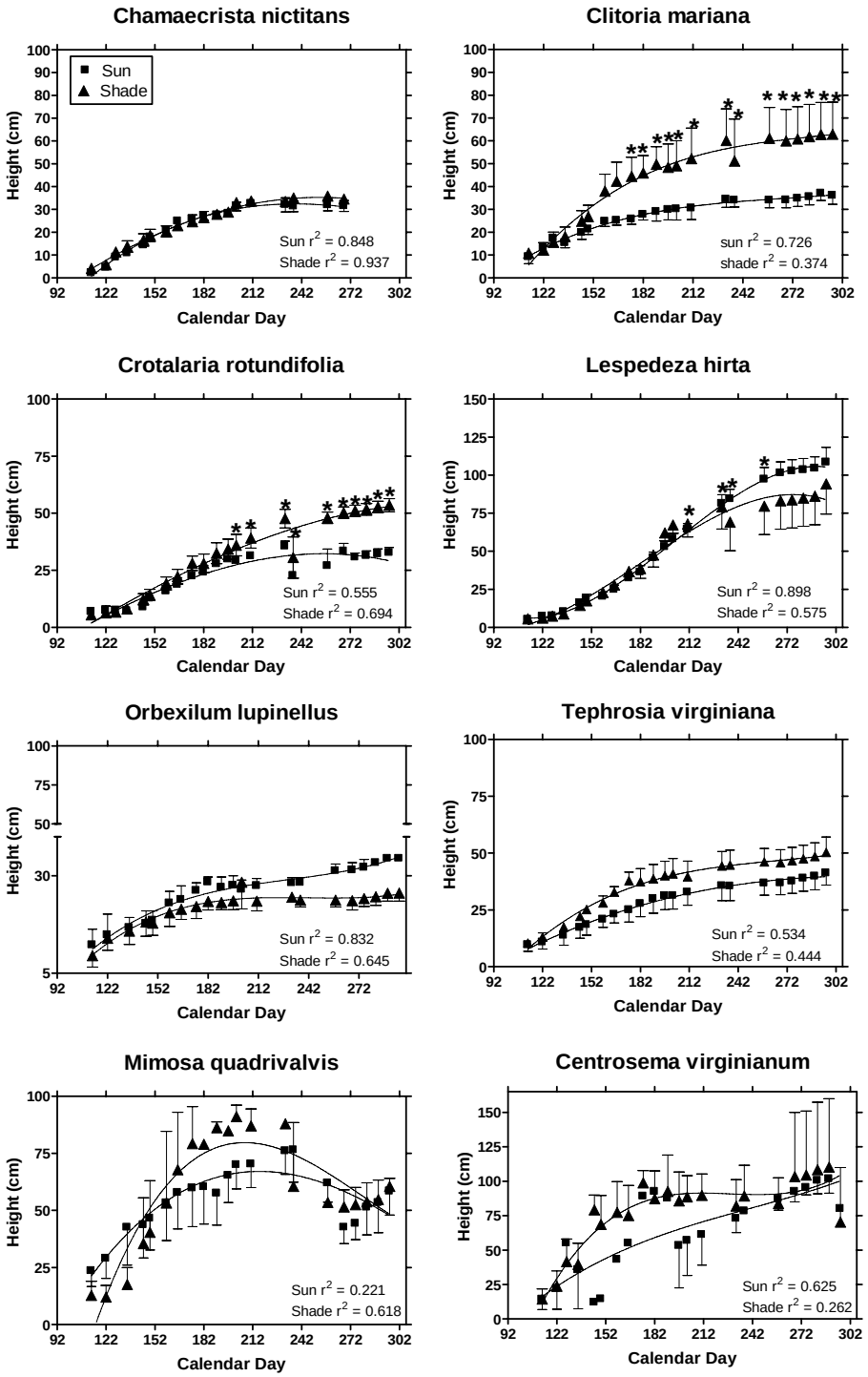


Figure 2-3. Stem elongation. Values are means +/- SE. ■ represents plants grown in the sun, and ▲ represents plants grown under the shade treatment. Goodness of fit values (r^2; α=0.05) are given for each curve that was fitted with a third-order polynomial.

Table 2-3. Regression equations for stem elongation curves of the form $y = Dx^3 + Cx^2 + Bx + A$. Coefficients are mean +/- SE.

		Equation Coefficients			
Species	***Light***	***A***	***B***	***C***	***D***
CANI	Sun	-107 ± 32.34	1.38 ± 0.54	-0.004 ± -0.002	$-3.8\times10^{-6} \pm 5.23\times10^{-6}$
CANI	Shade	-63.16 ± 28.22	0.74 ± 0.47	-0.001 ± 0.002	$-5.18\times10^{-7} \pm 4.52\times10^{-6}$
CLMA	Sun	-71.01 ± 37.33	1.09 ± 0.59	-0.003 ± 0.002	$4.84\times10^{-6} \pm 4.87\times10^{-6}$
CLMA	Shade	-155.60 ± 129.7	2.13 ± 2.06	-0.007 ± 0.010	$8.45\times10^{-6} \pm 1.70\times10^{-5}$
CRRO	Sun	-48.50 ± 44.59	0.48 ± 0.71	$-8.05\times10^{-5} \pm 0.003$	$-2.26\times10^{-6} \pm 6.00\times10^{-6}$
CRRO	Shade	-50.50 ± 51.62	0.49 ± 0.82	$-9.77\times10^{-5} \pm 0.004$	$-1.28\times10^{-6} \pm 6.95\times10^{-6}$
LEHI	Sun	186 ± 60.94	3.62 ± 0.97	0.02 ± 0.004	$-3.65\times10^{-5} \pm 8.14\times10^{-6}$
LEHI	Shade	80.9 ± 120.1	2.06 ± 1.94	0.01 ± 0.01	$-2.78\times10^{-5} \pm 1.65\times10^{-5}$
ORLU	Sun	-69.92 ± 26.73	1.18 ± 0.42	-0.004 ± 0.002	$7.10\times10^{-6} \pm 3.54\times10^{-6}$
ORLU	Shade	-76.76 ± 29.50	1.26 ± 0.46	-0.005 ± 0.002	$7.31\times10^{-6} \pm 3.90\times10^{-6}$
TEVI	Sun	-48.13 ± 58.98	0.65 ± 0.93	-0.001 ± 0.004	$1.004\times10^{-6} \pm 7.81\times10^{-6}$
TEVI	Shade	-120.4 ± 66.14	1.74 ± 1.05	-0.006 ± 0.005	$7.88\times10^{-6} \pm 8.70\times10^{-6}$

Table 2-4. Values given are slopes calculated from the derivative of the equations given in Table 2-3 at the mean for each coefficient.

		dh/dt			
Species	***Light***	***Day131 (14 May)***	***Day 181 (29 June)***	***Day 232 (19 August)***	***Day 296 (22 October)***
CANI	Sun	0.462	0.213	0.018	-0.141
CANI	Shade	0.384	0.232	0.070	-0.145
CLMA	Sun	0.319	-0.160	-0.455	-0.790
CLMA	Shade	0.677	0.350	0.148	0.080
CRRO	Sun	0.346	0.233	0.081	-0.157
CRRO	Shade	0.399	0.329	0.237	0.094
LEHI	Sun	0.294	0.799	0.749	-0.120
LEHI	Shade	0.497	0.721	0.520	-0.346
ORLU	Sun	0.263	0.106	0.569	0.151
ORLU	Shade	0.260	0.073	-0.003	0.061
TEVI	Sun	0.311	0.208	0.119	0.292
TEVI	Shade	0.500	0.239	0.095	0.087

dates. Slopes and heights for CANI were very similar for both sun and shade-grown plants throughout the growing season.

Most species reached maximum height near the end of the experiment (9 October to 22 October, Day 283 to 296). However, CANI (15 August, Day 228) and MIQU and ORLU (1 July to 5 September, Day 183 to 249) peaked earlier. CANI and MIQU experienced a slight decline in measured height after peaking due to some defoliation and stem breakage. Differences in maximum plant height were due to differences among species, as determined by analysis of variance ($p < 0.001$), and treatment effect was not significant due to the amount of variation between individual plants. Without regard to treatment, LEHI and CEVI were the "tallest" plants, followed by MIQU, CLMA, TEVI, CRRO, CANI, and ORLU, respectively (Table 2-5).

Leaf addition patterns for CEVI, CRRO (after 15 June, Day 167), and TEVI show that the plants grown in the shade tended to have more leaves on a given date than those grown in the sun. Due to large variability among individual plants, this pattern is only significant on a few dates for CRRO during the season (Figure 2-4A, B). CLMA, CRRO (before 15 June, Day 167), and LEHI patterns reveal the opposite effect, more leaf addition occurring on those plants grown in the sun than those in the shade. Again, due to large variability between individual plants, this effect is only significant for CLMA and LEHI on a few dates across the season (Figure 2-4A). ORLU, MIQU, and CANI did not show distinct patterns with regard to leaf addition over the season, and no statistical differences according to treatment were found on any dates.

Differences in root elongation patterns were difficult to detect due to the high mortality rates of specimens grown in the root tubes. In addition, due to the coloration of

Table 2-5. Maximum plant heights by species, regardless of treatment. Values are means ± SE. Different letters indicate significant differences (Duncan's post-test).

Species	***Day***	***Max. Height (cm)***	***n***
CEVI	289 (15 October)	53.2 +/- 27.6 a	5
LEHI	296 (22 October)	102.0 +/- 10.1 a	11
MIQU	225 (12 August)	81.3 +/- 9.5 ab	6
CLMA	293 (19 October)	53.2 +/- 9.7 bc	8
TEVI	296 (22 October)	46.6 +/- 4.6 c	10
CRRO	283 (9 October)	44.5 +/- 4.1 c	9
CANI	228 (15 August)	33.6 +/- 1.3 c	9
ORLU	249 (5 October)	31.4 +/- 3.3 c	4

A

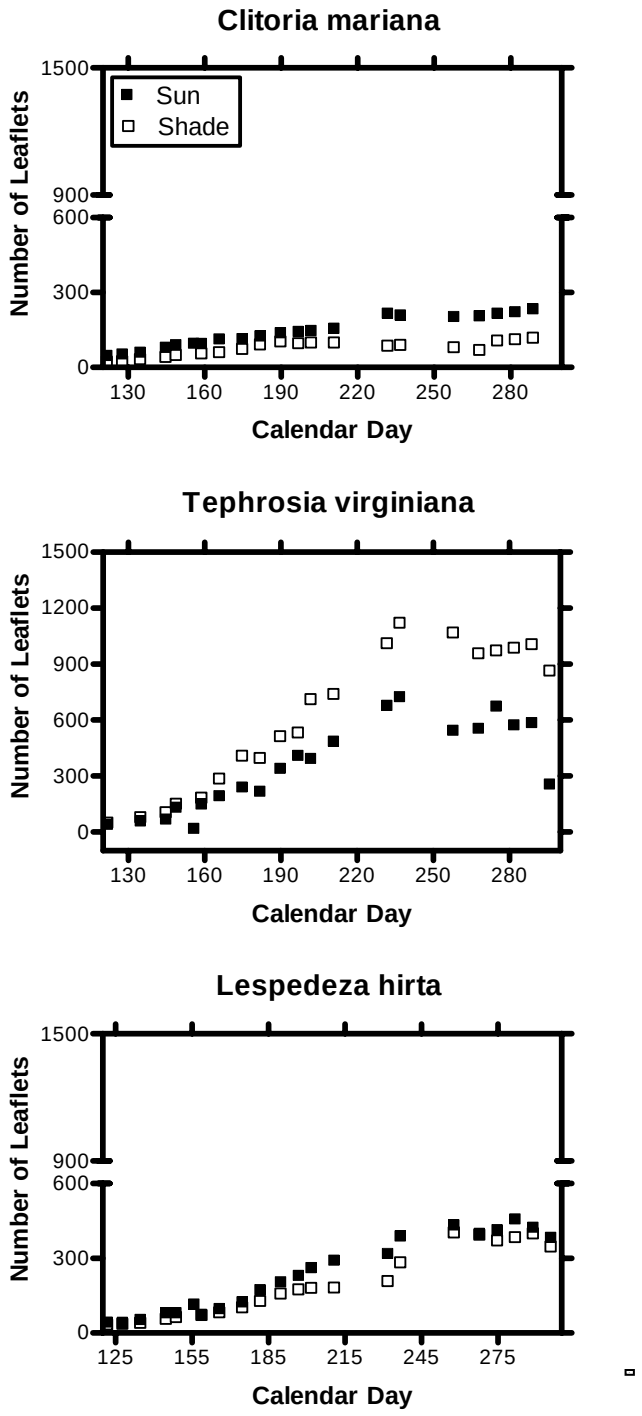


Figure 2-4. Leaflet counts and plant widths of sun and shade grown plants. Values shown are means ± SE. A) Leaflet counts for *Clitoria mariana*, *Tephrosia virginiana* and *Lespedeza hirta* plants grown in sun and shade. B) Width of *Crotalaria rotundifolia* plants grown in sun and shade.

B

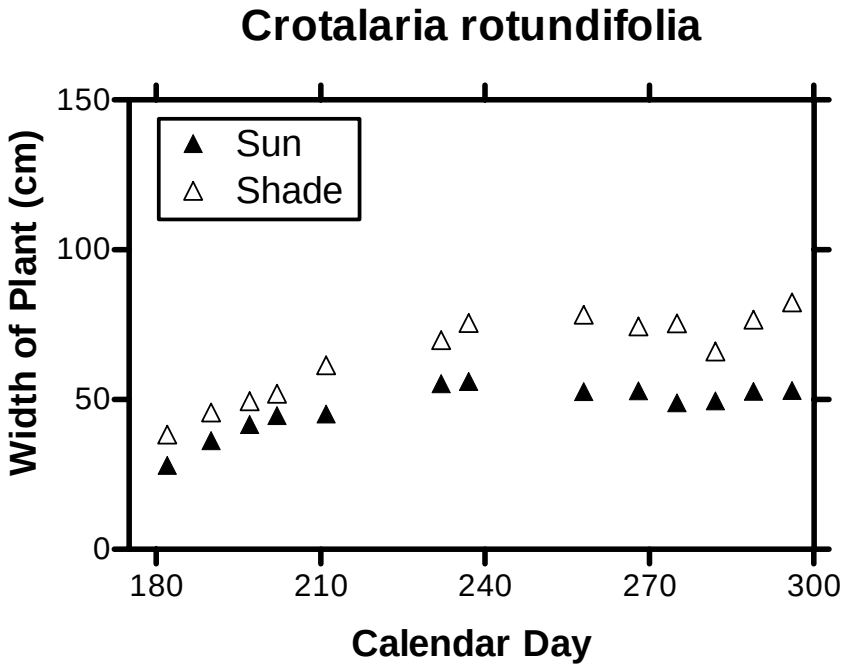


Figure 2-4. Continued.

the roots being close to that of the soil and the tendency of roots to not remain along the walls of the tubes, measurement were highly variable, and no differences according to treatment were significant. The rooting depth of the specimens in the sun and shade treatment were very similar for the first half of the growing season, although sun-grown plants tended to root slightly deeper in all three species shown (Figure 2-5). Shaded specimens appeared to have rooted slightly deeper than those in the sun at approximately 10 June (Day 162) for TEVI, and 30 June (Day 182) for LEHI and CRRO.

Biomass accumulation was not affected by light treatment for either total biomass, aboveground or belowground accumulation ($p = 0.3765$), therefore, additional values reported are pooled treatments by species. MIQU accumulated the largest amount of biomass over the season, followed by CANI and LEHI, then CEVI, CRRO, TEVI, and finally CLMA and ORLU (Figure 2-6).

Root to shoot ratios (R/S) showed no significant treatment effect, but species effect was highly significant ($p < 0.001$). MIQU and TEVI showed heavy allocation of biomass belowground (R/S>3, Figure 2-7), followed closely by CLMA, which had twice as much allocation below- versus aboveground (R/S>2). CEVI and CRRO had a mostly balanced allocation above- and belowground (R/S ~ 1). CANI, LEHI and RHRE favored aboveground allocation (R/S < 1). These patterns of biomass allocation did not reflect the same pattern as that of total biomass, but the different allocation patterns were represented in each of the groupings.

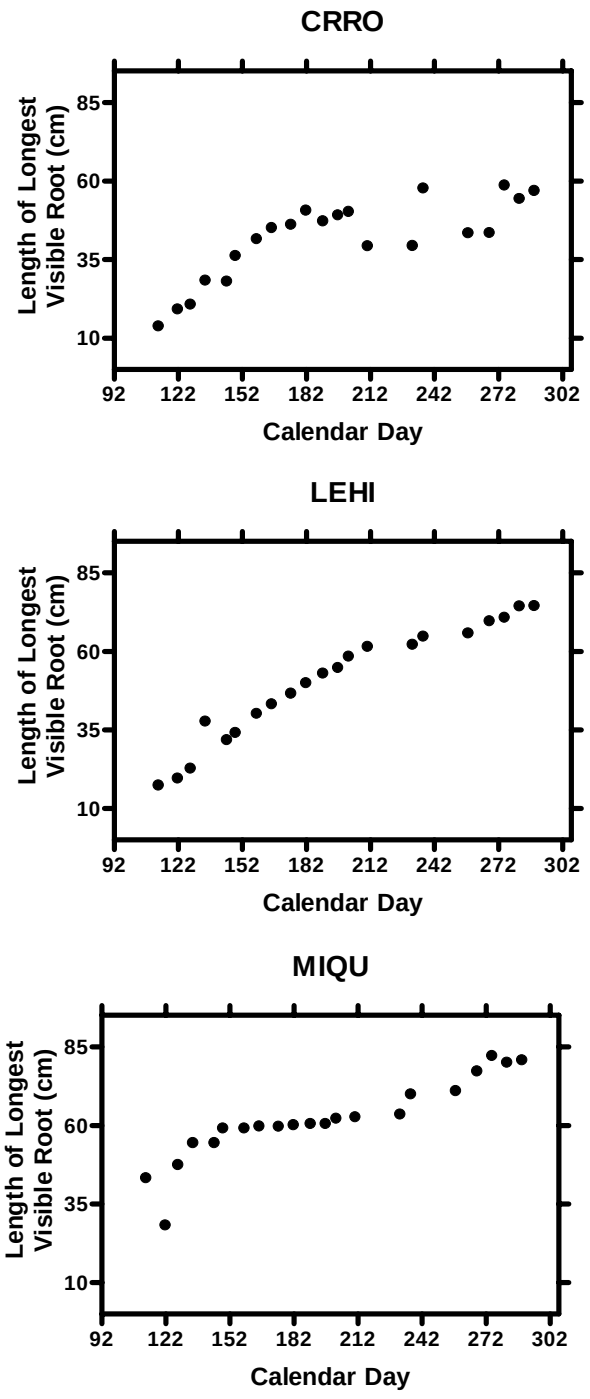


Figure 2-5. Elongation of roots grown in the sun and under shade treatment. Data was composited by species since light treatment effect was not significant. Values given are means ± SE.

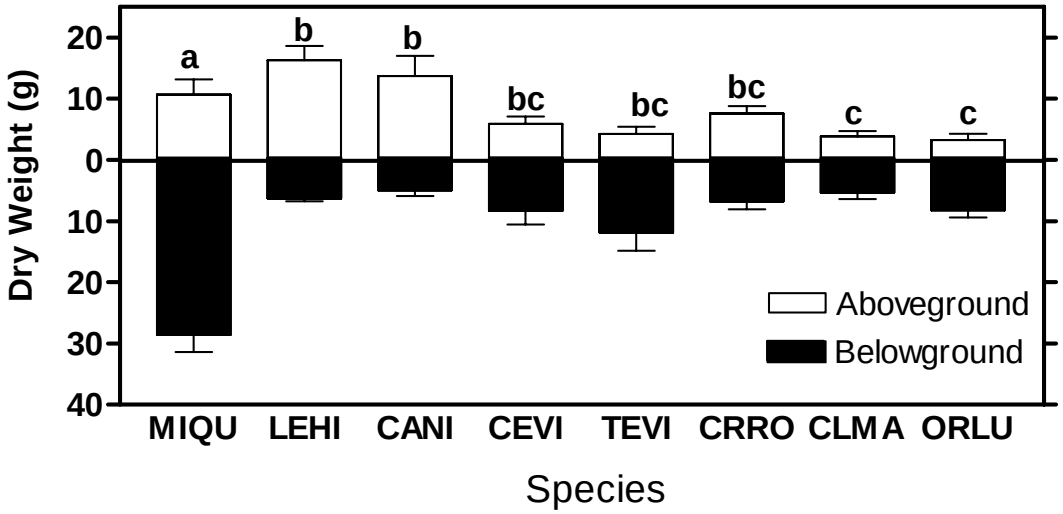


Figure 2-6. Aboveground and belowground harvested biomass. Values are means +/- SE. Data is composited by species because light treatment effect is not significant. Different letters represent significantly different total biomass (aboveground + belowground) values, and alphabetical order designates order of total biomass values, greatest to least (Duncan's post-test).

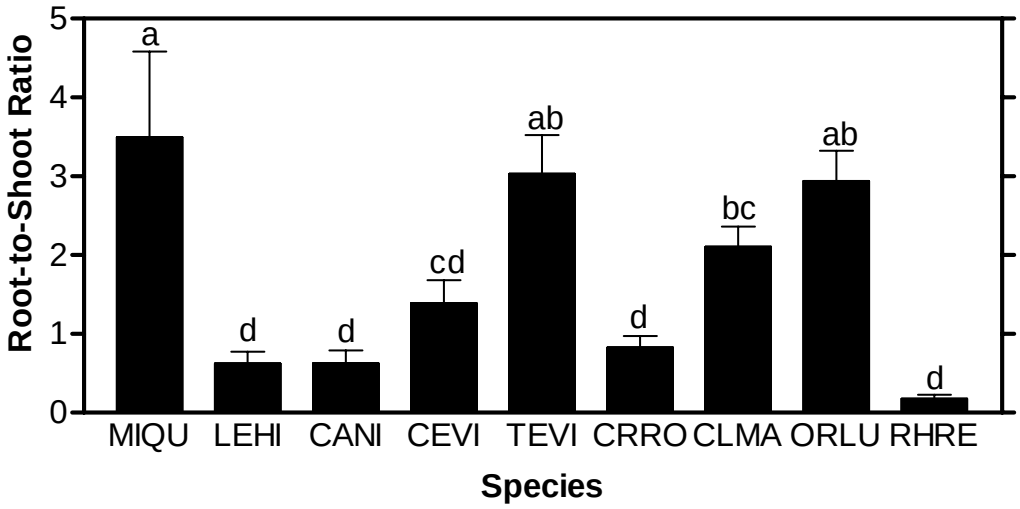


Figure 2-7. Root to shoot ratio of harvested biomass. Values are means +/- SE. Data are composited by species because light treatment effect is not significant. Different letters represent significantly different root-to-shoot ratios, and alphabetical order designates order of ratios, greatest to least (Duncan's post-test).

Discussion

Morphology and Phenology

The diverse native legumes in this study represented all three sub-families of legumes, as well as vines, erect herbs, and a semi-woody shrub. The life histories of these species represent a variety of strategies for overcoming shade, frequent fire and drought conditions. However, of the forty native legume species that grow extensively across the Ichauway reserve, only two are annual species: Cassia fasciculata and C. nictitans (Hainds et al.,1999). Perennial growth form is common in many frequently-burned ecosystems (Knapp et al., 1998; Morgan, 1999; Jacobs and Schloeder, 2002) and is apparently an effective adaptation to the frequently-burned longleaf woodland environment. The nodule morphologies of the species in this study follow the types described by Sprent (2002) for each of the subfamilies.

The majority of the species in this study began flowering during the late-spring, early summer. LEHI, like many of the other semi-woody *Lespedeza* and *Desmodium* species in the native woodland, flowered during the early fall (Chapter 3). All of the plants that produced fruit continued to flower and fruit until the time when they were harvested, except for CLMA. The indeterminate nature of flowering and fruiting represented by most of the species in this study is another adaptation to a fire-maintained ecosystem. Hiers et al. (2000) found that many legumes showed little change in duration or timing of peak flowering in response to the season in which they were burned, especially those that were fall-flowering or that matured multiple seed crops each year. Those plants that continuously produced seeds over the course of the season continuously contributed to the seed bank, and thus, did not need to re-grow and mature for a hastened seed crop after a late growing-season fire.

LEHI represented a fall-flowering plant that has determinant seed production in the wild, although it was not well represented in this study due to the timing of the harvest date. However, the number of seeds produced by LEHI in each cohort of seeds and the ease and range of its dispersal is much greater than that of the species which produce seed throughout the year. Unlike the other species in this study that rely on dehiscent pods or gravity for localized dispersion of seed, LEHI seeds adhere to fur (or clothing) of passing animals and are therefore widely dispersed. TEVI, which did not flower during this experiment, is an example of a species that flowers prolifically in the field in response to fire (Clark, 1971), although the flowering may be delayed by lightning-season fires (Hiers, 2000). Seed production (or lack thereof) in this study may not be indicative of production in the wild, due to unknown pollination factors that may not have been present in urban Gainesville, FL versus in the natural woodlands.

Growth Patterns

The shading treatment imposed in this study was 56 percent of full sun. Measurements of light quantity in the native woodland, below the tree canopy, but above the wiregrass canopy may be variable but is approximately 40 percent (Pecot et al., 2005), which is substantially higher than that typically reported for other forest types, including young pine plantations. Thus, shade tolerance among species in the longleaf woodland understory is not on the same scale of tolerance for understory species in other forest types that may only receive around 3 percent light infiltration (Battaglia et al., 2003). Light quality factors such as the red to far-red ratio may also be important to understanding responses of understory species, however, such analysis was outside of the scope of this study.

The species in this study all had variable growth responses to the two light environments, but shading did not have a significant impact on total biomass, or R/S allocation patterns (Figure 2-7). Although the small sample sizes due to high mortality in this study reduced our effectiveness to detect biomass shading effects, these effects were manifested in the later measurement dates for stem elongation (Figure 2-1). The effect of shading on height growth of CLMA, CRRO, and LEHI became was significant after 15 June (Day 167). CLMA and CRRO shade-grown plants were significantly taller than the sun-grown plants toward the end of the growing season, and LEHI grew significantly taller in the sun. The fact that tallest plants for individual species were not all located in the shade indicates that the shade-grown plants were not simply etiolated, but rather showed some degree of shade tolerance.

CRRO, which is a prostrate spreading plant, and CLMA, which is semi-erect to vining, both grow below and amongst the bunchgrasses of the native woodland, thus, their tolerance of shade is not surprising (Figure 2-1). LEHI, a tall semi-woody species, showed a more favorable growth response to sun than shade during the last weeks of the growing season. In the woodland, LEHI quickly outgrows the surrounding grasses and avoids mutual shading in the understory more aggressively than the smaller-stature species. CANI and TEVI, which showed no significant responses to shading, attain the same height as the surrounding grasses in the native woodland. The effects of shading on the two large vines, CEVI and MIQU, were not well defined in this study due to high mortality and the difficulties of measuring vines that intertwine and break easily. However, even if these vines have reduced shade tolerance, they still have the ability to climb over neighboring plants and to grow into sunflecked gaps in the savanna in order to

reach more direct light than smaller plants, such as ORLU and RHRE, for which adaptation to some shading would be more advantageous.

The variation in reported height among species, from 30cm to 100cm, represents the diversity of sizes and growth forms among these species. However, if the crown width of vining/spreading plants is considered, CEVI is the largest plant, spreading to over 2m, followed by MIQU, which grew to over 1m across, as well. CRRO also spread approximately twice as wide (Figure 4) as it grew tall, ~80cm versus ~45cm, respectively.

Keeping in mind the differences in plant morphology, the most decisive way to address comparisons of size of these species is by using total biomass. In an ecosystem that is frequently-burned and prone to frequent drought, significant allocation to belowground biomass is a positive survival adaptation (Knapp et al., 1998). These perennial species that can re-grow from reserves in the roots before surrounding plants will have a temporal and light-availability advantage over neighbors. MIQU and TEVI, both with R/S>3 (Figure 2-7), have also been shown to be significantly altered in phenology by fire (Hiers et al., 2000). Young MIQU plants excavated in the field had taproots exceeding 2 m long (personal observation). Plants that have higher R/S ratios and deep, branching root systems are better at enduring droughty conditions (Kramer and Kozlowski, 1979; Knapp et al., 1998), such as those that develop quickly in the coarse-sandy soils present in much of the longleaf-wiregrass ecosystem (Hainds et al., 1999). For a legume, an extensive root surface area also provides increased opportunity for rhizobial colonization.

The legume species in this study are difficult to generalize as a single group due to their diverse growth forms, reproductive phenologies, and life histories. Although the reduced early survivorship of our study population obscured some of our ability to document shade effects for all species, these data indicate that examples of legumes with some degree of shade tolerance can be found outside of the tropics (Sprent, 1999). Some species exhibited greater height growth with shading, but none of the species in this study demonstrated strong shade intolerance through reduced biomass responses to the 56 percent of ambient light level treatment. This light regime would certainly be representative of conditions in open longleaf pine savannas or in thinned young plantations with restoration plantings (Chapter 4), although a more heavily shaded treatment might have provided a greater effect upon several species. However, most of the life-history characteristics of the species examined in this study may be more strongly associated with adaptation to fire, N deficient soils, and drought than to light environment in longleaf pine savanna ecosystems.

CHAPTER 3
USE OF CORROBORATIVE METHODS TO ASSESS THE N_2-FIXATION OF NATIVE LEGUMES

Introduction

The fire-dependent longleaf pine- (*Pinus palustris* Mill.) wiregrass (*Aristida stricta*, Michx.) savanna ecosystem once dominated the southern coastal plain of the United States, covering as much as 37.2 million ha. Presently, less than 1.5 million ha of these ecosystems remain intact (Landers et al., 1995). However, restoration plantings reclaiming more than 283,000ha of former agricultural fields, pulpwood plantations, and other fire-suppressed lands are being established in the Southeast, 48,000ha of which are in USDA Conservation Reserve plantings in Georgia (Coffey and Kirkman, 2004). Groundcover reestablishment is the key to restoring wildlife habitat in these young longleaf pine plantings as well as providing continuity of pyrrhic fuels (Clewell, 1989; Kirkman, 2002). Legumes may also have a major role in maintaining N balance fire-maintained restored systems that are being established on depleted, former agricultural soils (Markewitz et al., 2002; Boring et al., 2004). Planting of native legumes would permit groundcover restoration without some of the problems that have occurred as a result of introducing non-native and agronomic legumes, including serecia lespedeza (*Lespedeza cuneata*) and *L. bicolor*, that have little shade tolerance and can be very invasive (Miller, 2003).

There is a need for species of legumes native to the longleaf-wiregrass ecosystem to be identified for use in groundcover restoration plantings that show strong potential for

N_2-fixation and that can make large, N-rich biomass contributions to depleted soil organic matter (Markewitz et al., 2002). In addition, adaptation to environmental factors such as nutrient deficiency, drought conditions, and reduced light under a young closed forest canopy must be considered along with an analysis of N_2-fixing potential. After initial screening, these species should be planted in field conditions for a better assessment of their physiological adaptations and N_2-fixing potential (Dreyfus et al., 1988).

Assessing N_2-fixation under field conditions is a difficult task, and most current methods measure fixed-N_2 indirectly. Traditional methods of assessment involve nodule excavation and other destructive biomass measures to assess N-fixation and cannot be performed repeatedly on the same plants. These methods can also be destructive when used in a woodland ecosystem because of the amount of disturbance caused by excavating root systems. The results of traditional methods of N_2-fixation assessment, such as nodule biomass measures, total-N comparison, and the acetylene reduction assay are not readily convertible into actual amounts of N_2-fixed. However, more direct methods, including the $\delta^{15}N$ natural abundance and ^{15}N enrichment methods (Virginia et al., 1989; Hiers et al., 2003), are integrated over time and can be used to calculate a quantitative estimate of N_2-fixed. These methods may eliminate the problems associated with instantaneous measures of fixation that may fluctuate over diurnal and seasonal conditions, such as the acetylene reduction assay (Boring and Swank, 1984; Halvorson et al., 1992). By combining methods of assessing N_2-fixation that include both instantaneous and cumulative measures and first applying them in a controlled study, a well-informed assessment can be made of the N_2-fixing capabilities of a legume species.

This study was designed to compare the N_2-fixation capabilities of nine species of legumes native to the longleaf-wiregrass ecosystem. Two additional objectives were also addressed: (1) to compare estimates of N_2-fixation capabilities as derived from five different methods of assessment: nodule biomass, the acetylene reduction assay, N transport and storage product analysis, $\delta^{15}N$ natural abundance, and total N content; and (2) to examine the effects of shading on their N_2-fixation activity.

Methods and Materials

Planting

Seedlings of nine native legume species were used in this experiment. They were propagated as described in Chapter 2, and were obtained from Dr. Kay Kirkman at the Joseph W. Jones Ecological Research Center, Baker County, Georgia: *Centrosema virginianum* (L.) Benth. (CEVI), *Clitoria mariana* L. (CLMA), *Crotalaria rotundifolia* J.F. Gmel. (CRRO), *Lespedeza hirta* (L.) Hornem. (LEHI), *Mimosa quadrivalvis* (L.) (MIQU), *Orbexillum lupinellus* (Michx.) Isley (ORLU), *Rhynchosia reniformis* D.C. (RHRE) and *Tephrosia virginiana* (L.) Pers (TEVI). Seeds for the annual *Chamaecrista nictitans* (L.) Moench (CANI) were obtained from Dr. Ken Quesenberry (Agronomy Department, University of Florida). Six specimens of each species were planted in pots that were specially designed to allow gas to flow through them as part of a closed system. These pots were constructed from a capped 35-cm long section of 10.5-cm diameter polyvinyl chloride (PVC) pipe.

Fourteen specimens from each species were transplanted into black tree-seedling pots, 35-cm tall x 80-cm^2 base (Stuewe and Sons, Corvallis, OR). Transplantation of seedlings was completed 11 February 2004. CANI was planted on 21 April 2004 from seeds that were germinated on moist filter paper. Due to the small numbers of plants,

destructive harvests of seedlings were not possible at the beginning of the experiment (T_0), but non-destructive measurements were taken. Plant heights at planting represented between 5.3 and 19.5 percent of the final heights. The smallest plants, such as ORLU and RHRE had the largest percentage of the final height present at T_0, and the larger plants, such as LEHI, were represented by the lower range of the percentages. Leaves were also counted at T_0 to determine percentage of leaves initially present. Percent leaves present at T_0 ranged from 1.5 to 8.7 percent for the small- to large-stature plants, respectively.

All plants were grown outdoors in Gainesville, Florida (29° 38' N, 82° 20' W) between April and November of 2004. A shade cloth enclosure was used to create an environment that provided a 0.54 fraction of total ambient light as determined using a Li-Cor Quantum Sensor, LI-185A (Chapter 2).

Plants were inoculated by introducing native soil to the pots. Two topsoils were collected at the Jones Center, from a fine-loamy, kaolinitic, thermic Typic Kandiudult (Orangeburg Series), and from a loamy, kaolinitic, thermic Arenic Kandiudult (Wagram Series). Each collection was made in an area with a diverse, thriving legume population. Soil collections were transported to Gainesville, FL where they were stored in a cool, dark room and covered with plastic to maintain moisture. Equal parts of each soil collection were mixed in order to provide inoculation for legumes that may be predominantly found in different locations. Pots were filled with purchased topsoil (Walmart Corp.) to within 6 cm from the top of the pot. Next, 2 cm of the native soil mixture were added, and finally, the plant was transplanted, using additional topsoil to

cover the roots as needed. Water was applied by drip-irrigation every 12 hours using a battery-operated timer (Rainbird).

Plants from acetylene reduction assay pots and black plastic pots were destructively harvested on 11 November 2004. Roots and nodules were washed free of soil, nodules counted, and a 2-cm section was collected from the base of each stem for analysis. All tissues were dried to constant weight at 80^{o}C.

N_2 Fixation Assessment

Measurements of ethylene production as a result of exposing legume roots to acetylene (C_2H_4) gas were taken every three weeks over the course of the experiment. Repeated assays using the same plant were made possible by the use of a non-destructive, flow-through system.

Specially-designed pots were used to allow the plants to be repeatedly assayed with minimal disturbance. The gas input line was attached to the bottom of the pot. A lid matching a 3.6L (20-cm tall x 17-cm diameter) plastic container (Rubbermaid Inc.) was permanently attached with wing nuts and screws to the flange that formed the top of each pot, and the joints were all sealed air-tight using weather-strip caulking. The center of each lid was removed to reveal the mouth of the pot. The plastic containers were inverted over the top of the plant and sealed to the stationary lid. On the bottom of each plastic container (the top of the apparatus when inverted), a port was created over which the gas line would fit tightly. Air-tightness was confirmed for each sealed pot before gas flow was initiated.

Ten percent acetylene was flowed into the pots at a rate of 1L min^{-1}. Gas samples for each pot were taken both from the lines running into the pots and those running out. The inflow samples served as a baseline for the amount of ethylene that might be present

in the acetylene source. The outflow samples, which flowed past the plant root systems, contained the ethylene generated from acetylene reduction as a result of nitrogenase activity. Duplicates of 1-mL samples were collected using syringes that were inserted into the tubing. Samples were transported to the laboratory and analyzed immediately. Analyses were performed using two gas chromatographs (Hewlett Packard 5710A and Shimadzu GC-8A).

Stem sections were cut from the basal 3cm of plants grown in PVC and black plastic pots as they were harvested (11 November 2004). Each stem was placed in a 20-mL glass vial and covered with a phosphate buffer solution, mixed according to Herridge (1984), except ethanol was used as the solvent rather than water at the suggestion of Izaguirre (personal communication). Vials with stems in solution were stored at 0^{o}C until extracted.

Stem sections were placed in a boiling waterbath (100^{o}C) for 25 minutes to extract stem contents. Stems were removed and dried to constant weight at 80^{o}C. Deionized water was added to each extract to a standard volume of 25 mL. Extracts were covered and stored at -30^{o}C between analyses to prevent evaporation. Aliquots of each extract were analyzed for ureide, NO_3, and α-amino acid concentrations using spectrophotometry (Beckman DU 640). Ureide concentrations were estimated as the phenylhydrazone of glyoxalate using allantoin as the standard, and α-amino acid concentrations were determined using a modification of the ninhydrin method (Yemm and Cocking, 1955), with asparagine as the standard; both analyses were conducted as described by Herridge (1984). NO_3 was extracted using the salicylic acid method as reported by Cataldo et al. (1975). Taking into account that ureides contain 4 N atoms per molecule, an index of the

relative abundance of ureide-N in each extract was calculated according to Peoples et al. (1996), where the bracketed variables indicate the molar concentration of each of the extract constituents:

$$RUI = 400[ureides] / (4[ureides] + [nitrates] + [\alpha\text{-amino-acids}]) \quad [3\text{-}1].$$

Use of $\delta^{15}N$ natural abundance technique in the field requires that differences between the soil and atmospheric ^{15}N pools be well established by also ascertaining $\delta^{15}N$ values of non-fixing reference plants and available soil N (Virginia et al., 1989). ORLU was selected as a non-fixing reference species for this controlled study was selected using corroborative assessments of N_2-fixation.

Stems and leaves harvested from plants grown in the PVC and black plastic pots were coarsely ground using a Cyclotec Sample Mill, and then ground to a fine powder using a Spex CertiPrep Mixer Mill 8000-D. Roots were not analyzed for $\delta^{15}N$ due to the amount of organic matter that remained attached after thorough cleaning. Finely ground tissues were analyzed for ^{15}N natural abundance and N content at the University of California, Davis (Stable Isotope Facility, Department of Agronomy, Davis, CA) using mass spectrometry. ^{15}N natural abundance is expressed as $\delta^{15}N$ (‰ ^{15}N depletion units):

$$\delta^{15}N = [(atom\%^{15}N_{sample} / atom\%^{15}N_{standard}) - 1] \times 1000 \quad [3\text{-}2]$$

where the standard is the atom percent ^{15}N concentration of atmospheric N_2.

Percent of total N derived from the atmosphere (% N_{dfa}) was calculated according to the equation:

$$\% N_{dfa} = 1 - (\delta^{15}N_{N2\text{-fixing plant}} / \delta^{15}N_{ref}) \quad [3\text{-}3].$$

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with species and light environment as main effects. If differences existed ($p<0.05$), Duncan's multiple

comparison test was used to determine which means differed significantly. The GLM procedure performed in the Statistical Analysis System (SAS, 2003) was used for ANOVA and post-tests.

ANOVA analysis did not show any significant treatment effects for any of the datasets in this study. Therefore, differences further discussed relate only to those among species, and means reflect a composite of both treatments.

Results

Survivorship

Of the six specimens for each species that were planted in the pots for the flow-through acetylene reduction assay, an average of three survived. LEHI and CRRO had the best survivorship, with five plants each, followed by CANI and TEVI with four, then CLMA with three. RHRE and ORLU had the worst survivorship, with no RHRE plants surviving, and only two ORLU, which did not permit statistical analysis of treatment effect on the acetylene reduction assay for these two species.

An average of four out of fourteen specimens grown in black tree seedling pots reached final harvest. LEHI, had the best survivorship with eight remaining, followed by TEVI and CLMA with six and five plants surviving until final harvest, respectively. CANI, CEVI, CRRO, RHRE, ORLU, and MIQU, had average or lower survivorship. ORLU and MIQU had only two and one plant surviving, respectively. However, since these plants were used in addition to the ones harvested from the PVC pots, statistical analysis was still possible for all species for most fixation indices.

Shade treatment effect was not significant for any of the N_2-fixation assessments in this study. Small samples and high variability contributed to the inability to detect statistical differences according to treatment. Ambient light for this study was also

impacted toward the end of the season by tropical storm occurrence. Detectable differences in plant height were developing around this period, and the lowered irradiance overall may have impeded the maximum effect of shading during this critical growth period. However, results from biomass and nodulation data did not show significant responses to shading.

Fixation Assessment

Species differences in nodule mass were significant ($p = 0.0006$). MIQU had the greatest nodule mass, but further statistical delineations were not detectable due to the large amount of variation between individual plants (Table 3-1). None of the small herbaceous species in this study had an average nodule mass of more than 1g.

Nitrogenase activity (acetylene reduction) was initially very low, overall, however, later in the season there were isolated peaks of substantial ethylene production from the older plants (Figure 3-1). CANI, LEHI, CEVI, and MIQU all showed an increase after the middle of the season, around 28 June (Day 180). CLMA showed the lowest nitrogenase activity across the season, never producing more than 0.3 μmol C_2H_4 hr^{-1} at any given date. ORLU also showed low activity, with only a single peak above 0.3 μmol C_2H_4 hr^{-1}. Comparison of late season maximum ethylene peaks using analysis of variance showed a significant species effect ($p = 0.0002$). The peak for MIQU was significantly greater than all other species maximums, but no other significant differences among the remaining species were detected (Figure 3-2).

The N transport and storage products extracted from stem sections of native legumes were dominated by ureides and α-amino acids. The relative concentration of α-amino acids (RAC) was approximately equal to that of the relative ureide concentration (RUC) for most species, followed by a very minute NO_3 concentration (Table 3-2).

Ureide appears to be an important transport/storage product molecule for this suite of species. The differences among species were not significant for RUC or RAC, but were highly significant for total extracted N and relative NO_3 concentration (RNC; $p = 0.0006$ and 0.0001, respectively).

The $\delta^{15}N$ values of the species were significantly different and ranged from -3.13 to -1.45 (Figure 3-3). CLMA had the $\delta^{15}N$ value closest to zero, followed by CANI, CEVI and TEVI, which all had values of approximately -2.3. ORLU and RHRE had the most negative values, approximately -3.2. However, only CLMA and CRRO were significantly different from ORLU, which was used as the non-fixing reference.

Percent N_{dfa} was calculated (Equation 3-3) using ORLU as the non-fixing reference. ORLU had very low nitrogenase activity (acetylene reduction) across the season, indicating very little, if any, N_2-fixation, in spite of having numerous small, but apparently ineffective nodules. Species effect was significant for percent N_{dfa} ($p = 0.0002$), and values ranged from 12.0 to 54.9 percent (Figure 3-4A). CLMA was determined to have the highest percent N_{dfa}, followed by CRRO ≥ TEVI ≥ CEVI ≥ MIQU ≥ LEHI ≥ RHRE.

Percentage of N ($gN\ g^{-1}$ tissue) in stem and leaf tissues ranged from nearly 3.0 percent to approximately 1.5 percent (Figure 3-4B). Species effect was significant for percent N ($p = 0.0001$). MIQU had the highest percentage of N (2.83 percent), followed by CANI, then CRRO ≥ CLMA ≥ CEVI ≥ ORLU ≥ TEVI, and RHRE. LEHI had the lowest percentage of N in its tissues (1.47%).

Total grams of plant accumulated N over the season was significantly different among species ($p = 0.0001$). CANI and MIQU had the largest amount of accumulated N,

Table 3-1. Nodule mass and number of nodules. Rankings are according to Duncan's post-test. RHRE not included in ANOVA (n = 1).

Species	***Mass (g)***	***# Nodules (per plant)***
MIQU	0.9114 a	218.60 a
CEVI	0.3303 b	59.43 b
TEVI	0.1316 b	51.40 b
CANI	0.1175 b	44.00 b
CRRO	0.0864 b	27.5 b
CLMA	0.0818 b	32.17 b
LEHI	0.0547 b	48.92 b
ORLU	0.0229 b	27.25 b
RHRE	0.0100	7.00

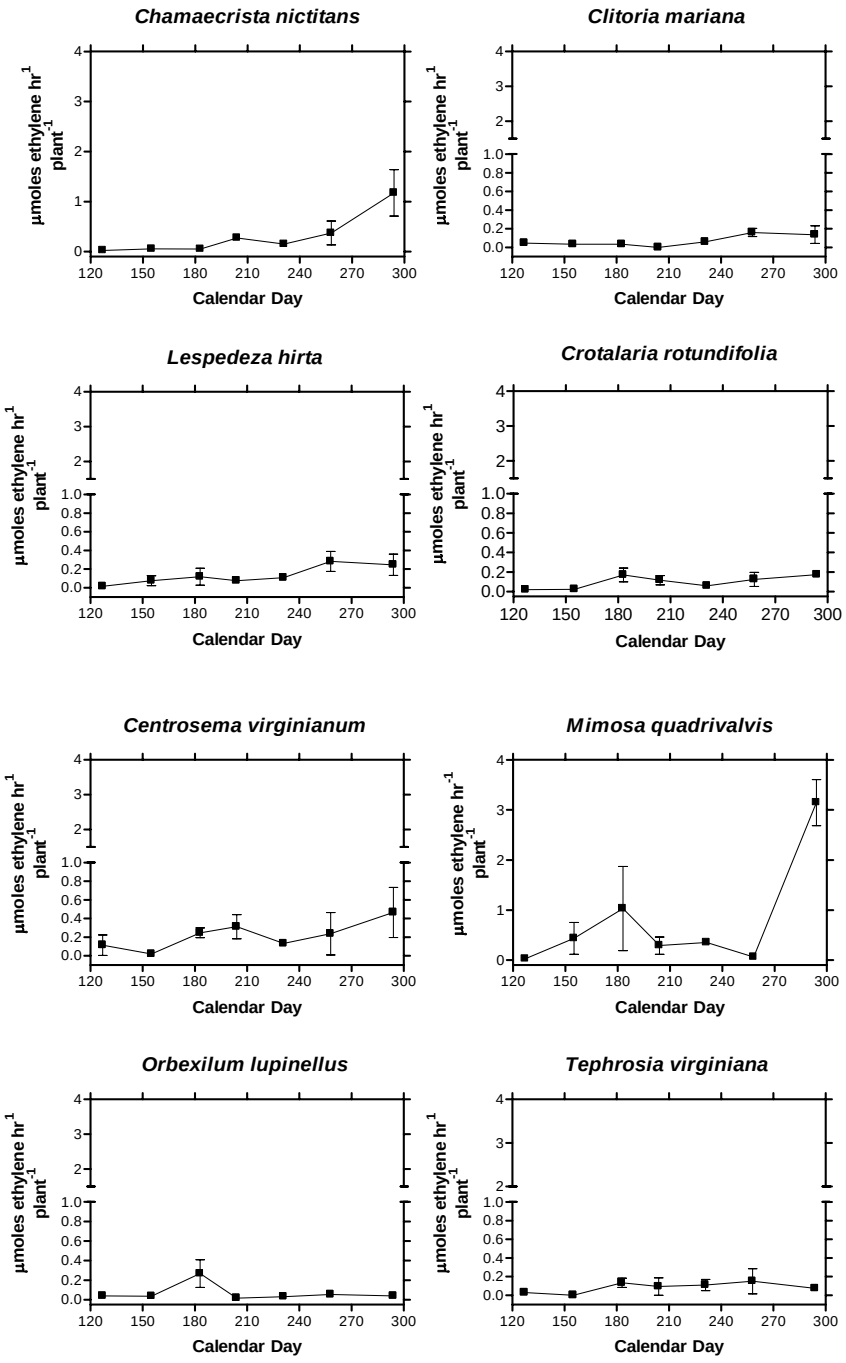


Figure 3-1. Ethylene production trends for the growing season by species. Data shown are means ± SE with treatments combined, and lines show mean trends.

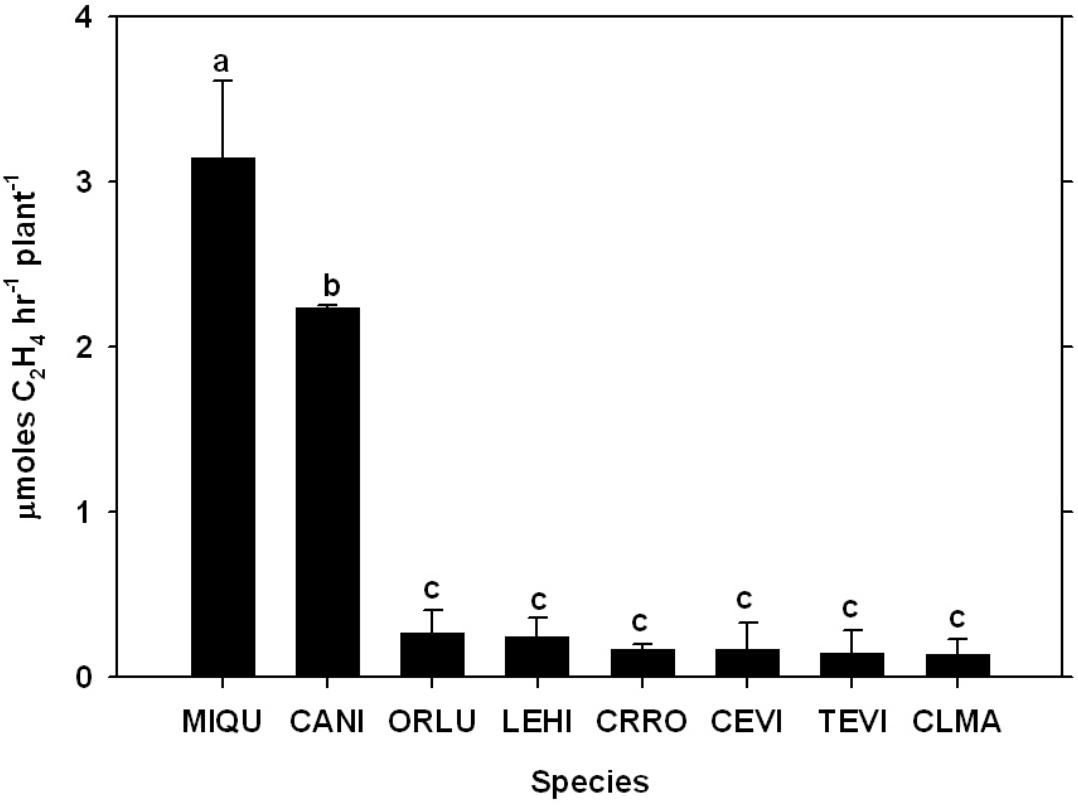


Figure 3-2. Maximum ethylene production (C_2H_4 reduction) peaks. Data shown are means ± SE. Different letters represent statistical differences (Duncan's post-test).

Table 3-2. N-transport/storage products extracted from stem sections. RUC, RAC, and RNC represent relative concentrations of ureides, α-amino acids, and NO_3, respectively. RUI and Total N are relative ureide index (Equation 3-1) and total extracted N (mmol N g^{-1} stem). Letters represent statistical differences within columns according to Duncan's post-test. Species effect was not significant, ns.

Species	***RUC***	***RAC***	***RNC***	***RUI***	***Total N (mmol N g^{-1} stem)***
CANI	45.68	54.28	0.032 a	73.01	0.0018 b
MIQU	34.26	65.72	0.008 e	62.11	0.0055 a
LEHI	27.15	72.82	0.020 bc	56.26	0.0014 b
CEVI	46.09	53.89	0.013 de	67.15	0.0049 a
CRRO	55.75	65.75	0.013 cd	67.15	0.0035 ab
TEVI	24.84	75.14	0.010 e	48.39	0.0035 ab
ORLU	51.83	48.14	0.026 b	79.79	0.0056 a
CLMA	44.23	55.75	0.013 de	67.15	0.0035 ab

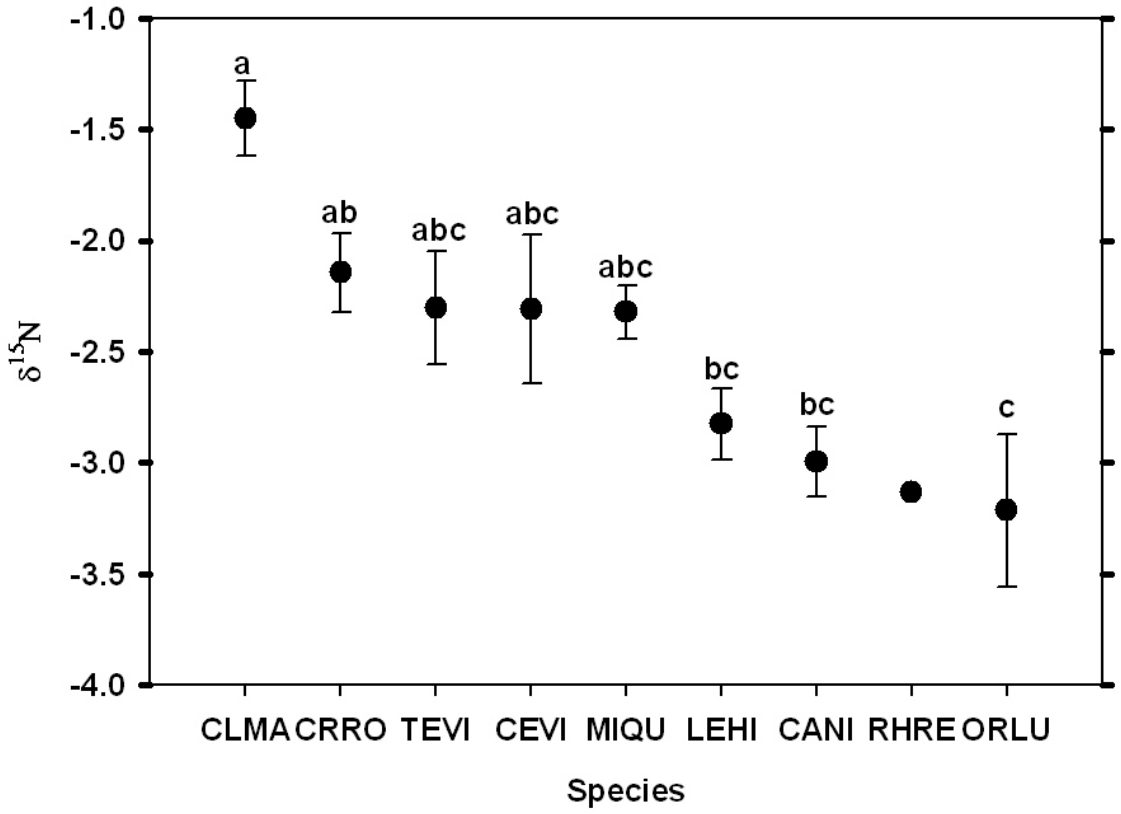
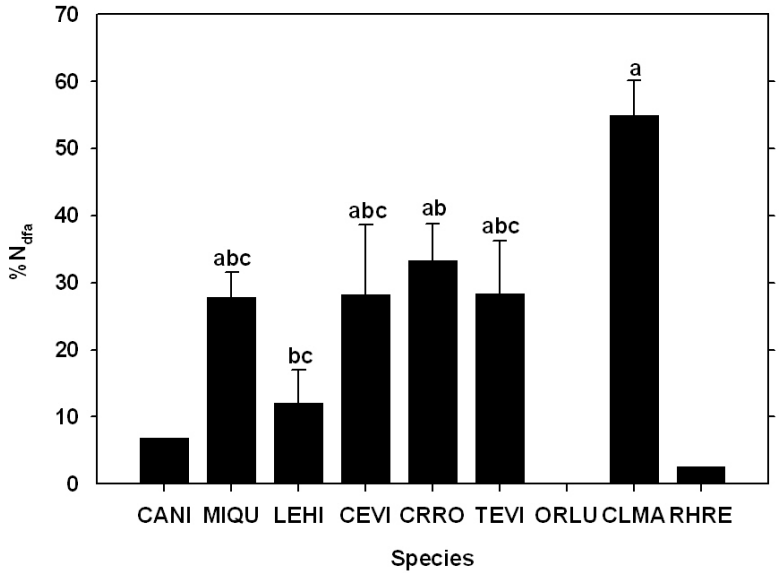


Figure 3-3. $\delta^{15}N$ values by species. Data shown are means ± SE. Different letters represent statistical differences (Duncan's post-test). RHRE, (n=1) was not included in the ANOVA.

A



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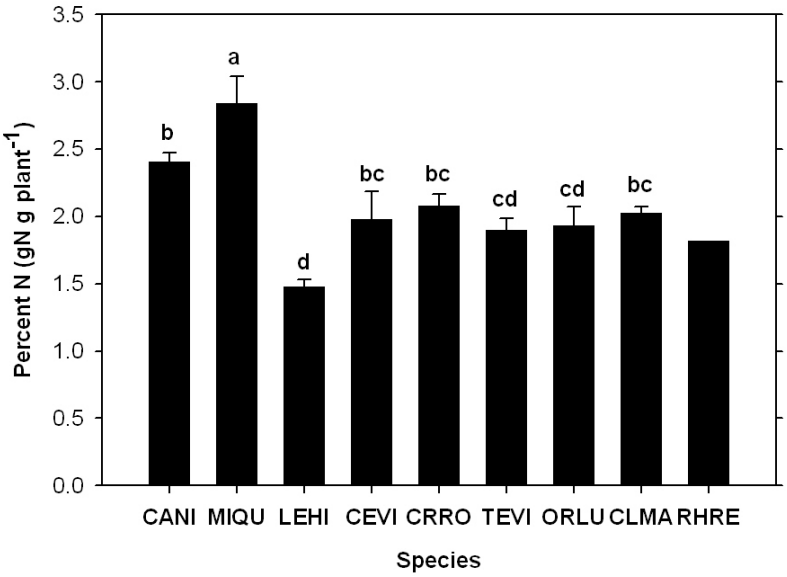

Figure 3-4. Mean % N_{dfa}, %N, and total N by species. Different letters represent statistical differences (Duncan's post-test). A) Percent of total N derived from the atmosphere. CANI and ORLU did not have adequate replication for inclusion in ANOVA, and ORLU was used as a non-fixing reference in the calculation of % N_{dfa} (Equation 3-3). B) Percent N in aboveground tissues. C) Total N content of aboveground tissues.

C

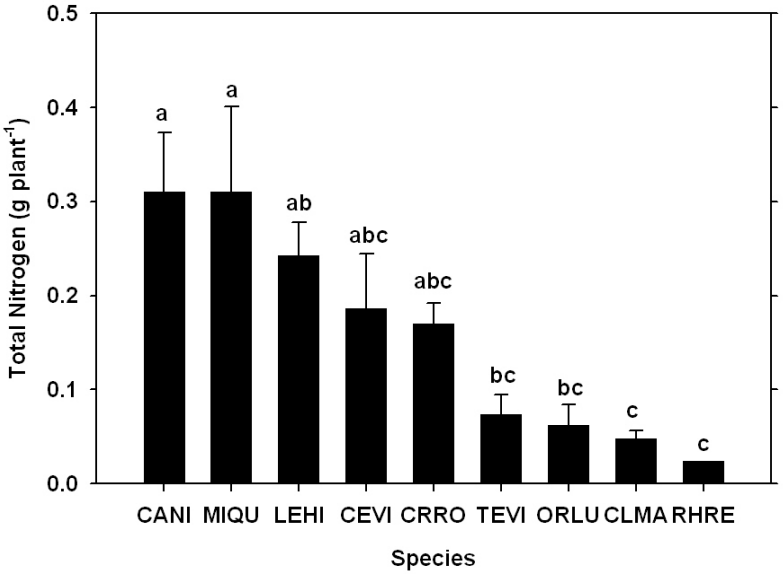

Figure 3-4. Continued

followed by LEHI, CEVI, and CRRO, and finally, TEVI, ORLU, CLMA, and RHRE (Figure 3-4C).

Discussion

Comparison of Methodology

Although there were some differences in the way the N_2-fixation capacity of the species were ranked by each assessment technique, the species could generally be assigned into high- and low-fixer categories. With little discrepancy between assessment results among methods, MIQU, CANI, CRRO, CLMA and CEVI showed higher N_2-fixation potential, and LEHI, TEVI, and RHRE showed relatively lower potential. The results for ORLU generally corroborated that this species did not form effective nodules in this study and thus had the least effective N_2-fixing capacity, approximately none (Figures 3-1, 3; Table 3-3).

The acetylene reduction assay, an instantaneous indicator of nitrogenase activity, is strongly affected by environmental stresses such as drought conditions and light reduction, which reduce photosynthesis rates. Therefore, seasonal and diurnal patterns in nitrogenase activity can generally be well documented with frequent acetylene reduction assays (Boring and Swank, 1984; Zitzer and Dawson, 1989; Halvorson et al., 1992; Peoples et al., 1996). This method of assessing N_2-fixation is also a quick and inexpensive, instantaneous first assessment of nitrogenase activity. However, it is necessary to follow temporal patterns of plant development to adequately index an annual pattern of nitrogenase activity, and even then, the assay is not a direct measure of N_2-fixation, since many species have nitrogenase systems with varying efficiencies and hydrogenase activities (Zitzer and Dawson, 1989).

Table 3-3. Specific nodule activities of species in this study and other comparative reports. Specific nodule activities for this study were estimated using the results of the final acetylene reduction assay (22 October 2004) and biomass of harvested nodules. Data from this study are ranges and means ± SE. [1]Hendricks and Boring, 1999; [2]Hogberg and Kvarnstrom, 1982; [3]Boring and Swank, 1984; [4]Zitzer and Dawson, 1989.

Species	***Specific nodule activity*** ***(µmol C_2H_4 hr^{-1} mg $nodule^{-1}$)***
Centrosema virginianum (CEVI)	0.272 ± 0.272
Mimosa quadrivalvis (MIQU)	0.297 ± 0.116
Other native legumes	0.002 to 0.007
Orbexilum lupinellus (ORLU)	0
Desmodium viridiflorum[1]	0.08
Lespedeza procumbens[1]	0.04
Leucaena leucocephala[2]	0.048
Robinia pseudoacacia[3]	0.05
Alnus glutinosa[4]	0.02 to 0.02
Eleaganus angustifolia[4]	0.002 to 0.01

Specific nodule activity comparisons between species in this study and other similar and larger species of legumes suggest that CEVI and MIQU have extremely high nodule efficiency (Table 3-3). However, these comparisons may not be completely accurate due to the difference in sampling techniques. The flow-through assay to measure nitrogenase activity (acetylene reduction) that was used in the current study, avoids many of the problems associated with field sampling, which was used in the comparative studies, such as limited nodule recovery and possible evolution of ethylene from excised plant parts. The disparity between values given for strong N_2-fixers in this study as compared to others may be partially due to these sampling differences.

The use of the relative ureide abundance technique for assessing N_2-fixation was limited in this study by the extremely low NO_3 concentration in the stems which shifted the relative ureide content (27-55%) higher than would have been expected according to results reported in other studies (RUC, 1-33%; Izaguirre-Mayoral et al., 1992; Sicardi de Mallorca and Izaguirre-Mayoral, 1993; Medina and Izaguirre, 2004). As a result, the RUI, which takes into account the 4:1 atomic ratio of ureide to NO_3 and is the value usually compared among species, was very high for all species in this study, making the use of delineation between high and low N_2-fixers according to RUI values as reported by Izaguirre et al. (1992; RUI > 60, high; RUI < 30, low) difficult to apply to this study. The most valuable comparisons for this study appeared to be between those species that appeared to be using a relatively high or low amount of soil NO_3, as distinguished by RNC values (Table 3-2).

The ^{15}N relative abundance technique is most useful in field studies with maturing plants, with adequate sample numbers of legumes, and with a verified non-N_2-fixing

species as a reference plant. In this controlled study, ORLU was selected as a reference species using corroborative methods to determine that it was non-N_2-fixing and could represent soil $\delta^{15}N$ uptake values. The strong $\delta^{15}N$ signature of CLMA is not easily understood in that this species had one of the lowest nitrogenase activities across the season. CLMA also had one of the lowest RNC indices, which would indicate that it was using less soil-N than some of the other species. It is possible that ephemeral periods of peak nitrogenase activity occurred within the three-week intervals between acetylene reduction assays, and it is also possible that since this species had very small biomass (Chapter 2), low levels of N_2-fixation could have a relatively stronger influence over the $\delta^{15}N$ value than the same levels of N_2-fixation in a larger plant. It appears possible the consistent, low levels of nitrogenase activity could have a strong influence on the $\delta^{15}N$ values of CRRO and TEVI, as well (Figures 3-1, 3-3).

Total N does not follow the same pattern as percent N because it also incorporates the total biomass of the plant which varied dramatically among the species (CLMA, 9 g to MIQU, 40 g). This measure follows the pattern of aboveground biomass accumulation very closely (Chapter 2). Although not completely applicable as a comparative measure of N_2-fixation, an estimate of total N for each of these species can be very useful for considering which should be included in restoration plantings. Those with the greatest biomass and tissue-N overall will contribute the most to building the N-pool, soil organic matter, and N availability in depleted soils (Markewitz et al., 2002). This estimate is also useful in determining which of the species could provide the greatest amount of N-rich material available for wildlife to browse (Hendricks and Boring, 1999).

This study points out some differences in N_2-fixation capabilities among the species of legumes native to the longleaf-wiregrass woodland ecosystem, as well as differences in results that can occur from using various assessment methods. The merits and problems associated with using a variety of methods to assess N_2-fixation in this controlled study were most evident when comparing results including some immature, small plants. Evidence from shorter-time-based measures such as the acetylene reduction assay and N-transport product analysis did not always corroborate definitively with cumulative measures for these small plants. Cumulative measures (total N, $\delta^{15}N$ approaches) may hold more insight for older, established perennial plants, especially field populations. Nodules are ephemeral and N_2-fixation rates will change diurnally and seasonally according to environmental conditions. Instantaneous methods may not be the most appropriate measures to estimate actual or maximum potential N_2-fixation, but can serve as a quick and inexpensive way to determine seasonal and diurnal-dependent patterns of nitrogenase activity. Instantaneous measures may be best used to document age of effective symbiosis and to define development of peak nitrogenase activity when the plant has accumulated effective leaf area, and to examine detailed responses of mature plants to varying environmental conditions and stresses (Dreyfus et al., 1988; Sprent, 1999; Vitousek at al., 2002).

When conducting an ecosystem field study, it is often important to use the least-destructive method possible as well as cumulative measures over long periods of time. For perennial species, especially in frequently-burned ecosystems, removing the aboveground portion of the plant does not totally remove the individual from the ecosystem, is similar to burning, and is much less destructive than the soil disturbance

caused by digging for roots and nodules. Additional error can also be introduced due to the fact that full root and nodule recovery is never assured in field conditions. Thus, methods utilizing aboveground plant tissues for N-transport product analysis, total N content and $\delta^{15}N$ natural abundance may be highly effective for use in long-term field studies when soil available N is decisively different from the atmospheric isotopic value (Virginia et al., 1989; Hendricks and Boring, 1999).

Species Differences

Species effects were significant for most of the N_2-fixation assessment techniques used in this study. This strong species effect echoes the great diversity of growth forms and life histories among the species in this study. Each of the three major subfamilies of *Leguminoseae* were represented, *Caesalpiniodeae*, *Papilionoideae*, and *Mimosoideae*. Two very different growth forms were also represented: spreading to vining plants (CRRO, CEVI, CLMA, and MIQU) and erect forbs (ORLU, CANI, LEHI, TEVI, and RHRE). LEHI becomes semi-woody by maturation at the end of a growing season. All of the species, except for CANI, in this study were perennial, as is common for species native to fire-adapted ecosystems (Morgan, 1999; Jacobs and Schloeder, 2002).

Rates of nitrogenase activity (C_2H_4 reduction) increased toward the end of the growing season, when most of the plants had accumulated substantial photosynthetic tissues, and had formed active nodules for N_2-fixation. CEVI and MIQU show exceptional nodule activity levels as compared to other legumes in the longleaf-wiregrass ecosystem, large woody legumes and actinorhyzal N_2-fixers (Table 3-3). In contrast, the other native legumes in this study showed much lower nodule activity levels than the two leading species, however, they are still within the range represented by other N_2-fixers. The differences in the groups of native species in this study likely indicate that the large

vines, CEVI and MIQU, reached a level of maturity and nodule development that some of the other species were not able to reach in an initial growing season. The pattern of acceleration in nitrogenase activity toward the end of the season might begin earlier, and be more rapid with older field populations of perennial plants that would readily establish photosynthetic tissues using carbon and N stored belowground from the previous year (Hendricks and Boring, 1999).

The two dominant constituents of the stem extracted-N, ureide and α-amino-acids, appear to have an inverse relationship for the species in this study (Table 3-2). The relationship between these two constituents is different from other studies where NO_3, was a much larger component (3.5 to 35.9%, Sicardi de Mallorca and Izaguirre-Mayoral, 1993; 18 to 70%, Peoples et al., 1996). Those studies had an inverse relationship between RUC and RNC. In this study, RUC and RAC were not significantly different among species, however, patterns among species according to RNC did show statistical differences, and it was elevated for those species with relatively low N_2-fixation capabilities as suggested by other corroborative methods. Those species with the highest NO_3 concentration, although very low compared to values in the other studies (3.5 versus 61.1%), were also among those with the lowest percent N and $\delta^{15}N$ values in aboveground tissues in this study indicating lower N_2-fixation. Sicardi de Mallorca and Izaguirre-Mayoral (1993) and Izaguirre-Mayoral et al. (1992) found a similar pattern when comparing low- to high-N_2-fixers. RNC is likely dependent upon soil NO_3 availability, which is very low under the typical field conditions where these native legumes are found (Wilson et al., 1999), and RNC values sampled in the native woodland could potentially be even lower than those reported in the current study.

The species examined in this study were again divided into two main groups of fixation activity using $\delta^{15}N$ values (high: CLMA≥CRRO≥TEVI≥CEVI≥MIQU; low: LEHI≥CANI≥RHRE≥ORLU), with CLMA indicating the least depletion of ^{15}N (Figure 3-3). Other reported $\delta^{15}N$ values for LEHI and CANI also indicate that these two species are relatively lower N_2-fixers (Hendricks and Boring, 1999). LEHI was not statistically different from the non-fixing reference in a study conducted by Hendricks and Boring (1999). The annual CANI was also identified as a low-fixer by the cumulative, $\delta^{15}N$ abundance assessment (Figure 3-3). This annual species would have relied on more soil-N uptake to become established than perennial species that were planted at the beginning of the growing season, and the RNC value in Table 3-2 indicated that it may be the species with the greatest rate of NO_3 uptake.

Percent N_{dfa} of the species in this study (12-55%; Figure 3-4A) were lower than values reported by Hendricks and Boring (1999) for other species of mature legumes from a similar ecosystem (54-88%). Species order according to % N_{dfa} is similar to %N, with the exception of MIQU, which had a large concentration of N, but a lower $\%N_{dfa}$.

Aboveground percent N also divided the species in this study into two categories of potential high- and low N_2-fixation, those species with >2% N (MIQU, CANI and CLMA) and those with <2% (CEVI, CRRO, TEVI, ORLU, LEHI and RHRE). Potential N_2-fixation rates indicated by %N agree with the acetylene reduction assay and $\delta^{15}N$ techniques for MIQU (high) and LEHI (low). However, comparatively large amounts of NO_3 uptake by CANI and CLMA compared to other species indicate that high levels of N_2-fixation are not related to high N concentrations in the tissues of these species. TEVI, which had lower percent tissue N in this study than that reported for Hiers et al. (2003),

also failed to flower within the growing season examined here (Chapter 2). TEVI has been shown to respond to fire with increased flower production (Hiers et al., 2000) and a significant elevation in percent N (Hendricks and et al., 2003). Thus, for this study, TEVI was probably lacking the maturity and perhaps stimulation by fire and subsequent phosphorus enrichment needed for maximum N_2-fixation to be detected (Christensen, 1977, Gholz et al., 1985).

Summary

Estimates of N_2-fixation capabilities of native legumes in this study were most conclusive for the two rapidly growing and apparently quickly maturing vine species CEVI and MIQU. These two species showed strong indices of fixation by both instantaneous and cumulative measures. The annual CANI and perennial LEHI accreted large amounts of N in biomass, but apparently from higher NO_3 uptake and low rates of N_2 fixation. The data were less conclusive for the remaining, slower-growing, smaller species that accreted N more slowly and had variable indices of lower but significant rates of fixation among the methods of assessment. However, these species represent only a small sample of the 40 species of legumes present in the longleaf wiregrass ecosystem, which likely also have varying capabilities for N_2-fixation (Hainds et al., 1999). For example, other large, semi-woody legume species of the genera *Lespedeza* and *Desmodium* within this ecosystem have shown higher potential for N_2-fixation than LEHI and should be investigated further (Hendricks and Boring, 1999). With further investigation of mature plants in the field, using a combination of total N content, $\delta^{15}N$, and N transport product analysis would provide for effective delineation of N_2-fixation for these species and others. Such an approach may provide more information to

facilitate using native legumes in restoration plantings for improving soil characteristics, wildlife habitat and forest productivity.

CHAPTER 4
GROWTH AND N_2-FIXATION OF NATIVE LEGUMES IN LONGLEAF PINE RESTORATION

Introduction

Conservative estimates of nitrogen inputs from biological fixation by native legume populations in pine woodland and grassland ecosystems have been grossly estimated from 5.2 to 9 kg N ha^{-1} yr^{-1} (Ojima et al., 1990; Hendricks and Boring, 1999). Native legumes are often found in high density populations across highly-variable light and water regimes in the longleaf pine- (*Pinus palustris* Michx.) wiregrass (*Aristida stricta* Mill.) ecosystem of Southeastern North America ranging from xeric sandhills to wet-mesic sites to edges of depressional wetlands (Hainds et al., 1999). However, species of legumes native to this ecosystem have been reported to have large variability in symbiotic N_2-fixation. Many factors may control N_2-fixation rates in woodland ecosystems, including water and nutrient availability, rhizobium populations, and especially light, given the requirement for large energetic costs to drive symbiotic N_2-fixation (Sprent, 1987; Vitousek et al., 2002).

Recent restoration initiatives on private and public lands in the southeastern U.S. coastal plain have resulted in the planting of approximately 283,000ha of former agricultural, pulpwood plantation and fire suppressed land back into longleaf pine stands through the USDA Conservation Reserve Program since 1996 (CRP; Coffey and Kirkman, 2004). These young longleaf stands grow vigorously on sandy, carbon- and N-depleted former agricultural sites (Markewitz et al., 2002).

Restoration of groundcover in young, planted longleaf pine stands is important for rebuilding soil organic matter and N, for providing wildlife food and cover, and for enhancing pyrrhic fuel continuity necessary to reintroduce frequent prescribed fires. Native grasses and legumes should be preferred over exotics for reintroduction, as has been made evident by the invasive nature of two species, *Lespedeza bicolor*, Turcz. and *L. cuneata* (Dum. –Cours.) G. Don, that have been previously introduced for soil improvement and wildlife forage (Miller, 2003). Field research is needed to help determine desirable native species for reintroduction on targeted sites. There are also issues related to the timing of groundcover species introduction in young pine stands, due to the large differences in light transmittance through developing longleaf canopies between planting and maturation, and following thinning operations associated with silvicultural practices (Mulligan and Kirkman, 2002).

The objectives of this study were to test for the differences in biomass accumulation and distribution, N-content, and N_2-fixation potential of six legume species planted in three levels of light under longleaf pine canopies. This field test is an important step in understanding growth and N_2-fixation of native forest legumes under shaded conditions.

Materials and Methods

Site Description

This common garden study was conducted at Ichauway, a property managed by the Joseph W. Jones Ecological Research Center (JWJERC), a 12,500 ha reserve located in Baker County, Georgia, USA (31^{o}19'N and 80^{o}20'W). The climate for this region is humid subtropical. Mean daily temperature during the study (10 May-7 November 1004) was 26.9^{o}C, and cumulative rainfall was 595mm. Although most of Ichauway lands were

under longleaf pine savanna when they were acquired in the mid 1930's, some of the land has been cultivated. Over the years, cultivated areas have been plowed, fertilized with N, P and K, and planted to crops including cotton (*Gossypium* spp.) and sorghum (*Sorghum* spp.) (Markewitz et al., 2002). The soil at the site was a fine-loamy, kaolinitic, thermic Typic Kandiudult (Norfolk Series).

The 14 year-old longleaf stand used in this study was planted on a formerly cultivated area according to CRP stocking recommendations of 1,235 trees ha^{-1}. Three canopy opening levels were initially located using a densiometer. Plots representing intermediate light levels (an average of 48% openness) were established in locations where a single tree had previously died, probably due to fire scorching or insect damage, leaving a small gap in the canopy. Closed canopy plots (an average of 9% openness) were located near the intermediate plots in an area with no missing trees. Open canopy plots were established at the edges of the stand. Trees within approximately 1 m of the open plots (3 trees per plot) were removed in order to achieve desired openness, and limbs below 2m height were removed adjacent to all plots for ease of access.

Canopy openness was more accurately quantified using a line quantum sensor on a clear, cloudless day (Li-Cor, Inc., Lincoln, Nebraska). Five light readings, taken after pruning and tree removal, were made on 5 May 2005 within each plot and in an adjacent open field between 12:39 and 15:13 EST. The proportion of light present in plots versus the open field, expressed as photosynthetically active radiation μmol $m^{-2}s^{-1}$ (PAR) was used to describe canopy openness.

Experimental Design and Planting

Four replicated 9 m^2 plots were arranged according to a completely random statistical design under the three light environments. Each plot was planted with five

plants from eight different species of 7 month-old native legumes: *Centrosema virginianum* (L.) Benth. (CEVI), *Desmodium ciliare* (Muhl. ex Willd.) DC. (DECI), *Lespedeza angustifolia* (Pursh.) Ell. (LEAN), *Lespedeza hirta* (L.) Hornem. (LEHI), *Mimosa quadrivalvis* (L.), *Orbexillum lupinelus* (Michx.) Isley (ORLU), *Pediomelum canescens* (Michx.) Rydb., and *Tephrosia virginiana* (L.) Pers (TEVI). Nomenclature follows Wunderlin and Hansen (2003). Seedlings were propagated at the JWJERC by Dr. K. Kirkman as described in Chapters 2 and 3. Plants were randomly arranged in rows that were spaced 60 cm apart, with 30 cm spacing between plants. Planting was completed 10 May 2004. Weeds were suppressed by mulching the plots with on-site pine straw and supplemented with more from the adjacent area.

Time Domain Reflectometry (TDR) rods were used to monitor soil moisture. 30- and 90-cm TDR rods were placed in the corners of each plot, and readings were taken every two weeks. Volumetric soil moisture for each plot across the season did not indicate any occurrence of severe drought conditions, nor did the plants undergo any periods of defoliation. Soil moisture was not significantly different among individual plots or between light treatments. Due to a dry period around the time of planting, plants were hand watered to aid root establishment (Figure 4-1). Approximately 1L was applied to each plant on a bi-weekly basis between 18 and 30 May, and once on 11 June.

Plants were destructively harvested from each plot at the end of the growing season, between 25 October and 7 November, 2004. Plant heights, except for CEVI, were measured from soil surface to the top of the stem before collection. Approximately 6 L of soil were removed with each plant by digging at a 10 cm radius around each stem

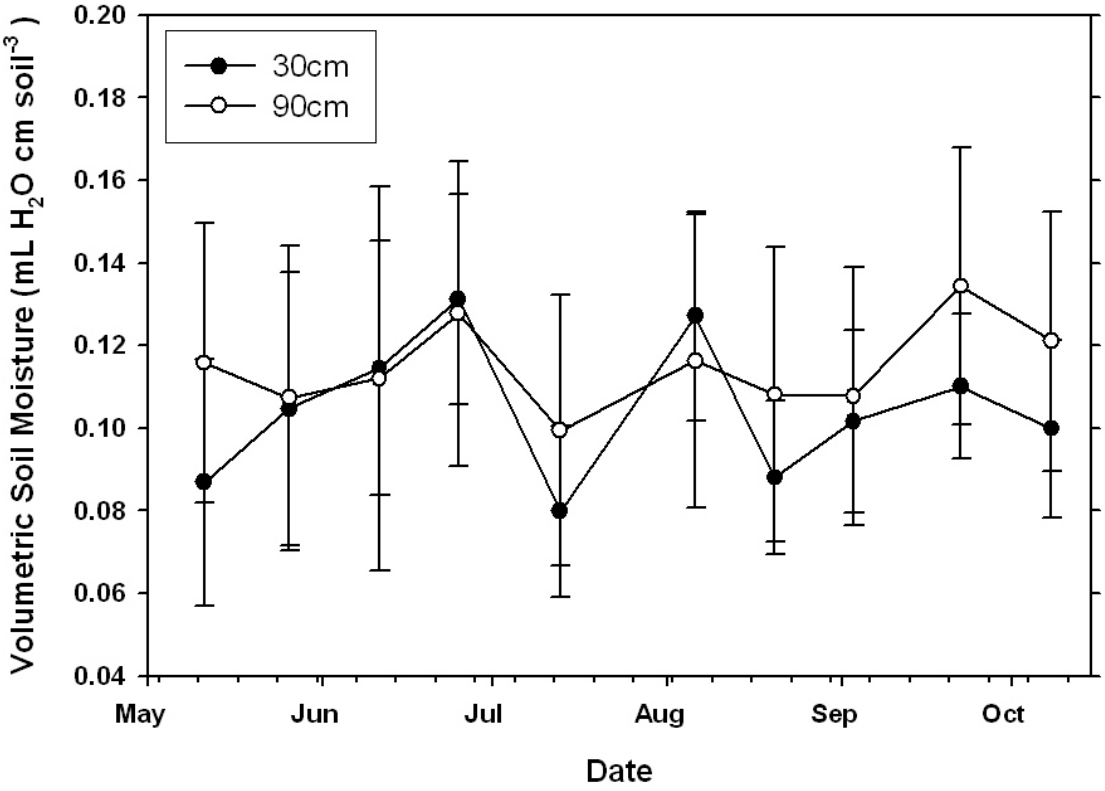

Figure 4-1. Volumetric soil moisture patterns for all plots. Readings were taken every two weeks between 11 May and 8 October, 2004. Data shown are means ± SE

to a depth of approximately 20 cm. Roots and nodules were taken to a field lab, washed free of soil, and collected. Leaves, stems, roots and nodules were separated, dried to constant weight at 70^{o}C and weighed. Due to the extremely small size of the plants in the closed light environments, the plant parts were weighed and recorded, but no further analysis was conducted on this set of samples (Figure 4-2). Leaves and stems from the plants harvested in the open light environment plots were composited and ground for further analysis. Plants harvested from the intermediate light environment were separated into leaves, stems and roots and ground for further analysis. Plants in this light treatment were also used for a related retranslocation study. Longleaf pine needles were collected from mid-canopy from trees near the experimental plots for use as a non-N_2-fixing reference for assessment by δ^{15}N. Needles were prepared for analysis in the same manner as the legume tissues.

The usefulness of the ^{15}N natural abundance technique for assessing N_2-fixation under field conditions has been verified through numerous agricultural and controlled studies. However, the utility of this technique in the field is dependent on comparison of δ^{15}N values of a non-N_2-fixing reference plant, to represents the ratios of isotopic nitrogen forms present in the soil with those of the legumes. Ground tissues from legumes and pine needles were analyzed for δ^{15}N natural abundance and total N content at the University of California, Davis (Stable Isotope Facility, Department of Agronomy, Davis, CA) using mass spectrometry.

Leaf and stem δ^{15}N values of plants harvested from the intermediate light environment were composited so that comparisons with the aboveground (leaf + stem) δ^{15}N values from the open light environment could be made. CEVI and ORLU did not

produce enough samples for this analysis. A weighted $\delta^{15}N$ value for the plants from the intermediate plots was calculated:

$$\delta^{15}N_{aboveground} = [(\delta^{15}N_{stem} \times \text{Total } N_{stem}) + (\delta^{15}N_{leaves} \times \text{Total } N_{leaves})] / (\text{Total } N_{stem} + \text{Total } N_{leaves}) \quad [4\text{-}1].$$

Using aboveground tissue values of $\delta^{15}N$ from intermediate and open light treatments, an estimate of percent N derived from the atmosphere ($\%N_{dfa}$) was calculated:

$$\% N_{dfa} = 1 - (\delta^{15}N_{N2\text{-}fixing\ plant} / \delta^{15}N_{ref}) \quad [4\text{-}2].$$

Statistical Analysis

Data were analyzed using two-way analysis of variance (ANOVA), with species and light environment as main effects. Where significant effects existed ($p<0.05$), Duncan's multiple comparison post-test was used to determine which means differed significantly. The GLM procedure performed in the Statistical Analysis System (SAS, 2003) was used for ANOVA and post-tests.

Results

Preliminary Results and Survivorship

PAR readings in the open field adjacent to the study site were 1938 ± 10.6 μmol $m^{-2}s^{-1}$, and plot readings were 370 ± 45, 1214 ± 103, and 1539 ± 61 for closed, intermediate and open plots, respectively. Canopy openness for each plot type, expressed as a fraction of adjacent field light levels were 0.085, 0.614, and 0.834 for closed, intermediate, and open, respectively. Differences in percent canopy openness among plot types were highly significant ($P < 0.0001$).

There was an average survival rate of 29% across species where LEHI > TEVI > DECI > LEAN > ORLU. CEVI had the lowest survival of only 15%. An average sample size of five for each species in each light environment remained except for CEVI

in the intermediate light environment (n=1). *M. quadrivalvis* and *P. canescens* were excluded from further analysis in this study due to poor growth and survivorship. *M. quadrivalvis* showed very little change in size at mid-season and final harvest date, therefore it was not harvested. Other field observations of *M. quadrivalvis* suggest that biomass accumulation may have been directed predominantly belowground. Taproots of maturing *M. quadrivalvis* in the field can be as long as 3m (personal observation). As in a companion study, *P. canescens* developed brown spots, defoliated and appeared to be dead by mid-season (Chapters 2 and 3).

Plants grown in the closed light environment accumulated very little biomass over the season as compared to plants grown in the other light environments (Figure 4-2). Due to the small growth response of plants in the closed light environment, plants harvested from this set of plots were not analyzed for N content nor assessed for N_2-fixation.

Growth

Total aboveground biomass showed significantly different species and light treatment effects (Table 4-1, 2). Light effect on biomass was Open > Intermediate > Closed. However, CEVI growth showed no statistical differences between open and closed treatments and was not adequately represented for intermediate. LEAN did not have significantly different greater biomass in the open environment versus the intermediate, but both were greater than the closed. In the open light treatment, the biomass was DECI > CEVI ≥ LEHI ≥ TEVI ≥ LEAN ≥ ORLU; only DECI had a significantly greater biomass than any other species (Figure 4-2). Biomass differences by species were not statistically different in the intermediate or closed light treatments.

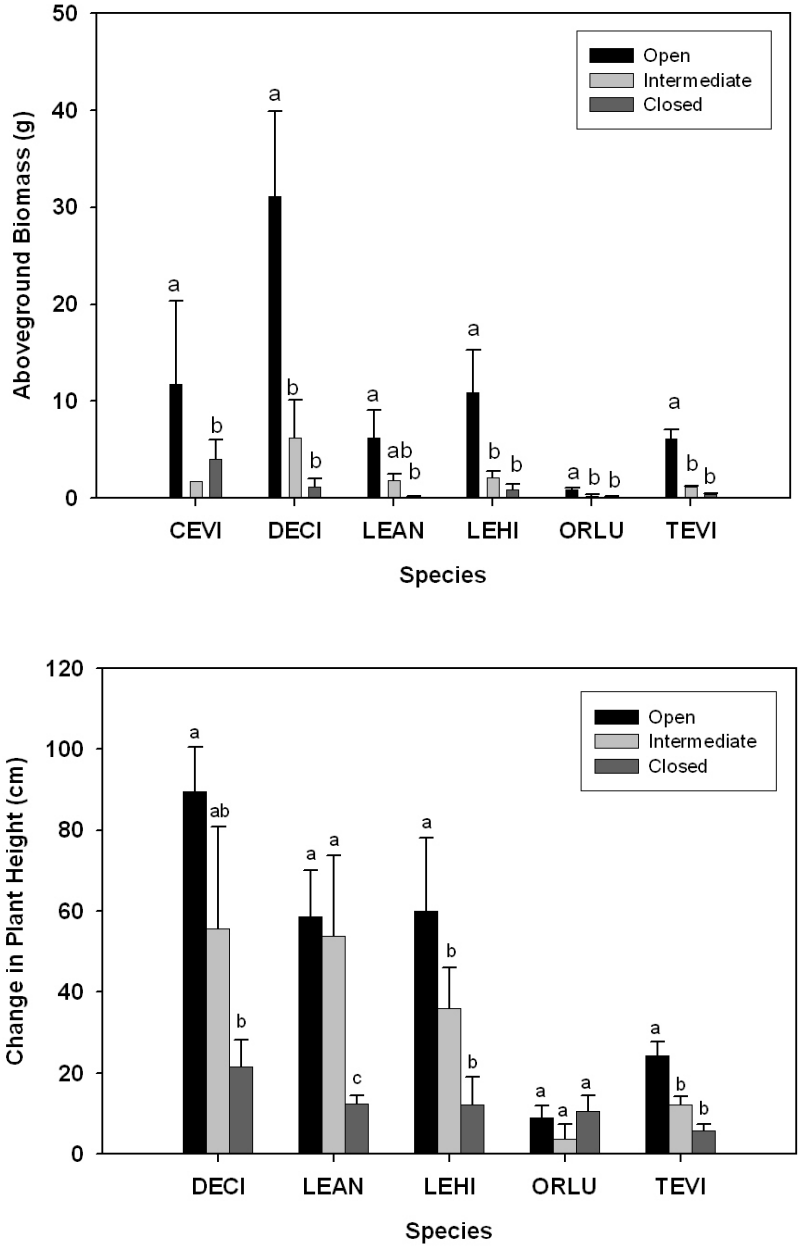

Figure 4-2. Aboveground biomass and change in plant heights from T_0 by species in each of the three light treatments. Data shown are means ± SE, and different letters within a species represent statistically different means (Duncan's post-test). Only one plant was harvested for CEVI in the intermediate light environment.

Table 4-1. Analysis of variance results for experimental variables. Light treatment and species were main effects. Values for total aboveground biomass (Total AB Biomass), root-to-shoot ratio (R/S) and nodule biomass (nodulation) represent differences across all three light treatments. Results for %N, $\delta^{15}N$, Total N, and %Ndfa represent differences among open and intermediate light treatments for aboveground tissues.

	Main Effects		
Variables	***Species***	***Light***	***Species x Light***
Total AB Biomass (g)	0.0094	<0.0001	0.0292
R/S	0.2247	<0.0001	0.2652
Nodulation (g)	0.0149	0.0023	0.5694
%N	<0.0001	0.0002	0.0421
$\delta^{15}N$	<0.0001	<0.0001	0.9063

Table 4-2. Total biomass (above- and belowground tissues, including nodules) per plant and aboveground values for %N, $\delta^{15}N$, and total N. Different letters within each column represent statistically different means.

Closed Light Treatment						
Species	***Total Biomass (g plant^{-1})***	***R/S***	***Nodule biomass (mg plant^{-1})***			
CEVI	5.38	0.51	98.2			
DECI	2.06	1.93	11.22			
LEAN	0.52	2.26	6.22			
LEHI	1.64	1.79	11.03			
ORLU	0.47	1.83	3.74			
Intermediate Light Treatment						
Species	***Total Biomass (g plant^{-1})***	***R/S***	***Nodule biomass (mg plant^{-1})***	***%N***	***$\delta^{15}N$***	***Total N (g plant^{-1})***
CEVI	2.50	0.46	22.80	-	-	-
DECI	7.43	0.90	9.93	1.82 b	-3.06	0.05
LEAN	2.86	1.07	13.42	1.68 b	-2.59	0.03
LEHI	3.29	0.76	17.23	1.96 b	-2.80	0.05
ORLU	0.67	1.65	15.40	-	-	-
TEVI	2.25	0.91	29.08	2.79 a	-2.20	0.03
Open Light Treatment						
Species	***Total Biomass (g plant^{-1})***	***R/S***	***Nodule biomass (mg plant^{-1})***	***%N***	***$\delta^{15}N$***	***Total N (g plant^{-1})***
CEVI	15.59 b	0.89	131.50 a	-	-	-
DECI	37.27 a	0.29	74.63 ab	1.33 b	-1.06	0.40 a
LEAN	8.60 b	0.55	37.57 b	1.76 b	-0.67	0.16 b
LEHI	13.53 b	0.54	18.77 b	1.21 b	-1.25	0.13 b
ORLU	1.82 b	1.16	16.00 b	-	-	-
TEVI	9.65 b	0.58	55.96 b	2.33 a	-0.46	0.15 b

Plants grown in the closed light environment were the shortest, overall. The lack of height growth is apparent when the heights of the plants at harvest are compared with the heights of the plants at time of planting (T_0). The largest change in height from T_0 was found in the open light environment, and the difference between these plant heights and those in the other treatments were significant overall, with few exceptions for individual species (Figure 4-2).

Root-to-shoot ratios (R/S) were also significantly different by light environment, but species effect was not significant (Table 4.1, 4.2). Most of the species showed the following pattern of significant differences in R/S across the three light environments: Closed > Intermediate > Open. However, CEVI, a vine, showed an opposite R/S pattern, Open > Closed. (The intermediate light level was not sufficiently represented.)ORLU showed no differences in R/S across light treatments, and DECI did not show a significant difference between the closed and intermediate treatments (Figure 4-3).

Nodulation, as measured by nodule biomass (mg), showed significant light treatment and species effects. Due to a large degree of variability in nodule biomass, the only species to show the significant light treatment effect was DECI ($p = 0.0532$) and followed the pattern: Open > Closed > Intermediate. General trends of nodule biomass accumulation for the other species were Open > Intermediate > Closed, although too variable to show significant differences. In all light treatments, CEVI, TEVI and DECI appeared to have the greatest nodulation (Table 4-2).

All species had a higher percentage of N concentration in aboveground tissues (stem + leaves) where grown in the intermediate light environment than in the open. Although species and treatment effects were significant for %N (Table 4-1), statistical

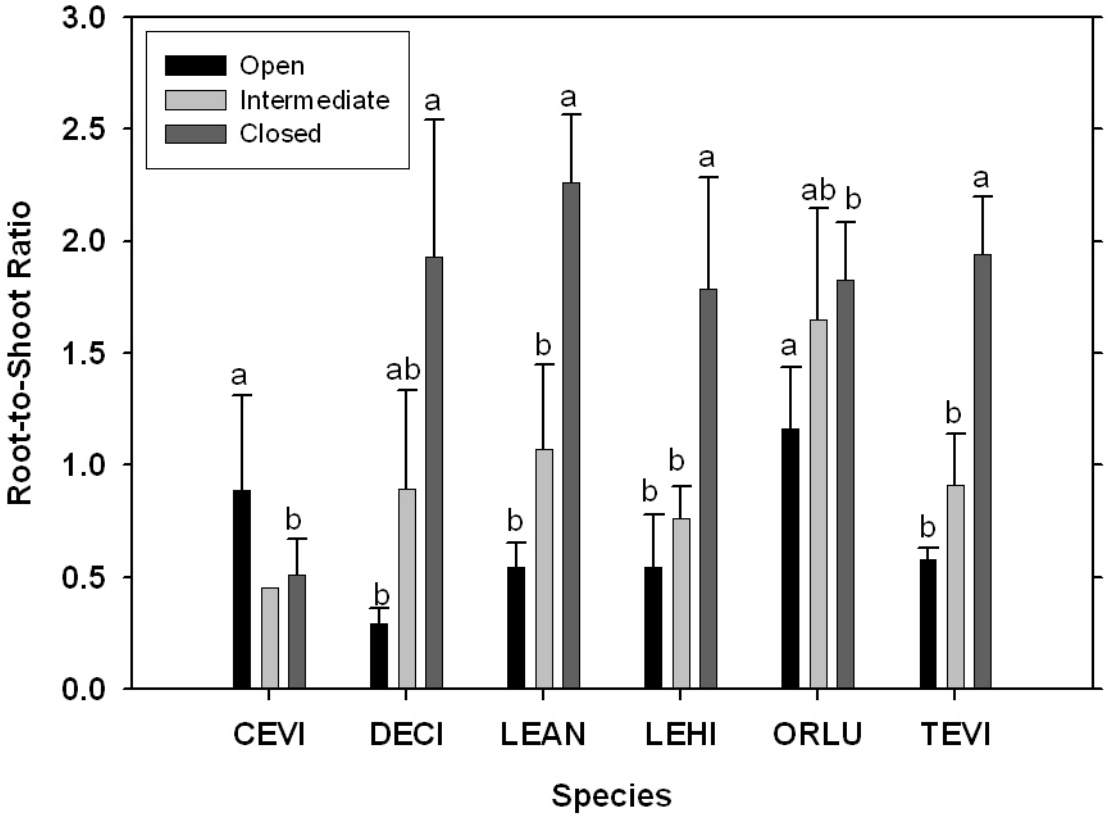

Figure 4-3. Root-to-shoot ratios by species in the three light treatments. Data shown are means ± SE, and different letters within a species represent statistical differences (Duncan's post-test). Only one plant was harvested for CEVI in the intermediate light environment.

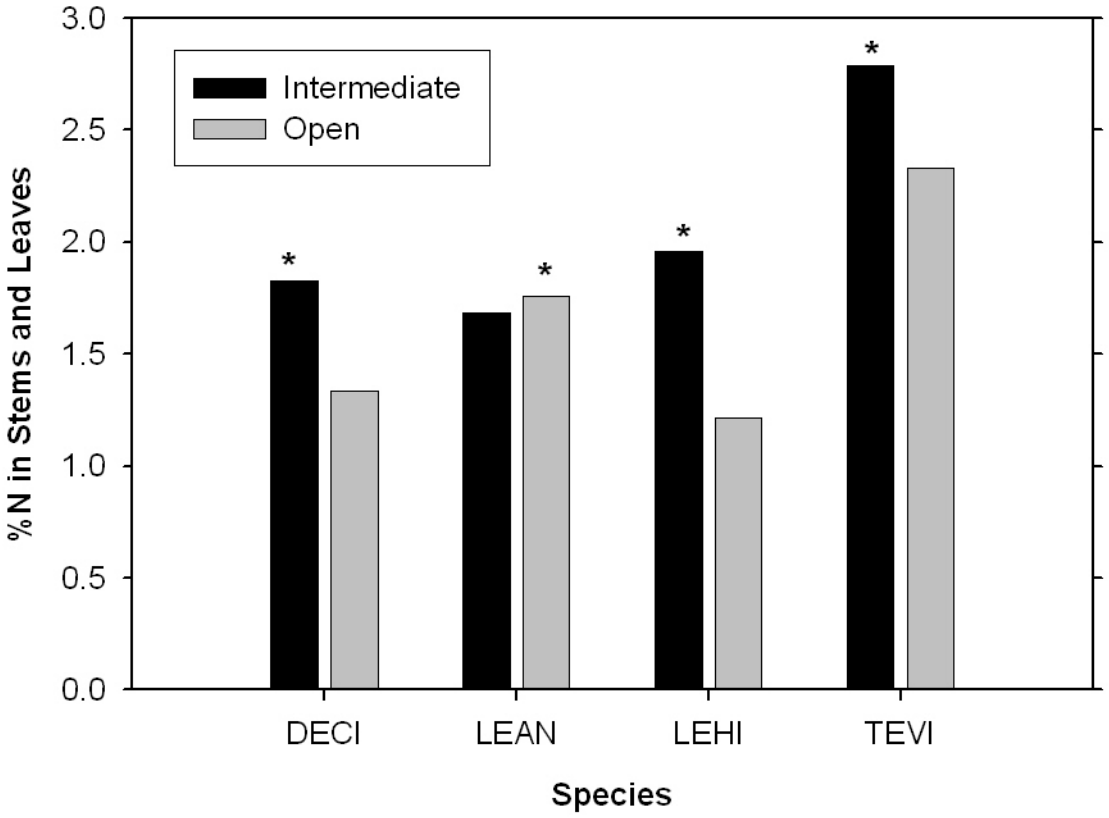

Figure 4-4. Percent N concentration in aboveground biomass (stem + leaves) by species in the intermediate and open light environments. Plants grown in the closed light environment were not analyzed for percent N. Data shown are means of four plots for each treatment. Statistically greater %N is indicated by * (Duncan's post-test).

differences in the two light treatments were only represented by DECI ($p = 0.0350$) and LEHI ($p = 0.0002$).

In both light treatments, TEVI had a statistically higher %N than the rest of the species followed by LEHI > DECI > LEAN for the intermediate treatment and LEAN > DECI > LEHI for the open treatment (Figure 4-4).

N_2-Fixation

Total N in aboveground tissues was significantly affected by light treatment (Table 4-2). However, this difference was only represented by DECI and TEVI, where plants grown in the open light treatment had statistically greater total N than those grown in the intermediate. Differences among species in the intermediate light environment were not statistically different, but in the open light environment they were ranked: DECI > LEAN ≥ TEVI ≥ LEHI.

Legume $\delta^{15}N$ values were not statistically different (Figure 4-5) from the non-N_2 fixing pine needle reference values in the intermediate light environment ($p < 0.1419$), but showed a significant difference from the reference in the open ($p < 0.0014$). $\delta^{15}N$ values of legumes were statistically affected by light environment ($p < 0.0001$), and this difference was represented in all of the species. For all legume species in this study, $\delta^{15}N$ values indicated that ^{15}N in aboveground tissues was less depleted (closest to atmospheric value) in those plants grown in the open light treatment than in the intermediate ($p = 0.0051 \pm 0.0044$).

Discussion

Shading Effects on Species

Plant growth was significantly greater, overall, in the open light environment than in the intermediate or closed light environments. The reduced level of PAR in the

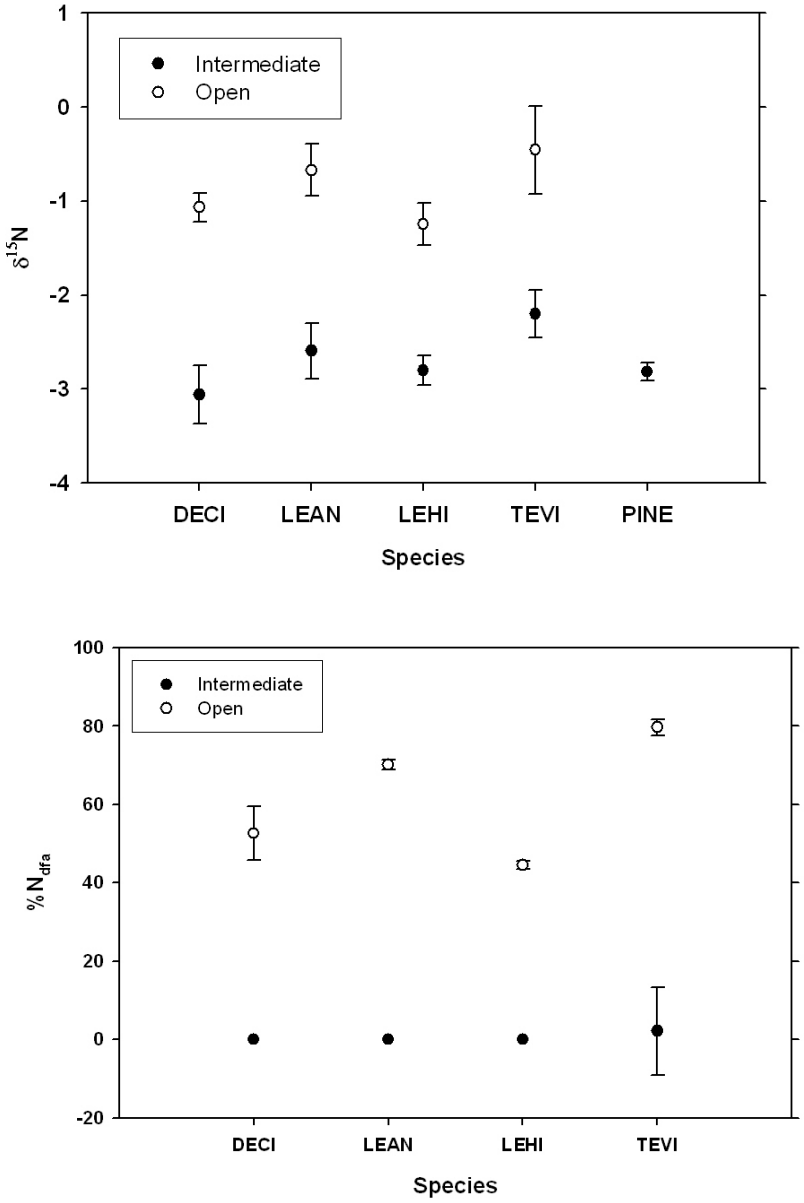

Figure 4-5. $\delta^{15}N$ and $\%N_{dfa}$ values by species for aboveground tissues in the intermediate and open light treatments. Data shown are means ± SE. Plants grown in the closed light environment were not analyzed for $\delta^{15}N$. Pine needles collected from adjacent to the plots were used as the non-fixing reference. $\%N_{dfa}$ was calculated using Equation 4-2.

intermediate (61.4% of incident) and closed (8.5% of incident) light environments compared to that of the open (83.4% of incident) had significant impact on growth and N_2-fixation rates of the legume species in this study. The change in height between T_0 and harvest for the species in this study may be due to etiolation and/or actual differences in growth. Lesser differences in elongation between intermediate and closed treatments for LEHI and TEVI may be more indicative of etiolation than growth, since biomass was not different between treatments for these species. DECI and LEAN, which did not show differences between plant heights in open and intermediate treatments, suggest that they were less etiolated. LEAN, which had smaller biomass accumulation differences among treatments showed further evidence that the differences in height between the intermediate and closed treatments reflected actual growth differences rather than etiolation.

There were significant differences in R/S between closed and open treatments for all of the species in this study, suggesting a stronger allocation of carbon to the roots in low light. Greater allocation to roots due to a variety of environmental stressors, including shade, drought and fire is a well-documented physiological response (Sprent, 1973; Knapp et al., 1998; Paz, 2003; Fernández et al., 2004) Allocation to roots may also be a response to increased competition from pine roots (Mulligan and Kirkman, 2002) and is a common adaptation to low-fertility soils (Paz, 2003). In the case of these legumes, a decreased rate of N_2-fixation due to shading, and a subsequent increased reliance on soil sources of N could lead to a greater allocation of biomass to roots as the plants forage and compete for N resources.

Smaller, non-significant differences between the R/S of plants grown in the open versus intermediate light environments may indicate that the threshold light level needed to initiate these allocation changes exist somewhere between the intermediate and closed light levels used in this study. R/S reported for CEVI, ORLU and TEVI in this study (0.89, open; 1.83, intermediate; and 0.91, intermediate, respectively) were much lower than those reported in a controlled, potted study (approximately 1.5, 2.7, and 3 for CEVI, ORLU and TEVI, respectively; Chapter 3). R/S of LEHI in the controlled study (0.8) was similar to the R/S in the intermediate light environment in the current study (0.76). Sampling differences are the most obvious cause for many of these differences, especially since CEVI and TEVI, have extensive, lateral branching root systems that were not harvested in their entirety in this study, but that would have been contained in the pots of the controlled study.

Dramatically different levels of N_2-fixation (as assessed by $\delta^{15}N$) in spite of a lack of difference in nodulation between the open and intermediate light environments (Table 4-2) indicates that nodule activity rather than biomass is more indicative of N_2-fixation in these species. For example, ORLU had a comparable amount of nodulation biomass to other species in a previous, controlled study, but was determined to have relatively much lower N_2-fixation rates than the other species in the study as assessed by both the acetylene reduction and $\delta^{15}N$ natural abundance assessments (Figure 4-4; Chapter 3). However, statistical differences in nodulation between light treatments were difficult to detect because of high variability between individual plants due to sampling area in the field and variable plant sizes. Nodules that were present under shaded conditions, due to

their relative expense versus effectiveness, may be highly susceptible to being sloughed by the legumes in response to stress (Gadgil, 1971).

Legume N_2-fixation under shade conditions has rarely been assessed due to the high energetic cost of nodule maintenance and the N_2-fixation reaction (Vitousek et al., 2002). A small number of available studies do indicate that nodulation and subsequent N_2-fixation by legumes is inhibited by shading (Gadgil, 1971; Sprent,1973). $\%N_{dfa}$ for the species grown in open light conditions in this study (44.5-79.7%) are similar to values reported for similar species by Hendricks and Boring (1999; 54-88%). The agroforestry legume species *Calliandra calothyrsus* and *Sesbania sesban* also have similar reported foliar $\%N_{dfa}$ values, 65-90%, respectively (Stahl et al., 2002).

Percent N concentration in leaf and stem tissues was greater in the intermediate light environment than in the open. The increased concentration of N in the shaded leaves may reflect the reduced amount of carbon assimilation occurring under lower light conditions, which is corroborated by the opposite pattern of N accumulation in the open light treatment. Sun leaves tend to be thicker and contain larger amounts of connective and suberized tissue leading to a higher lower N concentration than shade leaves (Kramer and Kozlowski, 1979). However, the larger overall size of the plants in the open light environment versus the intermediate explains a higher total N value for open-grown versus more shaded plants. Aboveground %N reported in this study for the large, semi-woody, LEHI, LEAN and DECI (1.21 – 1.76%) and for the less-woody, TEVI (2.33 ± 0.17%) in this study are within the same range as previously reported leaf %N levels for similar, semi-woody (1.6 – 2.3%) and smaller herbaceous legumes (2.4%; Hendricks and Boring 1992).

Ecological and Management Implications

Survivorship was relatively low in this study, across all species. One possible explanation for the plants poor survival is the time of transplanting. The small seedlings were planted in the spring when temperature, photosynthetic rates and associated transpiration rates were high. A similar planting of legumes conducted in the fall when temperatures and associated plant processes would have been lowered had a much higher survival rate (Kirkman, unpublished data). Based on this observation, transplantation should be conducted in the fall.

$\delta^{15}N$ values indicated a high level of N_2-fixation by most of these legume species in the open light environment. Differences between calculated percent N_{dfa} for the legumes (44-79%; Figure 4-5) were slightly lower than those reported by Hendricks and Boring (1999) from an older population of similar species in pine woodlands (54-88%). Both studies indicate potentially high growth and N_2-fixation rates under relatively open canopy conditions. However, many questions relating to ineffective nodulation and species survivorship highlight the need to examine other environmental and biotic controls on legume N_2-fixation, especially drought stress and rhizobium microsymbionts in older established field populations (Dreyfus et al.,1988).

Additionally, further field studies should examine the continued development of maturing legumes as this study only followed the first year of establishment. Further observations of remaining planted legumes from this study and from another continuing study under the 14 year-old longleaf pine plantation indicate that some of the species that were excluded from this study did not die, but did not establish during the first growing season after planting. Specifically *Mimosa quadrivalvis*, *Orbexillum lupinellus* and

Pediomelum canescens showed significant growth and maturation during the second year after planting (Kirkman, unpublished data).

The native legumes planted under the closed canopy of this 14 year-old longleaf pine plantation showed very little capacity for growth, N_2-fixation, or nitrogen accretion. Dense litter accumulation under this closed canopy can also heavily suppress the growth of other groundcover species, including wiregrass or Rubus sp. (blackberry) (Mulligan and Kirkman, 2002). However, mulching with pine litter could be an important tool to selectively encourage establishment of specific groundcover species under more open canopy conditions.

Growth and N_2-fixation of the legumes increased dramatically with increased canopy opening, advancing among the closed (8.5% of incident light), intermediate (61.4% of incident) and open (83.4% of incident) treatments. This pattern suggests that substantial legume populations, from the perspectives of wildlife food production and cover, and N contribution to the soil, will not be likely to grow or persist beneath dense young pine plantations until the trees are operationally thinned to a lower density. Even the relatively low stocking of pines in these plantations intended for enhancing wildlife habitat results in dense canopy development and little capacity for supporting native grasses and legumes. The findings from this study points to a recommendation that landowners plant legumes beneath plantations that have undergone an initial thinning after about 15-20 years of tree growth. At that point, light conditions should be more favorable for legume populations to grow and contribute a significant amount of fixed-N to the N and C depleted soils that are typical on converted agricultural sites.

CHAPTER 5
CONCLUSION

Conclusions from the Current Study

Introduction

The fire-dependent longleaf pine- (*Pinus palustris* Mill.) wiregrass (*Aristida stricta*, Michx.) savanna ecosystem once dominated the southern coastal plain of the United States, covering as much as 37.2 million ha. (Landers et al., 1995). Recent restoration initiatives on private and public lands in the southeastern U.S. coastal plain have resulted in the planting of approximately 283,000ha of former agricultural, pulpwood plantation and fire suppressed land back into longleaf pine stands through the USDA Conservation Reserve Program since 1996 (CRP; Coffey and Kirkman, 2004). Groundcover reestablishment in these stands is key to improving wildlife habitat and for restoring a continuity of pyrrhic fuels for frequent prescribed burning (Clewell, 1989; Kirkman, 2002). Legumes may also have a major role in maintaining N balance of these restored systems. Shade tolerance of groundcover species to be potentially reintroduced under young pine stands needs to be assessed before large-scale operations are undertaken (Mulligan and Kirkman, 2002).

Objectives

The overall objectives of this study were (1) to explore the impact of various degrees of shading on relative growth and N_2-fixation rates of legume species native to longleaf pine-wiregrass savannas, (2) to make initial observations of phenological development and nodule morphology for each species, and (3) to examine the

effectiveness of corroborative methods for assessing N_2-fixation. These objectives have not been previously addressed for most of these species. Controlled potted studies and a common garden experiment were used to assess species responses to shading under potted and field conditions.

The species examined in this study represented all three common subfamilies of the *Leguminoseae*, *Caesalpiniodeae*, *Mimosoideae*, and *Papilionoideae,* and represented three distinct growth forms: vines, erect herbs, and semi-woody erect herbs. Nodule morphology differences further confirmed the species subfamily designations. Each species had a unique response to shade, and each one represents a slightly different life history strategy that is apparent in growth habit and phenological development (Chapter 2). Adaptations to fire, low-fertility soils, droughty conditions, and a highly variable, but relatively open overstory canopy structure are manifested in the stress-tolerating and opportunistic nature of most of these legume species (Hainds et al., 1999).

N_2 fixed by legumes, although important to many ecosystems, is difficult to quantify. Due to the specific limitations associated with each assessment technique, corroborative assessments should be made in any study that seeks to quantify fixed-N. In this study, relatively higher levels of nitrogenase activity (acetylene reduction) for a species were corroborated by reduced soil NO_3 utilization, $\delta^{15}N$ signatures nearer atmospheric values, and higher tissue-N concentrations (Chapter 3). Even in a potted study with complete nodule recovery, nodule biomass was not a good predictor of N_2-fixing ability, since only one species had significantly different biomass from all of the others.

N_2-fixing capabilities were species dependent. Some of the species in the potted study did not appear to have fully developed during this short-term study. For example, *Tephrosia virginiana*, which was indicated as one of the strongest N_2-fixers in the common garden study (Chapter 4), did not reach a reproductive stage, nor was it very active in N_2-fixation in the potted study (Chapter 3). Additionally, *Orbexillum lupinellus*, which appeared to be well-nodulated in the potted study, was not active in N_2-fixation. This ineffective nodulation may indicate a slowly-developing plant, or it may indicate that the preferred symbiont for this species was not present (Chapter 3).

Field studies following more controlled studies can confirm initial findings and give more realistic estimates of legume N_2-fixing capacities (Dreyfus et al., 1988). The common garden experiment, which employed three levels of canopy opening, was designed to make such estimates of N_2-fixation capabilities under field conditions. Shade not only impacted the biomass and tissue-N allocation patterns of the legumes in the study, but was effective in producing light levels that provide critical limitations to growth and N_2-fixation rates (Chapter 4). This study also showed that *Lespedeza* spp., *Desmodium* spp. and *Tephrosia virginiana* are potentially very good candidates for inclusion in groundcover restoration projects due to their high tissue-N concentrations, N_2-fixation capabilities, and dominant growth forms that will provide adequate cover for wildlife. These findings should be considered when landowners make decisions about groundcover restoration. The final conclusion of this field study was that native legumes will not thrive or fix N_2 under a closed canopy and should be established in more open stands that have been recently thinned.

Directions for Future Research

This study examined only a small percentage of the legume population native to the longleaf pine-wiregrass ecosystem. Additional species should be considered for groundcover restoration, especially other species of the genera *Desmodium* and *Lespedeza*. Since the legumes predominantly represent a fall food source for bobwhite quail, perhaps other fall-flowering legumes should be considered for groundcover, such as *Dalea* spp. (Stoddard, 1931).

Further Application of N_2-Fixation Assessment Techniques

Of the N_2-fixation assessment techniques used in this study, only the N-transport and storage product analysis has not been used for field analysis of species in southern pine ecosystems. The portability and minimally-destructive nature of this technique may have great potential for field studies in the longleaf pine-wiregrass ecosystem, and may be especially valuable for a first-assessment of N_2-fixing capabilities of numerous species of legumes under field conditions when used in corroboration with the ^{15}N natural abundance technique.

Another interesting approach to quantifying N_2-fixation using the stem N transport/storage product analysis (relative ureide analysis) involves calibrating ureide indices with fixed-N_2 using a series of labeled $^{15}N_2$ assays and relative ureide analysis (Peoples et al., 1996). This method may have potential for allowing an estimate of N_2-fixed by a population of legumes with a quick and easy analysis that is easy to conduct on a variety of soils.

Further studies should also investigate the relative efficiencies of specific host-symbiont interractions, including tripartite interactions between mycorrhizae, rhizobium, and legumes. Relative efficiencies can be quite different between rhizobium strains, and

inoculation with highly effective strains could become important to native legume restoration if the land on which they are being planted has had heavy herbicide usage.

Future Research for Native Legume Utilization

Future research regarding groundcover restoration under longleaf pine should involve studies that investigate physiological properties of these and many other of the legumes native to the longleaf-wiregrass ecosystem and continue to ask questions relating directly to groundcover restoration objectives such as soil-N and organic material development and wildlife food quality. Additional studies should look at competitive interactions between native grasses and legumes planted in a restoration setting to determine when competition for soil and light resources becomes limiting for each type of plant. Fine root turnover, mycorrizhal associations, root exudates, and other belowground processes that contribute directly to building soil organic matter and N-availability should be further researched. Analysis of native legume and grass shoot and seed digestibility and nutritional composition could be important information for land managers seeking to attract and sustain wildlife populations on their property.

Imported legumes have been used for soil stabilization and game management purposes, and many of these species, including kudzu (*Pueraria lobata*), sericea lespedeza (*Lespedeza cuneata*) and shrubby lespedeza (*L. bicolor*), out-compete native vegetation in natural and roadside areas across the Southeast (Miller, 2003). Native legumes are a better choice for many soil preservation and land management purposes and are much less likely to become invasive. Many of the legumes native to the southeast region, such as *Centrosema virginianum* and *Dalea pinnata*, to name only a few, are also aesthetically pleasing and should be considered for wildflower plantings. The N_2-fixation capability of *C. virginianum* could possibly be marketed as an added

benefit to native plant collectors. More research is needed before other species can be designated as N_2-fixers.

Native legumes are important and poorly explored genetic resources. N is typically the most limiting plant nutrient in terrestrial ecosystems, and biological N_2-fixation is a "free" source of N-addition. In a time when organic farming practices are gaining popularity in the temperate parts of the world and agroforestry is increasing in feasibility and scientific recognition in tropical and subtropical parts of the world, legumes are becoming more valuable and marketable. The legume species-richness resources that are available in the Southeastern coastal plain should be actively examined for future development.

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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 served as an intern for the TN Department of Environmental Conservation (TDEC), Division of Natural Heritage, and assisted Dr. James Carpenter in a study of the endangered crayfish, *Orconectes shoupi*. Cathey also completed a senior honors thesis entitled "An Eradication Study of *Vinca minor*," which was undertaken in cooperation with the TDEC Division of Natural Heritage and the Lipscomb University Biology Department.

A strong interest in native plants and applied research led Cathey to take a position as research assistant in the cooperative program between the University of Florida and The Joseph W. Jones Ecological Research Center (Newton, GA) that supported the research presented in this thesis. With regard to the future, Cathey has research interests in applied plant and soil science, ecology and ecophysiology, and she has been awarded a fellowship by the College of Agricultural and Life Sciences at the University of Florida to pursue a PhD. Cathey's world view has been strongly influenced by her travel to undeveloped and developing nations (Haiti, Ukraine, and Peru), and she intends to pursue research that could have positive environmental and socio-economical implications for people in the U.S. and abroad. A desire to teach and conduct research will probably lead Cathey to pursue a faculty position at a university in the future.