BIOLOGICAL NITROGEN FIXATION IN TROPICAL DRY FORESTS OF COSTA RICA: PATTERNS AND CONTROLS

A Dissertation
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Jennifer S. Powers, Adviser

June 2014

Acknowledgements

It would not have been possible to develop this thesis without the support of my mentors, colleagues, friends and family. Above all, I would like to thank my advisor Jennifer Powers for her constant support and encouragement. She was present and deeply involved at every single step along this way but she also allowed and motivated me to work independently. She encouraged me to seek funding from numerous sources and gave me immediate advice whenever I asked for it. Most importantly, she constantly reminded me of the exciting, fun and collaborative aspects of science. Her friendship and mentoring were essential to the success of this research! I would also like to acknowledge the members of my thesis committee: Sasha Reed, Sarah Hobbie, Rebecca Montgomery, and Deborah Allan. They provided insightful advice during the last six years that significantly improved my work. I am thankful that they were willing to accommodate my frequent travel to meet with me. They enriched my research experience significantly by helping me think about my ideas from numerous angles. I consider myself extremely lucky and honored by having had the example, mentorship and the opportunity to work with these five amazing scientists!

I spent two years living and developing this research at the heart of Área de Conservación Guanacaste (ACG) in northwestern Costa Rica. Although this period included a great amount of hard work, I have only the happiest memories thanks to the many wonderful people who made it easy and above all, fun. I am most grateful to Milena Gutiérrez, Roger Blanco, and María Marta Chavarría in ACG for making both my research permits a smooth and easy ride. I would have not been able to endure such a long time living in a remote area without the help and friendship of Daniel Pérez, our field lab manager. I admire and thank Daniel for everything he taught me about the dry forests and the people of Guanacaste. He and his kind family made me feel at home and warmly included me in their lives like another family member. Many other wonderful guanacastecos helped me perform the fieldwork of this research, and I am very grateful to Dinia, Tomás, Gato, Raúl, Ronald, and Saúl. Geohanny Hernández from the nearby community of Cuajiniquil helped me collect most of the soil samples I analyzed for this dissertation. There was not a single day when he didn't smile, make jokes, or find the most positive angle of every kind of situation. I also thank my neighbors, Luis Cantillano and his family, for always keeping an eye on me in this isolated place.

Being able to work and share my time with to members of the Powers Lab made this experience even more enriching. Not only I am very grateful for their company, friendship and support but also many conversations with them helped and improved the quality of my work. I had a great time with you in Minneapolis and Guanacaste: Cathy, Justin, Joey, Heather, Sasha, Lisa, Yana, Kristen, Leland, Christina, and Cristina. I feel very lucky for having met so many close and lifelong friends that made my time in Minnesota the most enjoyable, including Ana, Rodrigo, María, Alejandro, Juan, Chepe, and Michelle. Six years ago, I would have never imagined calling home a place that is so many degrees of latitude away from the tropics and I owe this in great part to my dearest

friend Stephanie Lee. I will always thank her for sharing with me her contagious passion and perseverance at seizing and enjoying every single day, even the ones during winter.

I would like to take a moment to remember the loved ones who only saw me begin this experience and hold a very special place in my heart. Thank you Luis Diego Gómez Pignataro and Jorge Gómez Laurito for introducing me to the beauty and greatness of plants and to the most hidden and beautiful corners of Costa Rica, and for encouraging me to let myself be led by the passion for knowledge. “Conmigo vienen los de atrás”: my Tita Flory has been one of the most influential women in my life. She was the most supportive about any projects I would undertake in pursuit of my happiness, including this one. I find much strength and inspiration in thinking about her, Tito, Nonno, and Nonna.

The hill at the end of the climb to the tallest mountain in Costa Rica is so steep that is called “los arrepentidos (those who regret)”. But to me, the last hill of this dissertation was the easiest because I had the love and support of Scott, my beloved life partner and I will always be grateful for his patience and encouragement.

Muchas gracias,

Maga

Dedication

This dissertation is dedicated to my parents, Orlando and Gabriela, for their unconditional love, support and encouragement.

Abstract

In tropical forests, new nitrogen (N) inputs fuel a large proportion of global net primary productivity. However, global estimates of tropical N fixation are biased towards wet forests and other areas such as tropical dry forests are understudied. In the dry forests of Guanacaste, Costa Rica, N fixing legume trees are highly abundant throughout forest successional stages, thus I hypothesized that in tropical dry forests legume trees are critical regulators of ecosystem level N dynamics. I addressed this question from multiple approaches that included a shade house experiment and field surveys of N fixing legume trees in plantations or in diverse secondary forests using a common set of species: *Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*. Individual legume species had measurable influences on a number of soil properties, but this effect is more pronounced than the influence of legumes as a functional group. I observed species-specific variation in belowground foraging strategies and in the timing and degree of nodulation. In the shade house experiment, species differed in their nodulation effort and in how they regulated N fixation with respect to available resources. These five legume species could be arrayed along a continuum defined by strategies of nutrient conservation and nutrient acquisition, which coincided with degrees of fine-tuning of N fixation. In the field study, I did not find evidence of down-regulation of fixation with soil N. I hypothesized that the adjustment of N fixation to soil nutrients occurs indirectly and is mediated by water availability and its effects on nutrient pulses. My stand-level estimates N fixation by legumes showed that legumes are responsible for the largest contribution of new N inputs

to this ecosystem relative to other inputs such as free-living fixation or wet deposition, but which are modest relative to N recycling through leaf litter and fine root decomposition. Different legume strategies could represent different ways of dealing with the transient and seasonal water availability of this ecosystem. Collectively, my results suggest that the conceptual models of how N fixation works in tropical wet forests may not necessarily be the same in seasonally dry forests.

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Introduction

Nitrogen (N) is a vital nutrient for plant growth in terrestrial ecosystems. In tropical forests, new N inputs fuel a large proportion of global net primary productivity (Cleveland *et al*., 2013). As a result, biomass in tropical forests accounts for a significant proportion of the global carbon sink (Pan *et al*., 2011). Because carbon and nutrient cycles are intimately related (Reiners, 1986), ecosystem responses to increased atmospheric concentrations of carbon dioxide are constrained by the availability of N and other nutrients (Rastetter *et al*., 1997). In the tropics, anthropogenic N inputs are increasing rapidly (Austin *et al*., 2013) and our benchmark knowledge of how N cycles in intact forests is still far from complete.

The tropical biome is known for its biogeochemical heterogeneity (Townsend *et al*., 2008). Still, focal sites of tropical ecosystem research have been geographically restricted to wet forests and other areas such as tropical dry forests (TDF) are understudied. TDF comprise up to 42% of tropical forests (Murphy and Lugo, 1986). The assumption that biogeochemical processes function similarly between wet and dry tropical forests is untested, and likely incorrect, given fundamental differences between these two types of ecosystems. For example, in TDF water availability drives plant growth and phenology, which results in decomposition patterns drastically different than in wet forests.

My dissertation research examines the patterns and controls on N fixation in tropical dry forests of Guanacaste, Costa Rica (Figure 1). The questions that I asked

originated from a natural history characteristic of TDF: legume trees and lianas are highly abundant and diverse in these forests (Gentry, 1995; Gillespie *et al*., 2000; Pennington *et al*., 2009). Evidence of high gaseous losses of N (Davidson *et al*., 1993; García-Méndez *et al*., 1991) and high ecosystem soil N stocks (Jaramillo *et al*., 2003; Rentería *et al*., 2005) suggest that TDF are not N deficient. Given the high cost of fixing N, the ubiquity of N fixing legume trees in tropical forests is known as the N paradox (Hedin *et al*., 2009) but this paradox has been resolved by hypothesizing that individual trees down-regulate N fixation when soil N is abundant. Besides soil N, light energy and soil phosphorus are two other factors known to control symbiotic N fixation. In low light environments, N fixers can be energy limited due to high carbon costs for N acquisition and growth (Gutshick, 1987). Compared to non-fixing species, N fixers have high P requirements for building and maintaining the enzymes and proteins necessary for fixation and to balance the high metabolic use of N (Vitousek *et al*., 2002).

My central hypothesis was that *in tropical dry forests legume trees are critical regulators of ecosystem level N dynamics*. I addressed this question from multiple approaches that included a shade house experiment and field surveys of legume trees in plantations or in diverse secondary forests using a common set of species: *Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*. The four chapters of this dissertation address the following goals and hypotheses, to: 1) describe soil chemistry and nutrient availability under the crowns of legume trees compared to nearby non-legume species in a diverse secondary forest or in 18-yr old monoculture plantations and test the hypothesis that species effects are more

apparent in tree plantations, where they are "undiluted" by diverse litter chemistry in species-rich forests; 2) describe the seasonal and spatial variation in fine root stocks and nodulation over two years in plantations of four legume species, and test if soil moisture would track precipitation seasonally and correlate with fine root and nodule mass, and if nodule mass would scale positively to fine root biomass; 3) to determine how plant performance and N fixation respond to changes in light and nutrient availability in seedlings of a number of tropical species from throughout the legume phylogeny, and test if species that share functional trait values respond to resource variation similarly; and 4) to constrain the contribution of symbiotic N fixation rates to overall ecosystem N cycling in regeneration tropical dry forest in Costa Rica. Below I elaborate on each of the four chapter goals.

In the dry forests of Costa Rica, legume trees stand out as a different plant functional group since they have high leaf C and N concentrations and greater wood density than non-leguminous species (Powers and Tiffin, 2010). In *chapter 1*, I used legume and non-legume tree species to test the hypothesis that species influence soil physicochemical properties, creating a zone of influence under the tree canopy (Zinke 1962). Quantifying the effects that legumes have on soils was a required first step toward understanding how community composition and functional group assemblages influence soil processes and particularly N dynamics. The variables I measured included soil moisture, pH, labile carbon (C), inorganic N (NH_4^+ and NO_3^-), net N mineralization rates, total C and N, $\delta^{15}N$ signature and enzyme activity.

In tropical forests, fine roots are an important component of belowground carbon stocks. Fine root distributions are notoriously variable in space and time in forested ecosystems. This is why in *Chapter 2* I used forest plantations, with fewer tree species, in order to quantify spatial patterns in fine root stocks and nodulation of four dry forest tree species vertically through the soil profile and horizontally with distance from tree boles. My second goal in this chapter was to quantify seasonal patterns in fine roots and nodule production of these species and relate it to potential drivers, most importantly soil moisture and fine root mass. My study is one of the few to have monitored nodulation repeatedly in the same adult tree individuals through time, beyond one snapshot sampling.

In *Chapter 3*, I show results from a shade house experiment where I grew four N fixing legume species and one non-fixing legume for 6 months under different light environments and nutrient fertilization treatments. In this chapter, I tested the hypothesis that tropical legumes employ a facultative strategy of N fixation where they stop fixing when soil N is abundant and increase N acquisition in high light and with P additions. My goal was to determine how above- and belowground resources affect N fixation as well as biomass partitioning, performance, trait values and physiology, and how this varies among different legume species. Here, I quantified N fixation using three indices: nodule mass, the acetylene reduction assay and the ^{15}N natural abundance method, and compared their applicability. I also used a functional trait approach to determine whether several legume species showed different strategies of nutrient use and acquisition.

In *Chapter 4*, I explored whether N fixation in adult trees of legume species varied with respect to environmental controls, mainly soil nitrogen and phosphorus. I compared both nodulation and the ^{15}N natural abundance method to quantify N fixation. I also used the acetylene reduction assay technique to quantify free-living N fixation in litter and soils seasonally in a chronosequence of dry forest plots. I estimated the potential contribution of N fixing legume trees to ecosystem-level N cycling by constructing N budgets of N inputs and internal N transformations. Symbiotic fixation by legumes is thought to be a facultative process that decreases over successional time (Barron *et al*., 2010; Batterman *et al*., 2013; Sullivan *et al*., 2014), but evidence for this is restricted to tropical wet forests. In the dry forests of the Guanacaste region, legume abundance did not decrease with stand age across a chronosequence of 84 0.1 ha plots (Figure 2), which indicates that the contribution of legumes to the N budgets of this ecosystem may differ from that of wet forests.

Collectively, these four studies represent a significant advancement in our understanding of the contribution of legumes to ecosystem function in tropical dry forests. Throughout my dissertation I focused on the same species from seedlings to adult trees, from monoculture plantations to diverse forests, thus I was able to disentangle whether emerging strategies of different species were unique to an ontogenetic stage or not. My results build upon previous work on dry forest inventory plots and add new pieces of information that resolve uncertainties about the magnitude of N inputs to this ecosystem.

Some species in the genus *Acosmium* have been recently transferred to the genus *Leptolobium* and *A. panamense* is now known as *Leptolobium panamense* (Cardoso *et al*., 2012). Because Chapter 1 was published while *A. panamense* was still valid, in this dissertation I will refer to this species using the scientific name *Acosmium panamense*.

Figure 1. Map of Costa Rica showing the two conservation areas where I developed this dissertation: Parque Nacional Santa Rosa of Área de Conservación Guanacaste and Parque Nacional Palo Verde of Área de Conservación Tempisque.

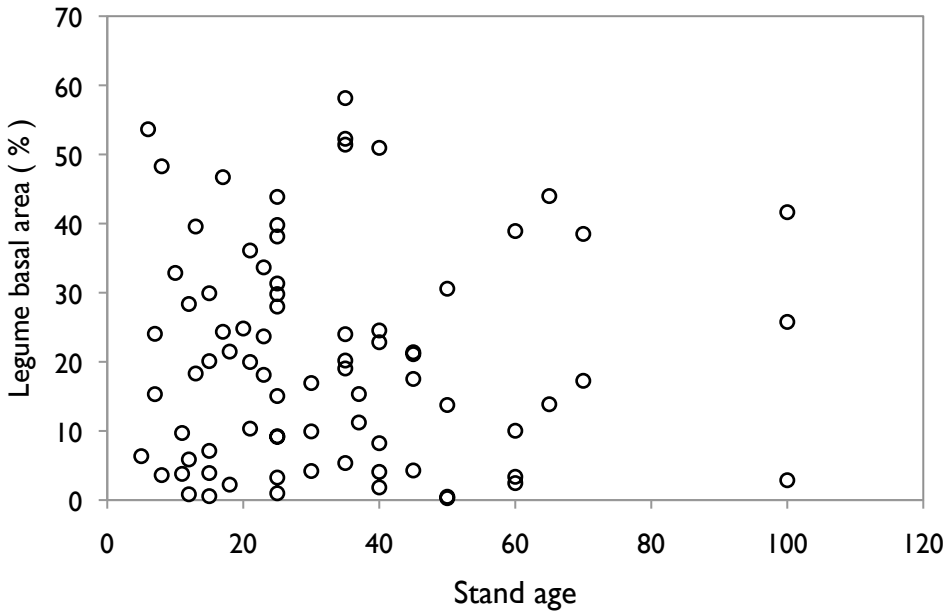

Figure 2. Proportion of basal area represented by legumes (Fabaceae) in 84 0.1 ha plots across Área de Conservación Guanacaste and Parque Nacional Palo Verde, Costa Rica.

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CHAPTER 1

Do legumes and non-legumes tree species affect soil properties in unmanaged forests and plantations in Costa Rican dry forests?

Gei, M. G., and J. S. Powers. 2013. Do legumes and non-legumes tree species affect soil properties in unmanaged forests and plantations in Costa Rican dry forests? *Soil Biology and Biochemistry* 57: 264–272.

Summary

Legume tree species, which are abundant in tropical dry forests, may be a critical regulator of soil nutrient dynamics because of their high foliar nitrogen (N) and potential for symbiotic N fixation. We investigated whether three legume tree species (*Acosmium panamense*, *Dalbergia retusa*, and *Gliricidia sepium*) have distinct soil chemistry under their crowns compared to nearby non-legume species (*Rehdera trinervis*, *Swietenia macrophylla*, and *Quercus oleoides*) when grown in two habitats: a diverse secondary forest or in 18-yr old monoculture plantations in northwestern Costa Rica. We quantified soil moisture, pH, labile carbon (C), inorganic N (NH_4^+ and NO_3^-), net N mineralization rates, total C and N, $\delta^{15}N$ signature and enzyme activity. We predicted that legumes would have higher soil nutrient availability under their crowns, but that this effect would be more pronounced in plantations, where tree species diversity is low. In the forest, soils under *Dalbergia* had the highest values of total C and N, and extractable nitrate, whereas soils under *Acosmium* the highest N mineralization rates. The activity of acid phosphatase

enzymes varied among the soils under different species in both habitats, with the highest activity in the soils under the legume *Acosmium* at the forest site. In the plantations, *Acosmium* had the highest values for total soil C and N, labile C, and potential N mineralization rates. We conclude that 1) the legume species did not have consistent effects as a functional group, possibly due to different amounts of nodulation in individuals within species, and 2) as hypothesized, the magnitude of the species effect was more pronounced in the plantations than in the diverse secondary forest.

Key words: legumes – species effect – soil chemical properties – nitrogen fixation – soil ^{15}N – tropical dry forest

Introduction

Variation in nutrient pools in above- and below-ground litter from different tree species help determine nutrient availability in the soil, as well as the quality of the substrate for soil microbes (Hobbie, 1996; Rhoades, 1997; Ushio *et al.*, 2010), and can contribute to species generated soil heterogeneity (Zinke, 1962). In turn, the existence of plant-generated soil heterogeneity, or patches of distinct soil conditions beneath tree canopies can impact nutrient fluxes into, out of, and within ecosystems (Rhoades, 1997). In their review, Hutchings *et al*. (2003) predicted that heterogeneous conditions in the soil change competitive processes and their outcome, with greater competition in nutrient-rich patches and less diversity as fast-growing species eliminate slow-growing species, compared to nutrient-poor patches. Nutrient-rich and nutrient-poor soil patches

are important in determining local and regional nutrient reserves and thus can potentially affect species composition at the community level (Rhoades, 1997; Binkley and Giardina, 1998; Hutchings *et al*., 2003; Vivanco and Austin, 2008).

Individual trees are known to influence soil physicochemical properties, creating a zone of influence under the tree canopy (Zinke, 1962). These "species effects" have been extensively studied in comparatively low-diversity systems such as temperate forests or tropical plantations, where different tree species have significant impacts on soil properties (e.g. pH, exchangeable cations, water content), on substrate quality for microbes (e.g. total C, N and P concentrations, and C:N ratio), and on nutrient availability (N mineralization, organic P concentration) (Hobbie, 1992; Finzi *et al*., 1998 a, b). One common mechanism invoked to account for these effects is the amount of nutrients recycled in each species' leaf litter, which in turn is affected by leaf nutrient concentration, the degree of nutrient resorption prior to leaf abscission, litterfall mass, or even by nutrients captured by tree canopies (McClaugherty *et al*., 1985; Rhoades, 1997; Binkley and Giardina, 1998).

Legumes (Fabaceae, Leguminosae) are a good test case for individual species effects on tropical soils as they are potential regulators of ecosystem nutrient dynamics (Fisher, 1995; Franco and de Faria, 1997; Wang *et al*., 2010). Legumes are the most diverse and widespread group of plants with the capacity of N fixation (Sprent, 1995; Sprent and Parsons, 2000), and are particularly abundant in tropical dry forests (Gentry, 1995; Gillespie *et al*., 2000; Pennington *et al*., 2009). Irrespective of their ability to fix atmospheric N_2, most members of this family have high foliar N (Fyllas *et al.*, 2009).

Moreover, in the dry forests of Costa Rica, legume trees stand out as a different plant functional group since they have high leaf C and N concentrations and greater wood density than non-leguminous species (Powers and Tiffin, 2010). Despite a general understanding of legumes as drivers of N dynamics, the magnitude of the effects of this group of species in tropical forest soils is poorly quantified. Defining the effects that legumes have on soils is a required first step towards understanding how community composition and functional group assemblages influence soil processes and particularly N dynamics. While a few studies have quantified the influence of tree species on soil characteristics in diverse tropical forests (Powers *et al.*, 2004, Reed *et al*., 2008), or plantations (Montagnini and Sancho, 1990, 1994; Fisher, 1995; Powers *et al.*, 1997; Warren and Zou, 2002) to date no studies have included species effects in both habitats.

This study was designed to elucidate the effect of legume tree species on soil chemistry and nutrient availability in both diverse secondary dry forest or alone in monoculture plantations. We focused on three legume species and compared their effects to nearby non-legume species. Because legumes typically have higher foliar and litter N concentrations compared to other species (McKey, 1994; Townsend *et al*., 2007), we predicted that legumes would have higher soil N availability under their crowns. A corollary to this prediction is that if legume trees are indeed increasing soil N availability under their crowns, the cost of the investment in the production of N-rich enzymes like acid phosphatases should be reduced (Houlton *et al*., 2008). Acid phosphatases hydrolyze the ester bonds of organic P compounds releasing phosphate in forms available to plants (Malcolm, 1983; Olander and Vitousek, 2000). Thus we hypothesized that acid

phosphatase activity would be higher under legume species compared to under non-legume species. In addition, we compared the stable N isotope composition ($\delta^{15}N$) of the soils under each species, expecting to find soils with ^{15}N values closer to zero under N fixers that would reflect inputs of atmospheric N_2. Alternatively, a relative enrichment in ^{15}N in soils under the N fixers could imply greater losses of N through trace gas emissions and hence fractionation or discrimination against the heavy N isotope, suggesting that N is in relative excess to microbial N requirements (Högberg, 1997; Martinelli *et al*. 1999). Finally, we predicted that the effect of legume species on soils under their crowns would be more pronounced for trees in the plantations compared to the forest, where a diversity of N-demanding non-leguminous species might be rapidly recycling the N resulting from the decomposition of legume species litter.

Materials and Methods

Site description

This study was carried out in Sector Pocosol and in Estación Experimental Horizontes of Área de Conservación Guanacaste (ACG; 10.84°N, 85.62°W) in northwestern Costa Rica. Established in 1971, ACG currently comprises 147,000 hectares (ha) of protected land (http://www.gdfcf.org). This region has a mean annual temperature of 25°C and a mean annual precipitation of 1575 mm with a large inter-annual range from 880–3030 mm and a 6 month dry season (Gillespie *et al.*, 2000). Pocosol is a mosaic of old pastures and dry forest of various ages and has been protected

from cattle grazing since 1988 (Gerhardt, 1993). Tree species richness (stems > 10 cm diameter at breast height) in 0.1 ha forest inventory plots in the region ranges from 1 to 21 species (Powers, unpub. data). Horizontes is located ~20 km from Pocosol and was established in 1991 as an experimental area of ACG for research in restoration and silviculture; it has 74 ha of forest plantations of native tree species embedded in ~7300 ha of lowland deciduous forest directly connected to ACG. Before 1991, Horizontes was a farm that had rice, sorghum and cotton fields, with cattle pastures as well (Gutiérrez, *pers. comm.*). In Pocosol, most of the soils are Entisols, Inceptisols, or Vertisols of volcanic origin overlying bedrock of volcanic ashes and pumice (Gerhardt, 1993). In Horizontes, soils are Inceptisols and of volcanic origin as well (Czarnowski, 2002).

Species selection and soil sampling

Species were selected based upon local abundance and presence in the Horizontes plantations. In the forested areas of Pocosol, we identified five individuals of each of the following tree species from the Fabaceae family: *Acosmium panamense* (Benth.) Yakovlev, *Dalbergia retusa* Hemsl., and *Gliricidia sepium* (Jacq.) Kunth ex Walp. In the same area, we chose five individuals of each of the following non-legume species: *Quercus oleoides* Schltdl. & Cham. (Fagaceae), *Rehdera trinervis* (S.F. Blake) Moldenke (Verbenaceae), and *Swietenia macrophylla* King (Meliaceae). All species are henceforth referred to by genus name only. The three legume species are known for their potential for nodulation and hence potential capacity to fix N (Corby, 1988; Sprent, 2001). Functional traits for these species have been previously collected (Powers and Tiffin,

2010) (Table 1-1). Non-legume species were chosen for their high foliar C:N ratios and were located in the same area as legume focal trees but at least two tree crowns away from any of them. Previous work has shown that many plant functional traits for these species, with the exception of the legumes, are not phylogenetically conserved (Powers and Tiffin, 2010). All individuals were chosen within a 20 ha area. To standardize for light environments, only individuals with direct access to light were included. In Horizontes, we targeted focal trees in 18-yr old monoculture plantations (1 ha in size) of the same three legume species sampled in the forest site, as well a plot of one of the non-legume species, *Swietenia*. The largest distance between two plots at Horizontes was 1km.

Beneath the canopy of each focal tree in both habitats, we collected eight samples from the top 10 cm of mineral soil using a 2.5 cm diameter corer after removing the litter layer. Cores were taken in each of eight cardinal directions from the bole and at different random distances between the bole and 100 cm before the crown drip line. Field moist soil from the eight individual cores was homogenized into one polyethylene bag per individual sampled. For up to three days following soil collection, samples were kept at 4 °C and taken to the University of Minnesota for subsequent analyses. Each sample was sieved (2 mm) to remove rocks and plant material. Four soil subsamples were removed: one for gravimetric analysis of water content, one for labile soil C and potential N mineralization determination, one for enzyme activity analysis that was stored at -80°C until processing, and one subsample was air-dried for subsequent physical and chemical analyses. Recent studies show that inorganic N fractions change rapidly and that

minimizing the transit time before processing is essential for obtaining accurate measurements of ammonium (NH_4^+) and nitrate (NO_3^-) concentrations (Turner and Romero, 2009). While we cannot rule out the possibility of rapid N transformations in our samples, all samples were handled identically and processed as rapidly as was possible given the logistic constraints of field work in a remote site, making our dataset internally consistent.

Soil characteristics

Gravimetric moisture was determined by oven-drying samples at 110 ˚C for 48 h. We measured pH in water in a 1:2.5 soil to solution ratio with an Oakton pH electrode on air-dried soils. Total C and N were measured on a COSTECH 4010 Elemental Analyzer at the University of Minnesota, and concentrations are reported on an oven-dried mass basis. In addition, the stable N isotope composition ($\delta^{15}N$) was analyzed on a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotopes Facility of University of California, Davis.

We used K_2SO_4 –extractable organic C as an index of labile soil C (Zhou *et al*., 2012). Subsamples of soils were shaken in 0.5 M K_2SO_4 for 1 hour, allowed to settle, and then the supernatant was filtered through Whatman No. 1 filter paper that had been pre-rinsed with 0.5 M K_2SO_4. Organic C in extracts was analyzed using a Shimadzu TOC-5050A total organic C analyzer (Shimadzu Corporation, Kyoto, Japan).

Extractable ammonium (NH_4^+) and nitrate (NO_3^-), and potential net N mineralization were measured using aerobic incubations of mineral soil under

standardized conditions in the laboratory following Kandeler (1996). Subsamples of 10 g of oven-dry equivalent of each soil were incubated in glass containers in a dark room at 25 °C for 28 days. The jars were covered with polyethylene film to allow air circulation but prevent excess evaporation. Each sample was wetted with deionized water to a moisture content of 30%. Initial soil samples were shaken for 1 hour in 50 mL of 2 M KCl solution to extract soil NH_4^+ and NO_3^-. After 28 days, the incubated samples were extracted as above. All extracts were analyzed for NH_4^+ and NO_3^- on a Bran-Luebbe AA3autoanalyzer at the University of Nebraska. Potential net N mineralization was calculated from the net change in the $NH_4^+ + NO_3^-$ concentrations, and net nitrification rates were calculated as the net change in the NO_3^- pool (Binkley and Hart, 1989). We calculated the ratio of nitrate to ammonium (NO_3^- : NH_4^+:) using the initial concentrations of NO_3^- and NH_4^+ at the time of sampling.

Enzyme activities

We measured the activities of the hydrolytic enzymes acid phosphatase (AP; EC 3.1.3.1), β- D-glucosidase (BG; EC 3.2.1.21), β-N-acetylglucosaminidase (NAG; EC 3.2.1.14) and cellobiohydrolase (CBH; EC 3.2.1.91) as indices of microbial activity. We followed standard methods described in Saiya-Cork *et al*. (2002) and Sinsabaugh *et al*. (1992). Subsamples of 1 gram were mixed with 125 ml of acetate buffer (50 mM, pH 5.0), and homogenized in a blender. Sixteen replicate soil suspensions for each sample per assay were then dispensed into 96 well microplates. Sodium acetate buffer, methylumbelliferone (MUB) standard, and labeled substrates were dispensed into plates

using a Precision 2000 robotic pipettor (BioTek Instruments). In total, 16 replicate sample wells (sample solution + substrate), eight replicate blank wells (sample solution + buffer), eight negative control wells (substrate + buffer), and eight quench standard wells (standard + sample solution) were used per assay. Prepared plates were incubated in the dark at 20°C for 0.5–20 h depending on the assay. Activity was measured as the fluorescence of the sample wells corrected for negative controls, blanks, and quenching. Enzyme activity was calculated as the nmoles of substrate converted per hour per gram soil dry weight.

Data analysis

Ideally, we would have located plantations and populations of the same species growing in close proximity. Unfortunately, we were unable to find such a situation, although the plantations and unmanaged populations that we sampled are both in the same conservation area, have parent material of similar origin, and experience the same temperature and precipitation regime. However, to avoid the possibility of confounding habitat type (plantation or unmanaged forest) with any underlying variation among sites in soil characteristics, we analyzed the data from each habitat separately. In order to compare the effect of individual species on soil chemical properties in either forest or plantation sites, we used one-way analyses of variance with "species" as the main fixed effect and Tukey post-hoc analyses to determine which means were significantly different from one another. When necessary, data were transformed to improve normality. We consider that the chosen taxa are statistically independent since we have

done extensive surveys of trait variation in this region, and found no evidence for phylogenetic conservatism of traits for all taxa, except for the legumes (Powers and Tiffin, 2010). Our focus on three legume species is supported by our interest on the effects of this functional group on ecosystem function and on the abundance of the species selected which can give us further insight into the role of these species at an ecosystem scale.

We then quantified the influence of legumes as a functional group by averaging soils under legume and non-legume species in each site and applying the same type of analysis. We computed pairwise Pearson's product moment correlation coefficients as a way to explore relationships among leaf traits, litter decomposition rates, and soil properties. Although plant traits were collected as a separate data set on different individual trees, Powers and Tiffin (2010) found that inter-specific variation was larger than intraspecific variation. Correlations were computed for forest and plantation habitat separately. All statistical analyses were done using JMP (JMP 9.0.0, SAS Institute).

Results

Soil chemical properties

The range of pH values was larger in the forest habitat (from 4.0 to 5.6) than in the plantation (from 4.6 to 5.7). However, the differences in soil pH among the species were not significant in either habitat. Interestingly, in both habitats, the highest values of soil pH were recorded under the legume species *Dalbergia* (5.6 and 5.7 in forest and

plantation respectively). Soil moisture varied among species significantly only in the plantation ($P < 0.0001$); the highest average was reported from the soils under *Dalbergia*.

Total soil C and N varied significantly among the species in the forest ($P < 0.05$ for C and $P < 0.01$ for N; Figure 1-1a) and in the plantations ($P < 0.0001$ for C and N; Figure 1-1b). There was a significant positive correlation between these two variables in both forest and plantations sites (Tables 1-3 and 1-4). In the forest, *Dalbergia* had the highest values of total soil C and N, and the lowest concentrations were found under the non-legume species *Quercus*. The plantation of the legume *Acosmium* had the highest concentrations of total soil C and N ($P < 0.0001$). When the species were grouped by functional type (legume or non-legume), soils under the legume functional type had significantly lower C:N ratios in both secondary forest ($P < 0.05$) and in plantation ($P < 0.05$).

Differences in labile soil C measured as K_2SO_4-extractable C were significant among different species in plantations ($P < 0.0001$; Figure 1-1d), with *Acosmium* having higher values than the other three species. In the forest these differences were marginally significant ($P = 0.059$; Figure 1-1c), however there was a pronounced difference between the largest average concentrations found in the soils under *Quercus* (130.8 ± 19.5 μg g^{-1}, standard error) compared to soils under the legume *Gliricidia* (77.98 ± 21 μg g^{-1}, standard error).

Soil inorganic N and N mineralization

Soil NO_3^- concentrations varied strongly among tree species at the forest site ($P < 0.001$; Figure 1-2a). Soils under *Dalbergia* had the largest NO_3^- pools and also the largest range of variation while soils under the non-legume *Quercus* had the smallest pools with least variation. In the plantations, there were significant differences in both soil NO_3^- and NH_4^+ concentrations among tree species ($P < 0.0001$ for both NO_3^- and NH_4^+, Figure 1-2b) with the highest NO_3^- pools under the legume *Gliricidia* and the lowest under the non-legume *Swietenia*. Interestingly, the ratio of extractable NO_3^- to NH_4^+ varied significantly among species in both habitats ($P < 0.005$ and $P < 0.0001$ respectively, Table 1-2). Across the habitats, the mean ratio of extractable NO_3^- to NH_4^+ ranged from 1.60 to 1.45 for *Dalbergia* and 1.06 to 5.85 for *Gliricidia*, i.e there was more NO_3^- relative to NH_4^+ in the soil. By contrast, the converse was true for the non-legumes *Quercus* and *Swietenia*, which had much lower concentrations of extractable soil NO_3^- relative to NH_4^+, with ratios from 0.11 to 0.35 (Table 1-2). The laboratory incubations revealed significant differences among species in potential net mineralization and nitrification in the forest and in plantations ($P < 0.0001$; Table 1-2) with *Acosmium* showing the highest net N mineralization at both sites, but immobilization in the soil collected from underneath *Dalbergia*, *Rehdera* and *Swietenia* in the forest and *Gliricidia* in the plantations. Net N mineralization rates and total soil C were negatively correlated in the forest and positively correlated in the plantations (Tables 1-3 and 1-4).

Soil ^{15}N

We found significant differences in $\delta^{15}N$ among the soils under different tree species at the forest site ($P < 0.05$, Figure 1-1e) as well as regional differences. In the forest, soils under the legume tree *Gliricidia* were the most enriched, with an average $\delta^{15}N$ of 4.64 ± 0.38 ‰; the most depleted soils were found under *Acosmium* with an average $\delta^{15}N$ of 2.91 ± 0.78 ‰. In the plantations, $\delta^{15}N$ was similar among the soils under different species but the average $\delta^{15}N$ value at this site was 2‰ higher than in the forest ($P < 0.0001$; Figure 1-1f).

Extracellular enzyme activity

The activity of acid phosphatase (AP) was significantly different among soils under different species in both habitats ($P < 0.05$ in the forest and $P < 0.005$ in plantations; Figure 1-1, g and h). AP activity was higher in the soils under the legume *Acosmium* at the forest site and equally high in the monocultures of *Acosmium* and *Swietenia*. There were no significant differences in the activities of the enzymes β- D-glucosidase, β-N-acetylglucosaminidase or cellobiohydrolase among different species in either habitat (results not shown).

Legume effect

When we averaged soil properties found under legume and non-legume species in each site, the only statistically significant results we found were that in both habitats soils

under legumes had higher NO_3^- concentrations and higher NO_3^- to NH_4^+ ratios than soils under non-legumes (Figure 1-2, c and d; Table 1-2).

In order to compare the magnitude of the species effect in the plantations and in the highly diverse secondary forest, we calculated the range of distribution of each soil property and the highest mean difference between any pair of species for each soil property in both habitats (Table 1-5). We found larger ranges of distribution and mean differences in the plantations than in the secondary forest for the following variables: total soil C and N, labile C and extractable NO_3^-.

Discussion

We investigated whether individual plant species and/or functional groups modify soil chemical properties, and whether this modification depends upon the context in which the trees are growing (i.e. in diverse forest or in monospecific plantations). Our results suggest that the effect of individual species is more pronounced than the influence of legumes as a functional group. We predicted that legumes would increase soil N availability through mechanisms such as N fixation as well as through decomposition of N-rich litter, and this would result in greater N availability under legumes than non-legume species. We found a large range of variation in soil properties under the three legume species, but little evidence for a consistent "legume effect". There were no differences in the activity of acid phosphatases by functional group. However, a species effect was discernible in a number of soil properties and the magnitude of this effect was

stronger in the plantations than in a highly diverse secondary forest. Below we discuss possible mechanisms that might account for these patterns and their implications for soil N cycling in tropical forests.

Species effects in relation to species traits

Differences in the quality of each species' leaf litter influence the rate at which this litter decays and makes mineral nutrients available to the microbial community (Hobbie, 1992). Nutrients in fresh foliage are generally a good predictor of leaf litter nutrient concentrations (Hättenschwiler *et al*., 2008), even after resorption of a portion of these nutrients (McGroddy *et al*., 2004). The differences we found in total soil C and N (Figure 1-1), in the total amount of extractable N in the soil (Figure 1-2), and in the relative amounts of NO_3^- vs NH_4^+ (Table 1-2) may reflect differences in detrital inputs to the soil, plant and microbial demand and uptake, as well as rates of microbial transformations of N. It is well known that these processes are affected by the chemical composition of organic inputs from plants, environmental factors like soil moisture (Binkley, 1995), and/or the composition of the microbial community (Porazinska *et al*., 2003). As shown in Table 1-1, Powers and Tiffin (2010) found traits in the leaves of *Acosmium* and *Gliricidia* that confer these species with the potential to have high quality leaf litter, i.e. high specific leaf area (SLA), high C and N concentrations. This may explain why, in the forest, we found the highest N mineralization rates in the soils under these species (Table 1-2). Not surprisingly, *Gliricidia* also has relatively rapid litter decomposition rates (Table 1-1). On the other hand, *Dalbergia*, and *Rehdera* share the

traits of relatively lower SLA and higher leaf C:N (Table 1-1). Greater C to nutrient ratio in leaf litter promotes the immobilization of nutrients in the microbial biomass during decomposition, and decreases rates of decomposition in the litter layer (Baillie *et al.*, 2006). Therefore, it is possible that these conditions promoted a microbial community that was more limited in N than in C and explain the fact that our laboratory incubations revealed immobilization in the soils under these species (Figure 1-1c). Finally, the oak species (*Quercus*) has leaf traits such as low SLA, leaf C, leaf N, and low decomposition rates (Table 1-1). These traits are consistent with the characteristics of the soils underneath those trees: low C and N, with the lowest extractable NO_3^- and NH_4^+, and low N mineralization and nitrification rates.

Greater species effects in plantations

As predicted, the magnitude of the species effect was more pronounced in the plantations than in the diverse secondary forest (Table 1-5). It is possible that in the forest, high species diversity has had a role in homogenizing individual species effects. Since age of trees, light environment, and soil physical properties were standardized when selecting sample trees, other processes like N uptake and resorption by the trees or understory vegetation may have contributed to the dilution of individual species effects and explain the lack of variation in soil properties and nutrient pools, especially in species-rich forests. For example, lianas may contribute to attenuating species effects by redistributing nutrients within the forest, since their canopies may be found meters away from the root zone (Powers *et al*., 2004). Also, a homogeneous tree canopy height can

influence subcanopy microclimate by reducing temperature maxima and evapotranspiration and by increasing relative humidity. In the plantations, the microenvironmental conditions could favor soil biotic activity, nutrient transformations, and improved physical conditions.

Implications for N cycling

In our study, the species effects at the plantation site are mainly due to the influence of one N-fixing species: *Acosmium*. We recorded high amounts of nodulation as well as higher root biomass in individuals of *Acosmium* in the plantation compared to the other planted species (Gei, *unpublished data*), suggesting that we sampled soils under trees that were actively fixing N. Soils under *Acosmium* trees had more resources (total C and N, labile C; Figure 1-1, b and d) and rates of N cycling (higher potential mineralization and nitrification rates; Table 1-2) that may result from high N inputs from fixation. We found high levels of both NO_3^- and NH_4^+ in this plantation (Figure 1-2b), which confirm that N is abundant for both microbial and plant uptake (Hedin *et al*. 2003). By contrast, in the forest, the soils under *Acosmium* had very low levels of NO_3^-, in the same range than the non N-fixing species *Quercus* (Figure 1-2a), but considerably faster rates of N mineralization (0.32 ± 0.36 mg N g soil day^{-1}). We propose that this difference in NO_3^- pools could be directly attributed to the process of immobilization of inorganic N into microbial or plant biomass but also to i) decreased N inputs by the lack of N fixation in *Acosmium* in the forest, and/or ii) increased N outputs through leaching, nitrification or biological uptake under *Acosmium* in the forest. We have additional reasons to believe

that nitrification might be an important pathway for N loss in this dry forest ecosystem: soils under *Dalbergia* and *Gliricidia* had consistently the highest ratios of NO_3^- to NH_4^+ in both habitats (Table 1-2). High NO_3^-:NH_4^+ ratios are indicative not only of excess N availability relative to plant demand (Neill *et al.,* 1997) but also of high NH_4^+ losses, and are correlated to high N_2O (or NO_x) fluxes (Erickson *et al*., 2002). Under these conditions, it is possible that nitrifier denitrification is also occurring and contributing in part to the production of N_2O (Wrage *et al*., 2001).

There are at least two mechanisms that explain the variation of the ^{15}N signature in soils: 1) N inputs from fixation alter the ^{15}N signature, 2) leaves and soils enriched in ^{15}N can be indicative of non-N conservative or "leaky" system where isotopically light N is lost from the ecosystem owing to fractionation during N losses (Amundson *et al*., 2003; Martinelli *et al*., 1999). Despite the fact that our study cannot resolve the causes of variation in our data, we can still provide possible explanations. We observed that the soils were more enriched in ^{15}N and had higher nutrient stocks in the plantations compared to the forest site (Figure 1-1). One possible explanation is that N is circulating in excess of plant demand.

Reasons for a lack of legume effect

A large body of evidence establishes that N-fixing species are able to increase soil fertility for the growth of other non-N-fixing species and maintain N availability in undisturbed forests (Franco and de Faria, 1997; Binkley and Giardina, 1998; Pons *et al*., 2007). This occurs mainly through the input of leaf litter high in N (McKey, 1994). This

high N-lifestyle increases N availability in the soil environment beneath them, and larger C and N stocks (Fisher, 1995; Macedo *et al*., 2008) and high rates of N transformations (Siddique *et al*., 2008) are generally found under N-fixing species. However, there are a number of mechanisms that might explain our finding that legume species did not behave in a similar way, i.e. act as a "functional group". Nitrogen fixation has been shown to work as a facultative process, which depends on environmental factors such as light, water and nutrient availability (Barron *et al*., 2010). If this is the case for our focal legume trees, then each individual could be fixing a variable amount of N, depending on the local environmental conditions. If so, our findings of the variation in soil N pools and dynamics among individuals and between legume species are not surprising.

Conclusions

This study shows great variation in the degree to which tropical legume species influence soil properties, which prevents us from drawing general conclusions about how legumes as a functional group affect ecosystem N cycling and soils. Instead, what we found were complicated patterns of effects on different aspects of soil N, which collectively suggest that N-inputs from fixation depend upon environmental conditions and context (i.e. forest versus plantation), that indices of soil N cycling such as ^{15}N are affected by both N input sources and outputs that fractionate (i.e. nitrification), and that the direction of correlations between potential N mineralization rates and variables such as labile C depend upon environmental conditions as well. It appears that in the plantations, N seemed to be cycling more in excess relative to plant demand, while in the

forest, the cycle was possibly more conservative and determined by the nutrient status of the microbial community. Future studies should consider adding microbial biomass or gross N mineralization to disentangle the role of plant and microbial demand and uptake in determining the patterns of N availability. We cannot rule out the possibility that gaseous N losses from this ecosystem are important. As predicted we found that the species effects were more apparent in the plantations, perhaps as a consequence of higher availability of resources like light, more simplified structure, and/or the lack of heterospecific trees that might "dilute" species effects. Future studies of this nature should include seasonal monitoring of soil properties as well as throughout forest regeneration, especially in seasonal dry forests.

Acknowledgments

This study was funded by a Summer Research Fellowship of the Department of Ecology, Evolution and Behavior of the University of Minnesota to M.G.G. and an NSF CAREER grant to J.S.P. (DEB-1053237). We acknowledge Milena Gutiérrez, Ronald Castro, Róger Blanco and María Marta Chavarría of the Área de Conservación Guanacaste for logistical support. We thank Daniel Pérez Avilés, Justin Becknell and Moana McClellan who provided excellent help in the field and Christopher Buyarski for his guidance in the lab. Finally, we thank Karl Ritz and the anonymous reviewers for their helpful and critical comments on the manuscript that greatly improved its quality.

Figure Legends

Figure 1-1. Soil chemical properties and enzyme activity beneath legume and non-legume tree species. (A) Total soil carbon and nitrogen in a secondary forest and (B) in plantations; (C) soil labile carbon in a secondary forest and (D) in plantations; (E) soil nitrogen isotopic composition in a secondary forest and (F) in plantations and (G) acid phosphatase activity in a secondary forest and (H) in plantations. Tree species are listed on the horizontal axis where A is *Acosmium panamense*, D is *Dalbergia retusa,* G is *Gliricidia sepium,* Q is *Quercus oleoides,* R is *Rehdera trinervis* and R is *Swietenia macrophylla*. Letters indicate significant differences by Tukey's HSD post-hoc test ($P < 0.05$). Bars indicate Standard Errors

Figure 1-2. Initial nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soils beneath different tree species in a secondary forest (A) and (B) in plantations, and beneath averaged legume and non-legume tree species in a secondary forest (C) and (D) in plantations. In (A) and (B) tree species are listed on the horizontal axis where A is *Acosmium panamense*, D is *Dalbergia retusa,* G is *Gliricidia sepium,* Q is *Quercus oleoides,* R is *Rehdera trinervis* and R is *Swietenia macrophylla*. Letters indicate significant differences by Tukey's HSD post-hoc test ($P < 0.05$). Bars indicate Standard Errors

Figure 1-1

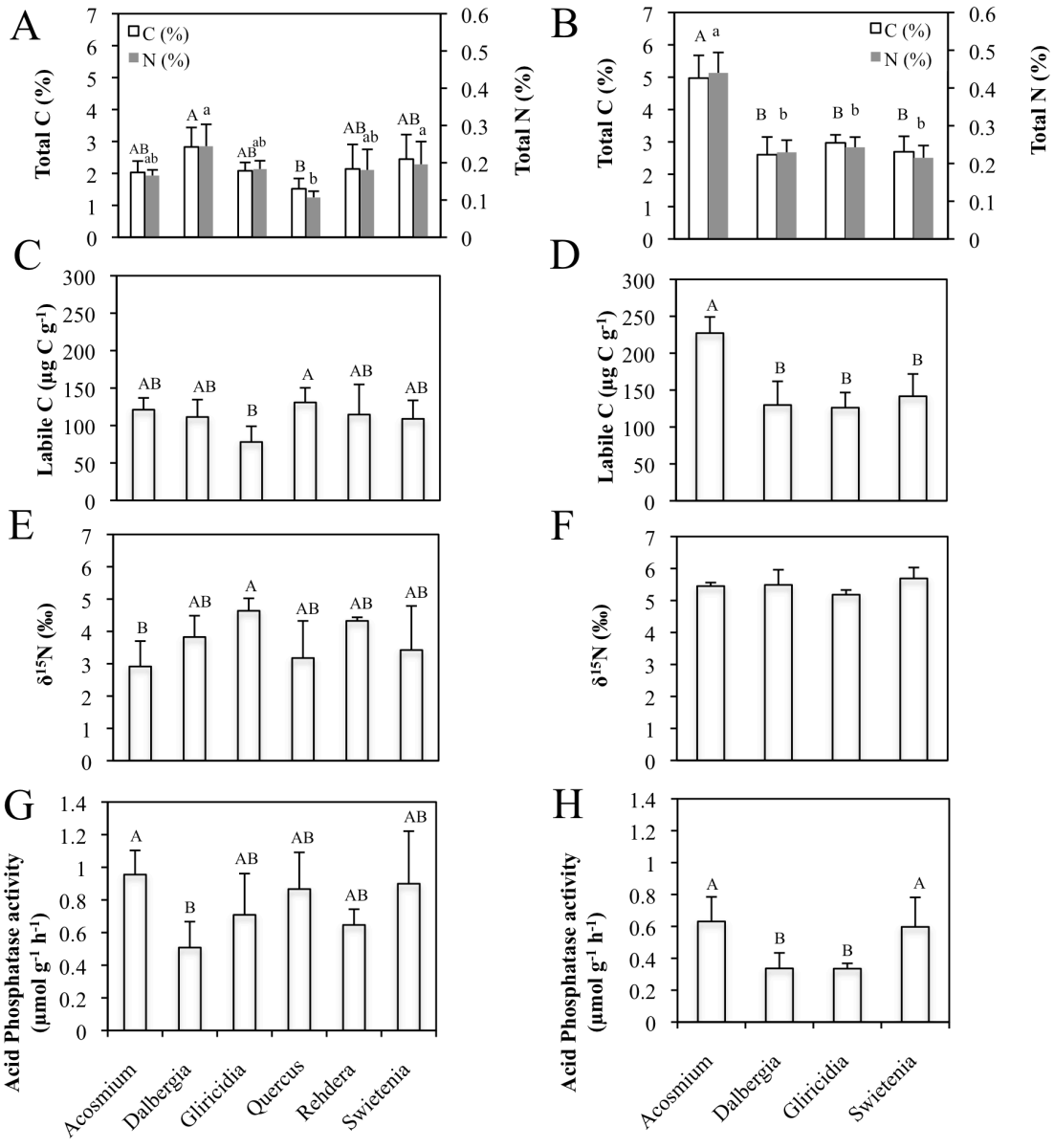
A
B
C
D
E
F
G
H
C (%)
N (%)
Total C (%)
Total N (%)
Labile C (µg C g-1)
δ15N (‰)
Acid Phosphatase activity (µmol g-1 h-1)
Acosmium
Dalbergia
Gliricidia
Quercus
Rehdera
Swietenia

Figure 1-2

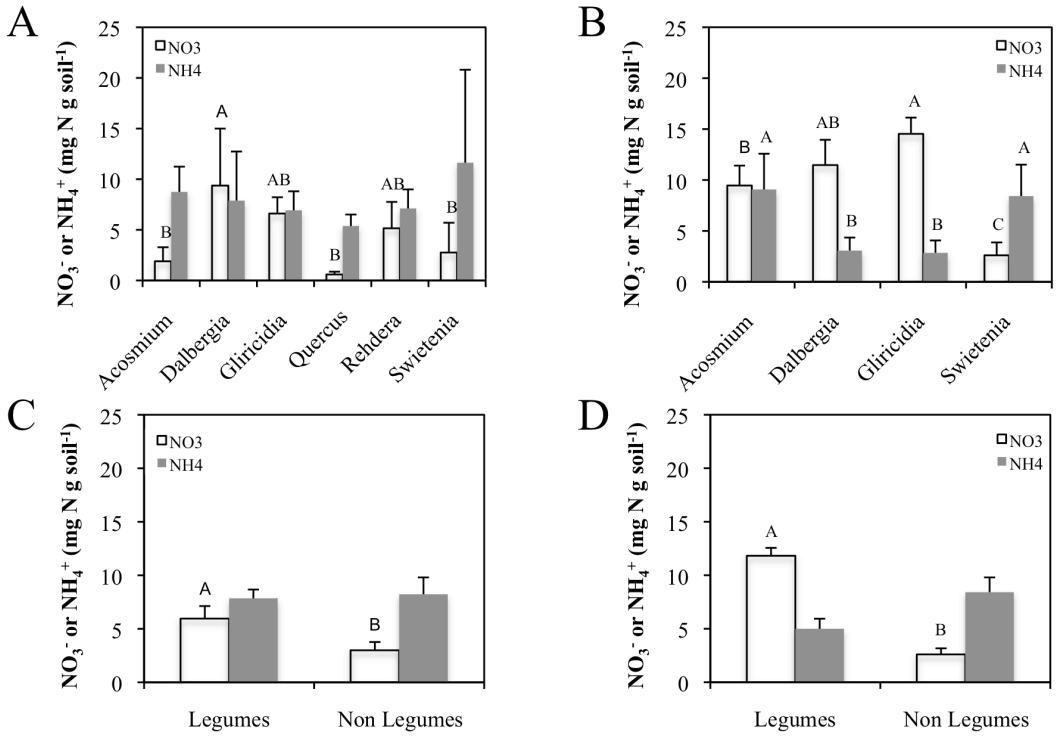
A
B
C
D
NO3
NH4
NO_3^- or NH_4^+ (mg N g soil^{-1})
Acosmium
Dalbergia
Gliricidia
Quercus
Rehdera
Swietenia
Legumes
Non Legumes

Table 1-1. Leaf characteristics and functional traits of six tree species found in Costa Rican tropical dry forests

Species	Leaf Habit	Functional Type	SLA ($cm^2\ g^{-1}$)	Wood Density ($g^1\ cm^{-3}$)	Leaf Water (%)	Leaf $\delta^{15}N$ (‰)	Leaf $\delta^{13}C$ (‰)	Leaf P ($g\ kg^{-1}$)	Leaf N ($g\ kg^{-1}$)	Leaf C ($g\ kg^{-1}$)	Decay rate * (yr^{-1})
Acosmium panamense	semideciduous	legume	92.19	0.77	58.26	-2.48	-27.86	0.7	29.3	489.6	n.a.
Dalbergia retusa	deciduous	legume	67.70	0.80	55.19	-1.70	-27.76	0.8	24.3	476.4	0.6
Gliricidia sepium	deciduous	legume	137.82	0.78	71.34	-1.06	-28.52	1.3	35.3	475.6	2.2
Quercus oleoides	evergreen	non legume	63.80	0.80	49.49	0.37	-28.27	0.7	14.6	478.9	0.5
Rehdera trinervis	deciduous	non legume	74.86	0.74	61.83	-0.55	-28.45	0.7	14.5	444.4	4.6
Swietenia macrophylla	deciduous	non legume	68.72	0.67	54.48	-0.79	-27.95	0.8	15.7	471.2	0.8

Data collected in 2008 from individuals in Área de Conservación Guanacaste (ACG). Values represent means of 1 to 7 trees, adapted from Powers and Tiffin (2010)

*Powers, *unpublished data*, decomposition rates are from one-pool models fit to data from a litterbag study. SLA: specific leaf area, n.a.: data not available

Table1-2. Nitrogen transformations and ratio of nitrate to ammonium in soils under six tree species found in Costa Rican tropical dry forests

Species	NO_3^-:NH_4^+		Net N mineralization		Net Nitrification	
	Forest	Plantation	Forest	Plantation	Forest	Plantation
Acosmium panamense	0.26 ± 0.26[b]	1.14 ± 0.39[b]	0.32 ± 0.36	0.51 ± 0.09[a]	0.26 ± 0.21	0.74 ± 0.20[a]
Dalbergia retusa	1.60 ± 1.21[a]	4.45 ± 2.16[a]	-0.02 ± 0.17	0.04 ± 0.23[b]	0.08 ± 0.30	0.12 ± 0.23[b]
Gliricidia sepium	1.06 ± 0.51[ab]	5.85 ± 2.29[a]	0.16 ± 0.13	-0.35 ± 0.19[c]	0.40 ± 0.13	-0.38 ± 0.21[c]
Quercus oleoides	0.11 ± 0.05[b]	n.a.	0.12 ± 0.15	n.a.	0.09 ± 0.08	n.a.
Rehdera trinervis	0.75 ± 0.39[ab]	n.a.	-0.09 ± 0.26	n.a.	0.14 ± 0.27	n.a.
Swietenia macrophylla	0.21 ± 0.13[b]	0.35 ± 0.23[b]	-0.03 ± 0.18	0.19 ± 0.29[ab]	0.20 ± 0.20	0.45 ± 0.32[ab]
P (Species effect)	< 0.005	< 0.0001	n.s.	< 0.0001	n.s.	< 0.0001
Legumes	0.97 ± 0.92	3.81 ± 2.65	0.16 ± 0.26	0.06 ± 0.40	0.24 ± 0.25	0.16 ± 0.51
Non Legumes	0.38 ± 0.37	0.35 ± 0.23	-0.02 ± 0.21	0.19 ± 0.29	0.15 ± 0.19	0.45 ± 0.31
P (Legume effect)	< 0.05	< 0.05	n.s.	n.s.	n.s.	n.s.

P values indicate one-way analyses of variance with "species" or "legumes vs non legumes" as the main fixed effect. Letters indicate significant differences by Tukey's HSD post-hoc test ($P < 0.05$). n.s. not significant, n.a. data not available data. NO_3^- : NH_4^+: ratio of initial extractable soil nitrate (NO_3^-) to ammonium (NH_4^+), Net N mineralization: net change in the $NH_4^+ + NO_3^-$ concentrations (mg N g $soil^{-1}day^{-1}$), Net Nitrification: net change in the NO_3^- pool (mg N g $soil^{-1}day^{-1}$). Values are means ± standard deviations

Table 1-3. Pearson's correlation coefficients of leaf traits, litter decomposition rates and soil chemical properties under six tree species in a secondary dry forest in Costa Rica (N = 6)

	SLA	Leaf N	Decay Rate	Soil total C	Soil total N	Labile C	$\delta^{15}N$	APA	Extractable NO_3^-	Extractable NH_4^+	N min	Nitrification	NO_3^- to NH_4^+
SLA	1												
Leaf N	0.852*	1											
Decay Rate	0.261	-0.045	1										
Soil total C	-0.120	0.136	-0.098	1									
Soil total N	0.036	0.266	0.017	0.982***	1								
Labile C	-0.835*	-0.668	-0.209	-0.302	-0.427	1							
$\delta^{15}N$	0.541	0.269	0.706	0.241	0.379	-0.772	1						
APA	-0.013	-0.087	-0.289	-0.573	-0.647	0.325	-0.691	1					
Extractable NO_3^-	0.245	0.385	0.160	0.756	0.843*	-0.566	0.690	-0.915*	1				
Extractable NH_4^+	-0.148	-0.062	-0.189	0.571	0.480	-0.105	-0.251	0.305	-0.041	1			
N min	0.493	0.648	-0.362	-0.465	-0.414	-0.001	-0.420	0.568	-0.394	-0.160	1		
Nitrification	0.932**	0.762	0.213	-0.118	-0.001	-0.773	0.351	0.268	0.026	0.154	0.526	1	
NO_3^-:NH_4^+	0.244	0.417	0.113	0.677	0.770	-0.508	0.651	-0.928**	0.986***	-0.177	-0.314	-0.015	1

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Units: SLA (Specific Leaf Area, $cm^2\ g^{-1}$), Leaf N (%), Decay rate (yr^{-1}), soil total C and N (%), Labile C ($\mu g\ C\ g^{-1}$), $\delta^{15}N$ (‰), APA (acid phosphatase activity, $\mu mol\ g^{-1}\ h^{-1}$), extractable NO_3^- and NH_4^+ (mg N g $soil^{-1}$), N min (net change in the $NH_4^+ + NO_3^-$ concentrations, mg N g $soil^{-1} day^{-1}$), Nitrification (net change in the NO_3^- pool, mg N g $soil^{-1} day^{-1}$)

Table 1-4. Pearson's correlation coefficients of leaf traits, litter decomposition rates and soil chemical properties under four tree species grown in monospecific plantations in Costa Rica (N = 4)

	SLA	Leaf N	Decay Rate	Soil total C	Soil total N	Labile C	$\delta^{15}N$	APA	Extractable NO_3^-	Extractable NH_4^+	N min	Nitrification	NO_3^- to NH_4^+
SLA	1												
Leaf N	0.867	1											
Decay Rate	0.995	0.843	1										
Soil total C	0.147	0.355	0.993	1									
Soil total N	0.102	0.355	0.786	0.995**	1								
Labile C	-0.081	0.126	-0.591	0.971*	0.970*	1							
$\delta^{15}N$	-0.914	-0.968*	-0.868	-0.118	-0.113	0.120	1						
APA	-0.369	-0.451	-0.401	0.608	0.569	0.742	0.609	1					
Extractable NO_3^-	0.689	0.909	0.612	0.064	0.097	-0.145	-0.924	-0.746	1				
Extractable NH_4^+	-0.391	-0.468	-0.430	0.600	0.563	0.739	0.627	0.999***	-0.755	1			
N min	-0.607	-0.418	-0.925	0.680	0.695	0.834	0.632	0.848	-0.563	0.856	1		
Nitrification	-0.640	-0.505	-0.870	0.620	0.627	0.789	0.703	0.889	-0.658	0.898	0.993**	1	
NO_3^-:NH_4^+	0.606	0.693	0.610	-0.391	-0.363	-0.577	-0.817	-0.955*	0.892	-0.961*	-0.846	-0.904	1

*P < 0.05, ** P < 0.01, *** P < 0.001. Units: SLA (Specific Leaf Area, $cm^2\ g^{-1}$), Leaf N (%), Decay rate (yr^{-1}), soil total C and N (%), Labile C (μg C g^{-1}), $\delta^{15}N$ (‰), APA (acid phosphatase activity, μmol $g^{-1}\ h^{-1}$), extractable NO_3^- and NH_4^+ (mg N g $soil^{-1}$), N min (net change in the $NH_4^+ + NO_3^-$ concentrations, mg N g $soil^{-1}day^{-1}$), Nitrification (net change in the NO_3^- pool, mg N g $soil^{-1}day^{-1}$)

Table 1-5. Mean, standard deviation, range, and highest mean difference of four soil properties in a diverse secondary forest and in monospecific plantations in Costa Rica

Variable	Forest				Plantation			
	Mean	**Standard deviation**	**Range**	**Highest mean difference**	**Mean**	**Standard deviation**	**Range**	**Highest mean difference**
Soil C (%)	2.17	0.65	2.56	1.31	3.31	1.10	3.58	2.37
Soil N (%)	0.18	0.06	0.23	0.14	0.28	0.10	0.30	0.22
Labile C (μg C g^{-1})	110.78	28.39	97.36	52.84	156.96	49.72	157.63	101.02
Extractable NO_3^- (mg N g $soil^{-1}$)	4.53	4.05	16.56	8.78	9.51	4.81	15.77	11.93

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CHAPTER 2

Surface fine root stocks are influenced by species and seasonality in plantations of four tropical legumes

Gei, M. G., and J. S. Powers. Surface fine roots are influenced by species and seasonality in plantations of four tropical legumes. *Plant and Soil*, in review.

Summary

Fine roots comprise an important and dynamic carbon pool in forests. Legumes, widespread in the tropics, have a specialized strategy of nitrogen acquisition; but the belowground dynamics of this group are still poorly understood. We studied the seasonal and spatial variation in surface fine root mass (FRM) and nodulation over two years in plantations of four legume species (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum* and *Gliricidia sepium*) in a dry forest in Costa Rica. We measured soil moisture, fine roots and nodule mass at two soil depths and at two distances from the tree bole (1 and 2 m). Mean FRM ranged from 10 to 17 g m^{-2} during the dry season to 84 to 115 g m^{-2} the following wet season. Species differed in belowground foraging strategies: *Acosmium* and *Gliricidia* had ~41% more roots in the surface layer, but in *Dalbergia* plantations, 44.3 % more roots were in the deeper layer. In *Acosmium* and *Gliricidia*, nodulation fluctuated seasonally, while the other species did not nodulate. FRM varied in synchrony with rainfall but also responded to interannual

precipitation anomalies. Thus, FRM is a sensitive component of the forest carbon pool, vulnerable to shifts in species composition and climate regimes.

Keywords: fine roots; nodulation; seasonality; legumes; tropical dry forests.

Introduction

Fine roots are plant organs with particularly high carbon (C) turnover rates in tropical forests (Mahli et al. 2009; Saatchi et al. 2011). Although fine roots may account for ~27% of net primary productivity (Mahli et al. 2011), fine roots are not as thoroughly understood as other components of terrestrial ecosystems. Fine roots, typically defined as those ≤ 2 mm in diameter, are known to be a resource-tracking organ, with biomass that typically increases under conditions of low nutrient or water availability (Espeleta and Clark, 2007; Nadelhoffer, 2000; West *et al*., 2004). In part, the dynamic nature of fine roots may contribute to the notoriously variable patterns in the spatial and temporal distributions of fine roots in forested ecosystems.

A number of studies have examined the spatial distributions of fine roots, both vertically and horizontally. Water and nutrients are distributed heterogeneously throughout the soil profile; however the most limiting nutrients to plants tend to be concentrated in shallow soils, which is reinforced by the biological cycling of nutrients coming from litterfall and throughfall (Jobbágy and Jackson, 2001). Studies from boreal to tropical forests confirm that fine root biomass is higher in the topsoil and decreases

with depth (Brassard *et al*., 2013; Castellanos *et al*., 2001; Powers *et al*., 2005; Yunusa *et al*., 2012).

The results from surveys of the spatial distribution of fine roots with distance to the tree trunk are not as consistent and include higher concentrations near the tree stem (Yanai *et al*., 2006) as well as a more homogeneous horizontal distribution of fine root biomass (Puri *et al*., 1994; Xiang *et al*., 2013). The variation in horizontal distributions of fine root biomass is likely due to a combination of extensive soil foraging by fine roots and avoidance of competition with neighboring trees. Another possible explanation for high horizontal variation in root stocks is the potential for overlapping root zones. Using DNA barcoding techniques, Jones *et al*. (2011) showed that adult canopy trees in diverse forests of Panama have high fine root overlap.

Fine root biomass also varies temporally and sometimes water availability is a cue for fine root growth. In irrigation experiments and field assessments in tropical forests, fine root biomass exhibited seasonal fluctuations triggered by changes in water availability during the transition from dry to wet season (Cavelier *et al*., 1999; Yavitt and Wright, 2001). In several highly seasonal tropical forests, fine root mass increased at the onset of the rainy season, persisted until the end of the rainy season but decreased progressively throughout the dry season (Kummerow *et al*., 1990; Kuruppuarachchi *et al*., 2013). However, not all studies have found a strong relationship between rainfall patterns and fine root stocks in seasonal tropical forests (Powers and Peréz-Aviles, 2013). Espeleta and Clark (2007) proposed a conceptual model where temporal changes in fine root stocks are more linked to seasonal nutrient pulses from aboveground litter

decomposition than to short term changes in rainfall and soil moisture, which explained intra-annual and multiyear variation in fine root biomass in a wet forest in Costa Rica.

Most studies of fine roots in forested ecosystems are conducted at the stand scale in forests with many tree species, and it is possible that trends in diverse forests obscure potentially large interspecific variation in the contribution of individual species to fine root mass. These differences result from variation among species in fine root production, turnover rates, and foraging strategies (Valverde-Barrantes *et al*., 2006). Forest plantations with fewer species are thus a valuable tool for determining fine root characteristics of individual tree species. Assessing the magnitude of the fine root belowground carbon pool, how it changes seasonally, and how this varies among species is critical to understand dry tropical forest ecosystem responses to changes in climate regimes and is also useful for designing forest and restoration strategies that maximize resource use.

In tropical forests, legumes (Fabaceae) are often one of the most abundant and species-rich plant families (Gentry, 1995; Gillespie *et al*., 2000; Pennington *et al*., 2009). Nodules are present in the roots of many legumes and house within them nitrogen (N) fixing bacterial symbionts (rhizobia). In spite of this symbiosis being a unique nitrogen acquisition strategy with important biogeochemical consequences at the ecosystem scale, repeated observations of nodulation through time are rare. The specific aims of this study were to: (1) quantify *spatial* patterns in surface fine root mass (live *and* dead) and nodulation of four dry forest tree species vertically through the soil profile and horizontally with distance from tree boles; and (2) quantify *seasonal* patterns in surface

fine roots and nodule production of these species and relate it to potential drivers by exploring the correlation between fine roots and soil moisture, and between nodule mass and soil moisture and fine root mass. To achieve those aims, we monitored soil moisture, surface fine root stocks, and nodule mass in plantations of four legume dry forest tree species in a seasonally dry region in Northwestern Costa Rica in a two-year study. Our study design allowed us to quantify spatial variation in addition to intra- and interannual changes in these patterns. We predicted that soil moisture would track precipitation seasonally and correlate with fine root and nodule mass, and that nodule mass would scale positively to fine root mass. In a previous study we found large differences in soil properties and biogeochemistry among legume species in these plantations (Gei and Powers, 2013), thus we hypothesized that surface fine root mass and nodulation would also differ among legume species.

Materials and methods

Study site

This research was conducted in several plantations of dry forest tree species located in Estación Experimental Horizontes of Área de Conservación Guanacaste (ACG; 10.84˚N, 85.62˚W) in northwestern Costa Rica. This region has a mean annual temperature of 25˚C and a mean annual precipitation of 1575 mm with a large inter-annual range from 880 to 3030 mm and a 6-month dry season that typically lasts from late November until May (Gillespie *et al*., 2000). We sampled during a year that received

above average precipitation (2552 mm during 2011) and a year during which rainfall was below average (1258 mm during 2012) (Figure 2-1; Gutiérrez, 2013). Horizontes was established in 1989 as an experimental area of ACG for research on native dry forest timber species directed towards restoration and silviculture (Elizondo and Blanco, 2010). In this area, 74 ha of forest plantations of native tree species are embedded in 7300 ha of lowland deciduous forest and abandoned pastures or fields. Before 1989, Horizontes was a farm that had sorghum, rice, and cotton fields, combined with cattle pastures (Gutiérrez, pers. comm.). These abandoned fields were cleared using a tractor and trees were planted as seedlings in blocks of 1 hectare in 1991. Soils are Inceptisols and Vertisols of volcanic origin (Czarnowski, 2002; Winters, 1995).

Tree species

We monitored belowground dynamics in 20-year-old plantations of the following species from the Fabaceae family: *Acosmium panamense* (Benth.) Yakovlev, *Dalbergia retusa* Hemsl., *Enterolobium cyclocarpum* (Jacq.) Griseb. and *Gliricidia sepium* (Jacq.) Kunth exWalp. All species are henceforth referred to by genus name only. The plantations of *Dalbergia* and *Enterolobium* consisted of 1 ha plots and those of *Acosmium* and *Gliricidia* 0.25 ha plots. The largest distance between any two plots was 1 km. The spacing between the trees is 3 x 3 m in the *Acosmium* and *Dalbergia* plots, and 3.25 x 3.5 m in the *Gliricidia* plot. In the case of *Enterolobium*, trees were planted at 6 x 6 m spacing since they were interplanted with *Dalbergia retusa*. At the end of this study, the mean diameter (at 1.3 m height) for *Acosmium* was 17.9 cm (standard deviation =

3.1), for *Dalbergia* 15.5 ± 5.1 cm, and for *Enterolobium* 49.6 ± 9.1 cm. In *Gliricidia,* which typically contained multiple stems, single stem mean diameter was 17.4 ± 3.6 cm. These species are all known for their ability to nodulate and hence their potential capacity to fix N_2 (Corby, 1988; Sprent, 2001), and in a previous study we documented soil characteristics in these plantations (Gei and Powers, 2013; Table 2-1). The understory vegetation mostly consists of grass that is regularly cut or managed with cattle grazing.

Fine root and nodule sampling

In each plantation, we chose three focal trees at random and took soil samples from below these canopies five times over a two-year period (2011 and 2012) during both dry and rainy seasons. Under each tree for each sampling period, two transects were randomly established in two directions (from a random number table) avoiding areas disturbed on a previous sampling event. In each transect, an 8 cm diameter corer was used to collect soil samples at two fixed distances from the tree trunk (1 and 2 m) and at two depths in the soil (0-15 cm and 15-30 cm), for a total of 8 cores per tree per sampling period. During our sampling in March 2012, it was not possible to collect samples from the 15-30 cm soil layer due to soil compaction and drying. In the plantation of *Enterolobium* which also had trees of *Dalbergia* interplanted, we sorted fine roots of *Enterolobium* based on root morphological features (color, width).

Fine roots (≤ 2 mm in diameter) were removed from each soil sample, carefully separated from nodules and rinsed with distilled water. After drying at 65˚C for 48 h, fine root mass and nodule dry weight were determined separately. Because we did not separate live from dead roots, we refer to fine root mass, FRM, not biomass. To measure soil moisture, a separate soil sample was taken using a 2.5 cm diameter corer at 0-15 and 15-30 cm depth within 20 cm from where root samples were taken. Gravimetric soil moisture was determined by oven-drying these samples at 110˚C for 48 h then weighing them.

Data analysis

We used multiway analyses of variance (ANOVAs) with species, sampling date, sampling depth and distance from the trunk as main effects to determine whether a) soil moisture, b) fine root mass and c) nodule mass showed species effects (differences among plantations), and differed temporally (among sampling dates) or spatially (horizontally among distances from the trunk and vertically among sampling depths) (JMP 9.0.0, SAS Institute). For each of these three models, the values of soil moisture, root and nodule mass measured in samples from the two transects were averaged for each tree, at each depth and distance from the tree. We only kept those effects that had a significant contribution to the explanation of the variation in our data. When necessary, data were transformed to improve normality. The data from March 2012, for which we did not have values from the 15-30 cm layer, were excluded to avoid model imbalance.

Finally, we examined whether soil moisture was a predictor of root mass or nodule mass, and if root mass was a good predictor of nodule mass using regression analysis. For these analyses, we pooled all samples taken under each species across all sampling points by species but not depth.

Results

There were large interannual differences in total rainfall (2192 mm in 2011 vs 1266 mm in 2012) and its distribution over the study period (Figure 2-1). During 2012, the rains started a month earlier than in 2011; however during the rainy season of 2012 (May to November) it rained during 10.9 ± 5.6 days per month compared to 18.6 ± 6.2 days per month in 2011 (Gutiérrez 2013).

As predicted, soil moisture showed strong seasonality and followed changes in precipitation in this dry forest ($F = 557.76$, $P < 0.0001$; Table 2-2, Figure 2-2). The minimum value of moisture was 5.3 % in soils under an *Acosmium* tree during the dry season in March 2012, and the maximum was 70.8 % under one of the *Enterolobium* focal trees sampled during the rainy season in June 2012. While there were significant differences among species for a given sampling date i.e. significant species by time interactions ($F = 8.75$, $P < 0.0001$; Table 2-2), seasonal differences in soil moisture were larger than species effects. Vertically, there were significant differences among soils sampled at different depths ($P < 0.0001$; Table 2-2). Soil moisture was on average 6.8 % (±17.5) higher in the deeper layer compared to surface soils. During the dry season of

2011, the difference in soil moisture between the shallow and deep layers was larger than during other times of sampling ($P < 0.01$), with the largest difference found in the *Dalbergia* plantation (68.4% higher in the deeper layer; Figure 2-2b). Although there were significant spatial patterns vertically, at both sampling depths soil moisture did not vary horizontally with distance from the tree bole in any plantation.

Mean fine root mass varied seasonally ranging from low values of 10 to 17 g m^{-2} during the dry season of 2011 to 84 to 115 g m^{-2} during the wet season of 2012 ($F = 9.35$, $P < 0.0001$; Table 2-2). In the wet season (June 2011, September 2011 and June 2012 samplings), the average FRM (0 – 15 cm) varied among species as follows: *Acosmium* > *Gliricidia* > *Enterolobium*, *Dalbergia* ($P < 0.0001$). In the dry season (March 2011 and March 2012), the average FRM was higher only in *Acosmium* compared to all other species ($P < 0.0001$), which did not differ from one another. In contrast to soil moisture, sampling depth and species effects were more important than season (Table 2-2). While species effects remained important at both sampling depths, the seasonal variation in fine roots was only significant in the 0-15 cm layer (Figure 2-2). The plantation of *Dalbergia* was an exception to this pattern: during the dry season of 2011, root mass peaked in the 15-30 cm layer (Figure 2-2b). The proportional difference of FRM between the two soil layers also varied among species ($P < 0.0001$). In *Acosmium* and *Gliricidia*, there were an average of 41.37 and 40.58 % more roots in the surface than in the deeper soil layer. In *Enterolobium*, this difference was only 3.20 % and in *Dalbergia*, we sampled 44.34 % more roots in the deeper layer than in the surface. We did not observe significant differences in the distribution of FRM with distance from tree trunks in any plantation.

Fine root biomass was not correlated to soil moisture (Figure 2-4a).

We found nodules in the roots of *Acosmium* and *Gliricidia* trees but not in the roots of *Enterolobium* or *Dalbergia*. Like root biomass, nodule biomass varied seasonally and was higher during the rainy season and almost absent during the dry season ($P < 0.05$, Table 2-2; Figure 2-3). Nodulation was higher in soils near the surface ($P < 0.05$, Table 2-2), with up to 6-fold more nodules in the upper than in the deeper soil layer. Contrary to our predictions, soil moisture was not a good predictor of nodule mass, even though these two variables had similar seasonal variation. Nodulation in *Acosmium* varied interannually: in June 2012, we found more nodules per area than during any sampling of the previous year. Nodulation in the top layer (0 – 15 cm) was higher during the dry season of 2012 compared to the dry season of 2011. Between the two sampling dates of June and September of 2011, we saw a shift in nodule mass between the two soil layers in *Acosmium*; in June, this proportion was higher in the deeper soil layer than in surface soils, but in September we observed the opposite (Figure 2-3a). Nodule mass was not explained by soil moisture or root mass (Figure 2-4b, c).

Discussion

In this study we quantified seasonal and spatial patterns in soil moisture, surface fine roots and nodulation in four plantations of legume tree species. Not surprisingly, our results support the prediction that the three variables showed strong temporal variation in this highly seasonal tropical dry forest. Our most striking results include large

differences in surface fine root stocks between June 2011 and June 2012 and species-specific variation in root depth profiles and nodulation. We hypothesize that contrasting patterns in rainfall between these two years explain the interannual differences in root mass.

The legume tree plantations that we surveyed had surface fine root stocks that were similar in magnitude to those sampled in nearby diverse dry secondary forests during the same period (Powers and Peréz-Aviles, 2013) and in other dry forests of the region (Castellanos *et al*., 2001), but our results add considerable detail to these general patterns from diverse forests. Our data show that species differed in their depth-related root foraging strategies. More specifically, the plantations of *Acosmium* and *Gliricidia* had not only higher surface fine root mass than the plantations of *Enterolobium* and *Dalbergia* but also fine roots with a more superficial distribution in the soil profile. This suggests that in a more heterogeneous tropical forest these species have the potential to exploit different belowground zones.

The species effects on the distribution of roots with respect to soil depth also extend to nodulation, as both *Acosmium* and *Gliricidia*, the two species with the highest superficial fine root distributions, were also the only two species that had nodules. In a previous study, soil from the top 10 cm in the same *Acosmium* plantation stood out from that in the *Dalbergia* or *Gliricidia* plantations with higher concentrations of labile carbon, higher N mineralization and nitrification rates, and higher phosphatase activity (Table 2-1; Gei and Powers, 2013). The leaves of *Acosmium* and *Gliricidia* have traits related to high foliar N demand i.e. high C and N concentrations (Powers and Tiffin, 2010), and the

need to maximize N acquisition may help explain why these two species exhibit marked differences in belowground traits (active nodulation and superficial roots). FRM and its superficial distribution in *Acosmium* and *Gliricidia* also reflect a response to the mineralization of nutrients from a high quality litter layer that decomposes quickly. Finally, some studies have suggested positive correlations between forest basal area and fine root mass (Finér *et al*., 2011), but our data are not consistent with this, as the rank order of fine root mass in our species did not correspond to that of basal area.

While we found strong species-specific patterns in the spatial distribution of roots through the soil profiles, we found no evidence that fine roots varied systematically with distance from the tree trunk for any of the species we studied. Because trees in the *Enterolobium* plantation were interplanted with *Dalbergia*, we were expecting to find higher horizontal variation in FRM; but that was not the case. Other studies have found that the lateral spread of the root system is more asymmetrical in closely spaced plantations than with wider spacing (Puri *et al*., 1994) but additional data would be required to determine the root spread of individual trees in these plantations.

Our data also allow us to examine a critical aspect of legume function, nodulation. Nodules are dynamic structures with life spans that range from weeks to months (Sprent, 2007) and the degree to which a tree is nodulating is commonly interpreted as an indicator of N fixation activity. Very few studies in the tropics have monitored nodulation repeatedly in the same adult tree individuals through time, beyond one snapshot sampling. The nodulation status of the trees in this study not only fluctuated throughout the year but also among species, which highlights important differences in the

contribution of legume species as a functional group to the N cycle of an ecosystem. In tropical forests, the plant-rhizobia symbiosis is thought to be a facultative process where nodulation occurs depending on a suite of local environmental conditions such as the availability of light or soil nutrients (Barron *et al*., 2010; Hedin *et al*., 2009). In these plantations, all trees have access to sufficient light, which suggests that N fixation was not energy limited. It is possible that in the plantations of *Dalbergia* and *Enterolobium*, where we did not find nodules N fixation was limited by other nutrients or down-regulated by soil N.

In our study, fine root mass, soil moisture and nodulation showed significant seasonal variation. For example, at the beginning of the 2012 rainy season fine root mass was much larger than at the same time during the previous year. We hypothesize this increase in FRM can be attributed to patterns in rainfall and to nutrient pulses in this tropical dry forest. June of 2011 received an additional 150 mm of rainfall compared to June 2012, and in June 2011 there were 26 days with rain compared to 15 days in 2011 (Gutiérrez, 2013). The year 2012 was considerably drier overall than the year 2011 and this lower water availability could be the reason for higher root production and deployment. Similarly, Green *et al*. (2005) found that standing root biomass was positively correlated with rainfall of the preceding month. Part of the fine root mass that we recorded in 2012 may represent roots that survived the dry season of 2012 as a result of the high rainfall of October 2011, one of the rainiest months recorded at the Estación Experimental Horizontes (902 mm, Figure 2-1). Surface fine roots are responsible for plant nutrient uptake and thus reflect the seasonal variation in nutrient availability in this

forest. In fact seasonal changes in fine root mass are know to be directly related to leaf litter production and decomposition (Espeleta and Clark, 2007). Perhaps, the excessive precipitation during the wet season of 2011 promoted unusual rates of nutrient leaching from the topsoil such that the high FRM that we observed in June 2012 could represent a response to pulses of nutrients after the first rains following a period of nutrient scarcity.

Soil moisture influences the establishment of a successful relationship between the plant and rhizobia by ensuring bacterial proliferation, survival, and root colonization (Zahran, 2001). This translated into a synchrony between nodulation and soil moisture in the dry forests of Chamela, Mexico (González-Ruiz *et al*., 2008). In our study, nodulation was most active during the wet season and tended to disappear during the dry season (Figure 2-3). In *Acosmium*, the interannual variation in nodule mass, most notable when comparing nodulation during June of the wet year 2011 and June of the dry year 2012, suggests that in these plantations of tropical legume tree species, water availability is an important control over fine root mass. Our study provides additional evidence of strong seasonality in soil moisture, surface fine root production, and N fixation in dry forest legumes, and identifies water as an important driver of ecosystem processes in this biome. In spite of the obvious synchronicity between fine root and nodule production with rainfall, the correlations between soil moisture and fine roots or nodules were weak (Figure 2-4). It is possible that our results reflect the influence of soil moisture on these processes through its ultimate impact on nutrient pulses and on nutrient availability in general (Espeleta and Clark, 2007). Alternately, it is possible that our snapshot measurements of soil moisture operate on a slightly different timescale than fine root

growth and decomposition, and thus are not directly coupled when sampled intermittently. The study of Powers and Peréz-Aviles (2013) in a nearby diverse tropical dry forest points to differences in soil fertility as an important potential factor determining fine root dynamics. Other factors that we did not account for during this study may also help explain the variation in FRM and nodulation to a greater degree.

Conclusions

In summary, our data show important differences in how fine roots exploit space belowground within a group of species that share a unique nutrient acquisition strategy (N fixation), the legumes. Of the four legume plantations that we studied, trees in only two plantations were actively fixing N, which suggest that N limitation in the other two plantations has been alleviated. Tree species differed in belowground foraging strategies, and this may contribute to the dynamic nature of surface fine roots in time and space in diverse tropical forests. During two years with contrasting rainfall patterns, surface fine root mass and nodulation not only changed in synchrony during the two typical seasons of dry forest (dry and wet), but also responded to antecedent conditions including precipitation anomalies, extreme events, and deviations from average. We conclude that surface fine root stocks are a sensitive component of the forest carbon pool that can be affected by shifts in species composition and changes in climate regimes.

Acknowledgements

This study was supported by a L'Oréal-UNESCO Co-Sponsored Fellowship for Young Women in Life Sciences to M.G.G and an NSF CAREER Grant (DEB-1053237) to J.S.P. We thank Daniel Peréz-Aviles, Tomás Gutiérrez, Geohanny Hernández and Ronald Castro who provided excellent help in the field. We also thank Milena Gutiérrez for providing land use history and climate data of Estación Experimental Horizontes and María Marta Chavarría of the Área de Conservación Guanacaste and Roger Blanco for logistical support. Sarah E. Hobbie and Deborah Allan provided helpful comments that improved previous drafts of this manuscript.

Figure Legends

Figure 2-1. Monthly precipitation values during 2011 and 2012 from a weather station at Estación Experimental Horizontes of the Área de Conservación Guanacaste, Costa Rica (Gutiérrez, 2013)

Figure 2-2. Seasonal changes in fine root biomass (columns) and soil moisture (triangles) at two different soil depths (0 – 15 cm in black and 15 – 30 cm in grey) in plantations of four legume species in Estación Experimental Horizontes, Costa Rica. N = 6

Figure 2-3. Seasonal changes in nodule mass (columns) and soil moisture (triangles) at two different soil depths (0 – 15 cm in black; 15 – 30 cm in grey) in plantations of a) *Acosmium panamense* and b) *Gliricidia sepium* in Estación Experimental Horizontes, Costa Rica. N = 6

Figure 2-4. Variation of fine root biomass with soil moisture in plantations of four legume species in Estación Experimental Horizontes, Costa Rica (a). Variation in nodule biomass in plantations of *Acosmium panamense* and *Gliricidia sepium* with (b) soil moisture and (c) fine root biomass

Figure 2-1

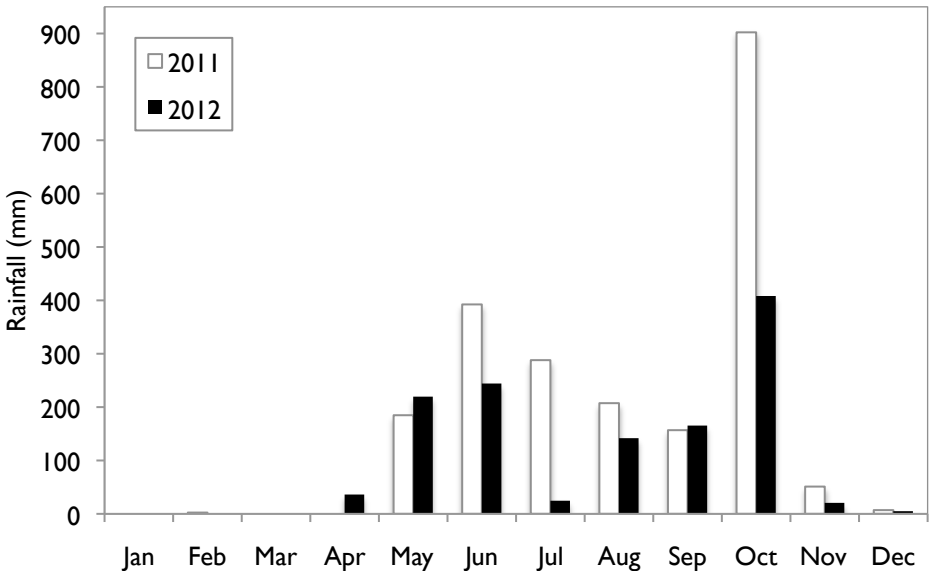
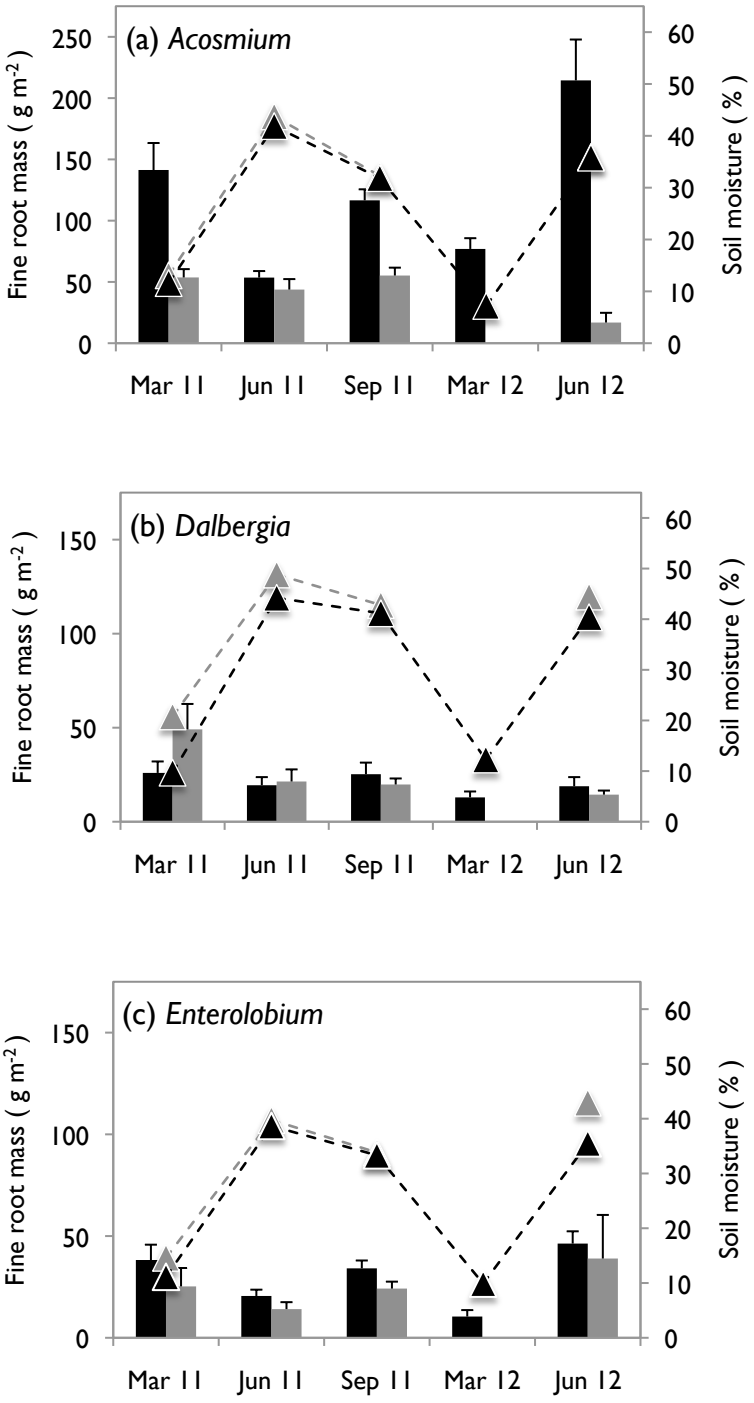

2011
2012
Rainfall (mm)
900
800
700
600
500
400
300
200
100
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 2-2


(a) Acosmium
(b) Dalbergia
(c) Enterolobium
Fine root mass (g m-2)
Soil moisture (%)
0
50
100
150
200
250
0
10
20
30
40
50
60
Mar 11
Jun 11
Sep 11
Mar 12
Jun 12

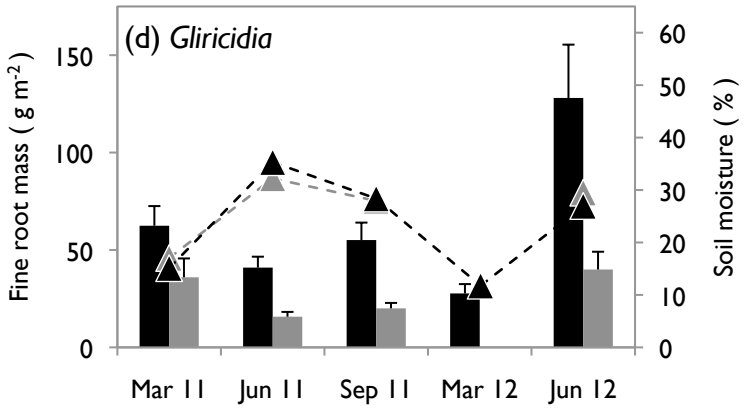

(d) Gliricidia
Fine root mass (g m-2)
Soil moisture (%)
150
100
50
0
60
50
40
30
20
10
0
Mar 11
Jun 11
Sep 11
Mar 12
Jun 12

Figure 2-3

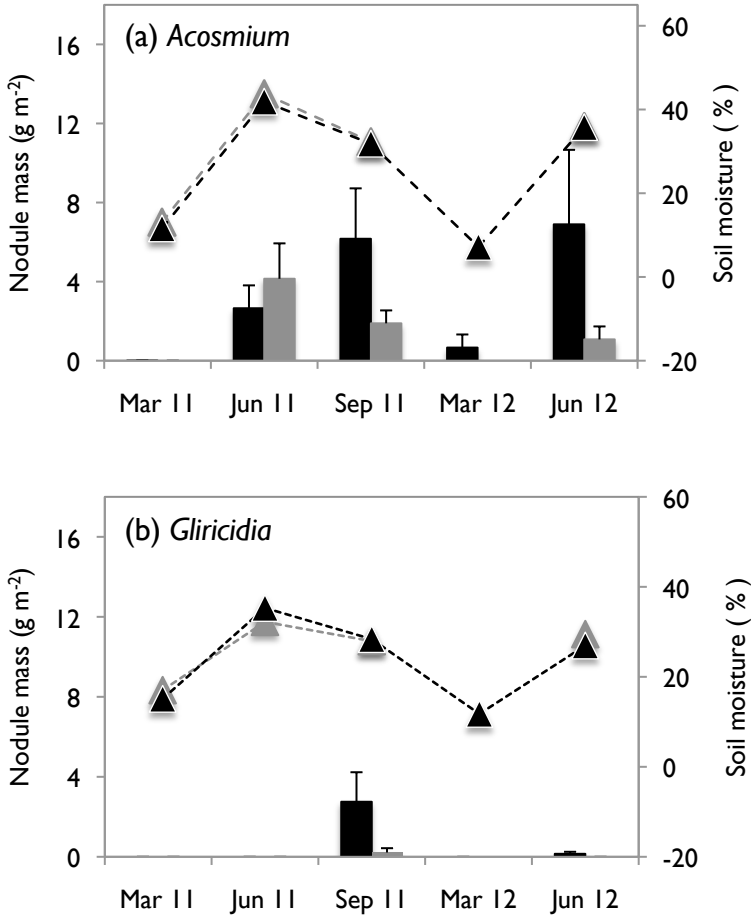
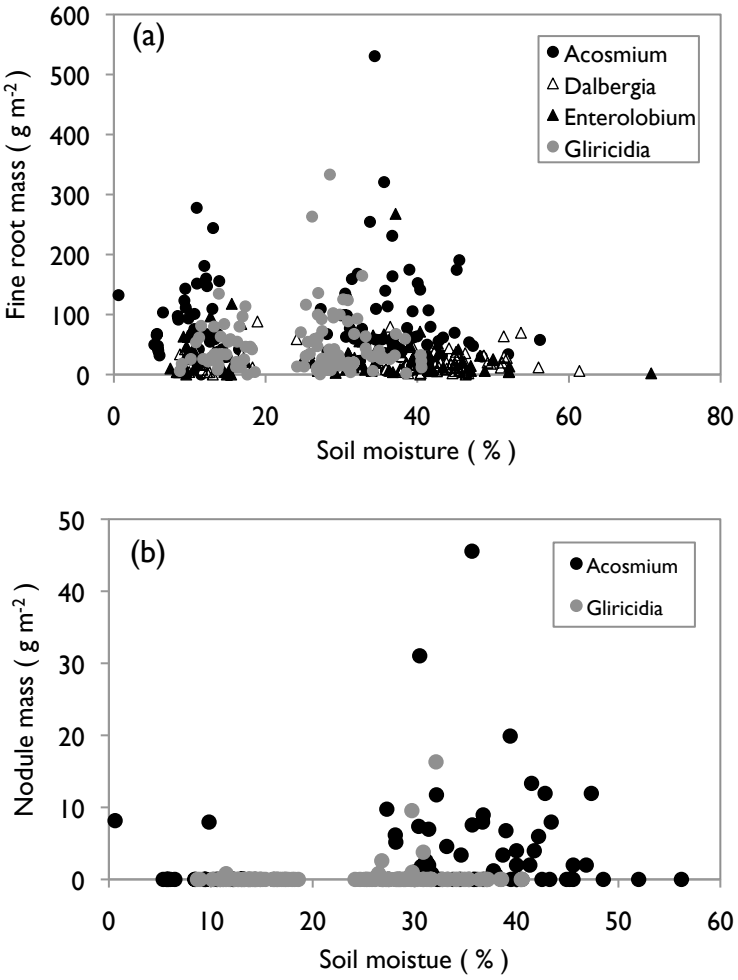

(a) Acosmium
Nodule mass (g m-2)
Soil moisture (%)
16
12
8
4
0
60
40
20
0
-20
Mar 11
Jun 11
Sep 11
Mar 12
Jun 12
(b) Gliricidia
Nodule mass (g m-2)
Soil moisture (%)
16
12
8
4
0
60
40
20
0
-20
Mar 11
Jun 11
Sep 11
Mar 12
Jun 12

Figure 2-4


(a)
Acosmium
Dalbergia
Enterolobium
Gliricidia
Fine root mass (g m-2)
600
500
400
300
200
100
0
0
20
40
60
80
Soil moisture (%)
(b)
Acosmium
Gliricidia
Nodule mass (g m-2)
50
40
30
20
10
0
0
10
20
30
40
50
60
Soil moistue (%)

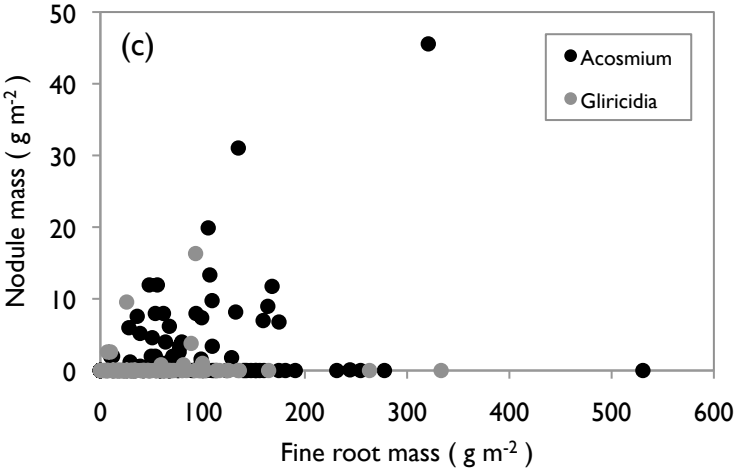

(c)
Acosmium
Gliricidia
Nodule mass (g m-2)
Fine root mass (g m-2)
0
10
20
30
40
50
0
100
200
300
400
500
600

Table 2-1. Soil properties beneath tree canopies in 1 ha, 20 yr old plantations in Estación Experimental Horizontes, Costa Rica (Gei and Powers 2013). N = 3

Plantation	pH	Soil total C	Soil total N	Labile C	$\delta^{15}N$	APA	Extractable NO_3^-	Extractable NH_4^+
Acosmium panamense	5.04	4.97 ± 0.70	0.44 ± 0.05	227.19 ± 21.88	5.45	0.63 ± 0.15	9.46 ± 1.96	9.07 ± 3.51
Dalbergia retusa	5.44	2.60 ± 0.55	0.23 ± 0.03	129.77 ± 32.09	5.49	0.34 ± 0.10	11.47 ± 2.48	3.07 ± 1.28
Gliricidia sepium	5.16	2.97 ± 0.25	0.24 ± 0.03	126.17 ± 20.52	5.18	0.33 ± 0.03	14.53 ± 1.60	2.84 ± 1.22

Units: total C and N (%), Labile C (mg C g^{-1}), $\delta^{15}N$ (‰), APA (acid phosphatase activity, mmol g^{-1} h^{-1}), extractable NO_3^- and NH_4^+ (mg N g $soil^{-1}$). Values are means ± standard deviations

Table 2-2. Results of analyses of variance of soil moisture, root and nodule biomass as functions of species, seasonality and spatial variables in four different plantations of tree species native to dry forests of Costa Rica

Model	R^2	df	Sum of Squares	F ratio	P-value
Soil moisture	0.92				
Time		3	33.99	557.76	<.0001
Species		3	1.64	26.98	<.0001
Depth		1	0.5	24.5	<.0001
Time*Species		9	1.6	8.75	<.0001
Time*Depth		3	0.73	11.97	<.0001
Species *Depth		3	0.33	5.42	<.01
Time*Depth*Species		9	0.51	2.77	<.05
Error		156	42.67		
Root Biomass	0.62				
Time		3	11.32	9.35	<.0001
Species		3	46.74	38.63	<.0001
Depth		1	17.18	42.60	<.0001
Species *Depth		3	8.39	6.93	<.001
Error		156	62.92		
Nodule Biomass	0.59				
Time		3	7.97	7.72	<.0001
Species		3	42.11	40.81	<.0001
Depth		1	1.89	5.50	<.05
Time*Species		9	15.36	4.96	<.0001
Time*Depth		3	3.64	3.52	<.05
Error		160	55.03		

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CHAPTER 3

One size does not fit all: variation among tropical dry forest legume species in nitrogen fixation and responses to above- and belowground resources in a shade house experiment

Summary

Tropical nitrogen (N) fixing legumes are assumed to finely tune N fixation to local environmental variables, yet only a few studies have tested this assertion. I examined how plant biomass and growth, and N fixation responded to light and nutrient availability in five tropical species from across the legume phylogeny, and whether legume species that share functional trait values responded to resource variation similarly. I grew four N fixing legume species and one non-fixing legume in a shade house experiment for 6 months under different light environments and nutrient fertilizations. I found that different species adjusted N fixation to environmental resources to varying degrees, showing a continuous range of phenotypes between obligate and facultative. N fixation was constrained by phosphorus (P) but species differed in their response to added P. Plant performance and N fixation showed similar levels of energy limitation where higher light promoted higher biomass accumulation, N fixation, and maximum carbon assimilation rates. These legume species were arrayed along a continuum defined by strategies of nutrient conservation and nutrient acquisition, which coincided with degrees of regulation of N fixation (obligate and facultative). Our

results show significant functional trait variation within what is considered a single, well-defined functional group (the legumes).

Key words: legume – nitrogen fixation – tropical – dry forest – nitrogen – phosphorus – ^{15}N – nodulation

Introduction

Symbiotic nitrogen (N) fixation represents the largest source of new N that fuels net primary productivity in tropical forests (Cleveland *et al*., 2013). Large losses of N from this biome (Brookshire *et al*., 2012) suggest that tropical forests are not N deficient. This implies that the ubiquity of N fixing legume trees in this region is a paradox (Hedin *et al*., 2009). This paradox has been resolved by hypothesizing that individual trees down-regulate N fixation when soil N is abundant, yet there are few studies that have tested this assertion. Studies from humid forests in Panama show that the most common legume species, particularly from the genus *Inga*, have a facultative strategy where trees actively fix N in disturbed or young forests or gaps, and down-regulate N fixation in mature forests (Barron *et al*., 2010; Batterman *et al*., 2013b). Thus, N fixation acts as a mechanism by which ecosystems overcome N limitation over successional time. The extent to which this is valid in other tropical ecosystems such as tropical dry forests is not known. Legumes are one of the most prevalent and species-rich families in tropical dry forests (Gentry, 1995; Gillespie *et al*., 2000), yet with a few exceptions (Pearson and

Vitousek, 2001; González-Ruiz *et al*., 2008; Freitas *et al*., 2010), field assessments of N fixation from tropical dry forests are scarce.

The environmental variables known to control N fixation in tropical wet forests may be different for seasonally dry forests. First, these forests are characterized by pulsed nutrient mineralization and availability as a consequence of a seasonal rainfall distribution (Lodge *et al*., 1994) and this may have implications for the regulation of N fixation. Surveys of nodulation in dry forests suggest that N fixation is indeed highly seasonal (González-Ruiz *et al*., 2008 and Chapter 2). Second, N fixers face high "operational" or carbon costs for N acquisition and their growth and persistence can be energy limited in low light environments (Gutshick, 1987). This may be less constraining in short-statured and more open-canopy seasonally dry forests (Ewel, 1977). Last, nitrogen fixers are also known to have higher phosphorus (P) requirements than non-fixers for building and maintaining the enzymes and proteins necessary for fixation and to support the high metabolic use of N (Vitousek *et al*., 2002). The availability of soil P in dry forests also fluctuates seasonally (Read and Lawrence, 2003); therefore P and water could potentially interact and constrain N fixation in these ecosystems.

McKey (1994) has argued that the energetic cost of symbiotic nitrogen fixation has been overemphasized, while the possible benefits of a nitrogen-intensive strategy are under-recognized. On ecological timescales, these benefits include the production of thin, protein-rich leaves that allow for high photosynthetic rates and rapid turnover of photosynthetic tissues in response to shifting water, light and even nitrogen resource conditions. In tropical dry forests of Mexico, those characteristics allowed N fixing

legumes to thrive in hot and dry early-successional forests (Lebrija-Trejos *et al*., 2010). On evolutionary timescales, it is possible that nitrogen-intensive traits have equipped legumes with an evolutionary advantage allowing them to adapt to a variety of environments. Because of their global distribution and higher than average diversification rate over the last 60 million years, legumes are considered one of the most successful lineages of flowering plants (LWG, 2013).

While the legume family is the third largest angiosperm family and most diverse in terms of morphology and life history (McKey, 1994), species are often treated as equivalents. For example, modeling approaches to quantify N fixation at a biome scale make the distinction between legumes with the potential to fix N and non-fixing legumes, and are parameterized solely based on the abundance of N fixing legume species in a particular ecosystem (Rastetter *et al*., 2001; Wang *et al*., 2007). Sometimes N fixing legumes are divided into obligate or facultative functional groups and all tropical legumes are assumed to have a facultative strategy (Menge *et al*., 2009). However, in seasonally dry forests the strategy to fix N may not be the only axis of trait differentiation for legumes, and other adaptations such as water use efficiency could be acting as environmental filters. Having a better understanding of the variation in resource use strategies and functional traits in legumes could make such models more accurate.

In this study, I examined how plant performance and N fixation responded to light and nutrient availability in a number of tropical species from across the legume phylogeny, and whether species that share functional traits responded to resource variation similarly. I tested this by growing four N fixing legume species (*Acosmium*

panamense, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and one non-fixing legume (*Caesalpinia eriostachys*) in a shade house experiment for 6 months under different light environments and nutrient fertilization treatments. My specific goals were threefold: *first,* to determine how above- and belowground resources affect biomass partitioning, growth rates, and leaf traits such as maximum photosynthetic rates, water use efficiency, and leaf N, and three indices of nitrogen fixation, and how this varies among species; *second*, to compare the three most common techniques for indexing N fixation (quantifying nodulation, the ^{15}N natural abundance method, and the acetylene reduction assay). At present there are no methods for quantifying N fixation *in situ,* and researchers have relied on a variety of indirect methods. However, very few studies simultaneously quantify N fixation using three independent methods. My *third* objective was to determine whether different legume strategies can be distinguished based on functional traits.

Under low light availability plants can be energy-limited to pay the carbon cost of N fixation, thus I expected that legumes would fix more N in the high light treatment. I expected that added N or P would influence N fixation by repressing nodulation in N-fertilized seedlings and enhancing it in P-fertilized seedlings. Adult trees of these species growing in tree plantations have different belowground foraging strategies (Chapter 2), thus I expected that species would show a diversity of responses to variation in above- and belowground resources. I further predicted that there is a continuum among N fixing species from obligate to facultative strategies, depending upon plant N requirements and environmental factors.

Methods

Site and Species

I grew four N fixing legume species (*Acosmium panamense* (Benth.) Yakovlev, *Enterolobium cyclocarpum* (Jacq.) Griseb., *Gliricidia sepium* (Jacq.) Kunth ex Walp., and *Lysiloma divaricatum* (Jacq.) J.F. Macbr.) and one non-fixing legume species (*Caesalpinia eriostachys* Benth.) in a shade house in Área de Conservación Guanacaste (ACG) in northwestern Costa Rica for 6 months. All species are henceforth referred to by genus name only. *Caesalpinia* was planted a month later and was grown for only 5 months. This region has a mean annual temperature of 25°C and a mean annual precipitation of 1575 mm with a large inter-annual range from 880–3030 mm and a 6 month dry season (Gillespie *et al.*, 2000). These species are known for their nodulation capacity (except *Caesalpinia*) and high local abundance in ACG and other dry forests of the Mesoamerican region (Table 3-1). I collected seeds from at least three adult trees of each species in distinct areas of ACG and planted them in ~4 L bags in a mixture of 2/3 sieved forest soil and 1/3 sand. Soil was collected from the top ~30 cm of mineral soil from a nearby secondary forest of Área de Conservación Guanacaste – Sector Santa Elena, and sieved to remove rocks, large seeds, and organic debris. Prior to planting, the seeds were scarified in hot water (100°C) for 5 min and left in cold water overnight. To provide the plants with a source of N fixing bacteria, I collected soils from under several adult trees of the target species, combined all samples into slurry and inoculated seedlings

with this mixture. All seedlings of the four N fixing species received the same inoculum when they were 1 week old.

Growing conditions and fertilization regime

The shade house was covered with two types of neutral density shade cloth that resulted in two light environments. The high light side of the shade house (referred to as "light") received an average of 58 % of full sun (standard error = 0.29), while the low light side (referred to as "shade") received an average of 25 % full sun (standard error = 0.17) (Powers, unpublished data). After planting, and during the first three months of the experiment, I rotated the seedlings within the shade house every other week so that they would receive similar amounts of light. When rainfall was insufficient, plants in both light environments were watered until saturation via an overhead sprinkler system. At three months following germination, I implemented fully factorial fertilization treatments with nitrogen (N) and phosphorus (P) (+NP, +N, +P, or no nutrients = control). The different fertilizers were applied weekly as liquid (20 mL) ammonium nitrate (NH_4NO_3) and sodium phosphate (Na_2PO_4) at commonly used rates (150 kg N ha^{-1} yr^{-1} and 50 kg P ha^{-1} yr^{-1} respectively). The concentrations of nutrient solutions were scaled down to the period of fertilization (12 weeks) and to the surface area of each bag (113 cm^2). Control plants received an equivalent volume of water. The sample size for each species was ten plants per light and fertilization treatment. In order to calculate the $\delta^{15}N$ value for each species cultivated in the absence of soil mineral N supply, I grew an additional set of 10 plants of each species in pure river sand in both light environments.

Morphological and physiological measurements

I took monthly measurements of stem height and counted the leaves of each seedling throughout five months of the experiment. Relative growth rate of height (RGRh) was calculated as follows:

$$RGRh = (\ln \text{height at } t_2 - \ln \text{height at } t_1) / \#\text{ of days} \quad \text{(equation 1)}$$

Two weeks before harvesting the seedlings, I quantified photosynthetic rates with a portable photosynthesis system (LiCor 6400; Li-Cor Inc., NE, USA) on the three healthiest looking seedlings in each treatment of the four N fixing species. Immediately following harvest, roots were washed free of soil and all nodules carefully removed from the root system. Plants were then dried at 60 ºC for at least three days. After drying, I separated aboveground tissue into stems and leaves, and all fractions (leaves, stems, roots, and nodules) were weighed separately. All leaves were ground to fine powder on a ball mill for subsequent analyses. The stable N isotope composition ($\delta^{15}N$) of leaves along with leaf C, the stable isotope of carbon, $\delta^{13}C$ (commonly interpreted as an integrated measure of water use efficiency), and leaf N were analyzed on a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility of University of California, Davis.

Soil analyses

Before planting, a subsample of the soil plus sand used in this experiment was air dried and then analyzed for total elemental concentration of nutrients by quantification

with ICP-AES (Perkin Elmer Optima 3000DV) at the Research Analytical Laboratory at the University of Minnesota (Table S3-1). One week before harvesting, I took a 10 g soil sample from the top 5 cm from 3 seedlings of each species in each light and nutrient fertilization treatment. Immediately after collection, the samples were shaken for 1 h in 50 mL of 2 M KCl solution to extract soil NH_4^+ and NO_3^-. All soil extracts were analyzed for NH_4^+ and NO_3^- with spectrophotometry following Doane and Howarth (2003). Gravimetric moisture was determined by oven-drying subsamples at 110 ºC for 48 h.

Estimates of nitrogen fixation

As there are currently no direct ways to quantify nitrogen fixation *in situ*, I used three indirect approaches to calculate the reliance of seedlings on fixation: final nodule dry mass of each seedling, nitrogenase activity as indexed by acetylene reduction activity (ARA) of nodules, and the ^{15}N natural abundance isotopic method. The ARA method quantifies activity of the enzyme responsible for nitrogen fixation, nitrogenase, and takes advantage of the fact that this enzyme also reduces ethylene to acetylene (methods for ARA follow Reed *et al*., 2007 and Barron *et al*., 2010). In brief, during the harvest, root segments with attached nodules were sealed into 45-mL clear acrylic tubes, injected with enough acetylene to create a 10% headspace concentration by volume (through a lid fitted with a septum), and vented to the atmosphere to equilibrate pressure in the tubes. Samples were incubated for 30 min and then sample headspaces were mixed, subsampled, and injected into pre-purged 10 ml Vacutainer tubes. Ethylene

concentrations were measured using a Shimadzu 14-A Gas Chromatograph equipped with a flame ionization detector (330 ºC) and a Poropak N column (110 ºC; Supelco, Bellefonte, Pennsylvania, USA). I accounted for the ethylene production by nodules in the absence of acetylene and for the ethylene produced by the injected acetylene. I reported hourly rates of acetylene reduction based on dry mass of nodules and also extrapolated them to N fixation rates to facilitate comparison with other studies. I applied the theoretical conversion ratio of 3:1 for C_2H_4:N_2 rate conversions.

The ^{15}N natural abundance method is based on different N sources having different isotopic signatures, and thus requires that the ^{15}N abundance of non-fixing reference species deviates significantly from the atmospheric abundance, and that reference and tested species extract N from the same soil sources and have similar phenology (Shearer and Kohl, 1986). In theory, N fixing plants relying solely on the atmosphere as an N source will have $\delta^{15}N$ signatures close to that of air (set to 0), while plants relying solely on mineral N sources from the soil will have ^{15}N values close to the mineral N pool. To estimate N fixation using the ^{15}N natural abundance method, I used the following mixing model after Shearer and Kohl (1986):

$$\%Ndfa = (\delta^{15}N_{reference} - \delta^{15}N_{N\text{-}fixing}) / (\delta^{15}N_{reference} - B) \times 100 \qquad \text{(equation 2)}$$

where Ndfa is the percentage of nitrogen derived from the atmosphere, $\delta^{15}N_{reference}$ is the average $\delta^{15}N$ of the non-fixing legume *Caesalpinia* grown in the same light environments with the same fertilization treatments as the N fixing species; $\delta^{15}N_{N\text{-}fixing}$ is the average ^{15}N abundance of the N fixing legume species, and B is the average $\delta^{15}N$ for each species

grown in sand, i.e. when plants are relying only on N fixation to meet all of their N requirements. This mixing model takes advantage of the difference in nitrogen isotopic composition of fixed N coming from air and the nitrogen isotopic composition of soil mineral N, estimated using non-fixing reference plants. The N isotopic composition of the legume will then depend on how much of its nitrogen was fixed or supplied by soil.

The ^{15}N abundance of the non-fixing species (*Caesalpinia*) was significantly different than that of three of the N fixing species (*Acosmium*, *Gliricidia* and *Lysiloma*, $P < 0.0001$) and was on average 2.11 ‰ higher (Figure 3-8) thus validating our use of the ^{15}N natural abundance method. Because the $\delta^{15}N$ of the non-fixing legume *Caesalpinia* differed significantly between the two light treatments, I used average *Caesalpinia* $\delta^{15}N$ values from the high or low light environments to calculate the %Ndfa of legumes in each light treatment. Any negative estimated Ndfa values were set to zero, and any higher than 100% were set to 100.

Data analysis

To understand how plant performance and N fixation varied under different resource conditions (Goal 1), I used a full factorial analysis of variance (ANOVA) model with fertilization treatment ('nutrient'), light regime ('light'), and 'species' as fixed effects and the following variables as responses: total dry mass (TDM), leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), nodule mass fraction

(NMF), leaf nitrogen and carbon content (N%, C%), leaf N isotope composition ($\delta^{15}N$), light saturated photosynthetic rate (A_{max}), water use efficiency ($\delta^{13}C$), ^{15}N natural abundance (Ndfa), and nitrogenase activity (ARA). To evaluate whether species differed in their monthly relative growth rates of height over time and across treatments, I ran mixed linear models with 'species', 'nutrient' and 'light' as fixed factors and time as a random effect, using the nlme package in R (Pinheiro *et al*., 2012). This approach is analogous to repeated measures ANOVA, but is more robust for unbalanced datasets. Then, to compare relative height growth rates through time I used a linear regression model and Tukey post hoc analyses to determine month-to-month differences. The three different methods of N fixation were compared using correlation analysis (Goal 2). I computed Pearson's product moment correlation coefficients for each pairwise comparison among all species, and also for each species separately to determine whether these correlations differed among species. Last, I examined N fixation strategies and correlations among traits (Goal 3) using several approaches. Preliminary analyses suggested that Ndfa was better correlated with plant traits and biomass partitioning than the other two N fixation methods, thus, I focused on this metric of N fixation. I used a discriminant analysis to predict the classification of the five legume species along a canonical axis that summarizes the variation in several morphological and physiological traits (total dry mass, leaf mass fraction, root mass fraction, final stem height, leaf N, water use efficiency). I repeated this analysis with the four N fixing legume species including maximum photosynthetic capacity and N fixation traits (Ndfa).

Results

Effects of light

Light had significant main effects on all response variables but ARA (Table 3-2), and while the light by species interactions were significant for all of the variables except ARA and Ndfa, there were no significant fertilization by light interactions for any response variable (Table 3-2). Seedlings grew from 27 to 78 % more total biomass in the high light side of the shade house ($P < 0.0001$); but the effects of light were more apparent belowground (Figure 3-1). The light treatment explained 35% of the variation in root mass fraction, with species allocating proportionally more biomass belowground under higher light conditions (Figure 3-1d), although this pattern varied among species (i.e. significant species by light interactions, $P < 0.0001$). By contrast, biomass allocation to aboveground tissues of stems and leaves were both slightly higher under low light conditions ($P < 0.001$ for both LMF and SMF). Overall, rates of photosynthesis were significantly higher in high light conditions (Figure 3-3); but again, this differed significantly among the four N fixing legumes with the interaction explaining 32% of the variation in the model. Seedlings of *Enterolobium* and *Gliricidia* grown in high light had higher area-based photosynthetic rates than plants grown in the shade ($P < 0.0001$ and $P < 0.01$) but this pattern was opposite for *Acosmium* ($P < 0.05$). Leaf N concentration was significantly higher in seedlings grown in low light ($P < 0.05$) but most of the variation in this trait was driven by large differences in % N among species (Figure 3-2a, Table 3-2). At the end of the experiment, available soil ammonium (but not nitrate) was significantly

higher in seedlings grown in low light ($P < 0.001$) and also varied among species (Figure 3-2d). Average levels of soil nitrogen ranged from 4.02 μg NH_4^+ g $soil^{-1}$ in *Enterolobium* pots in low light to 0.92 μg NH_4^+ g $soil^{-1}$ in *Lysiloma* pots in high light.

The values of $\delta^{13}C$ varied by 5 ‰ among species, ranging from -32.96 ‰ in one *Enterolobium* plant grown in the shade to -27.98 ‰ in one *Caesalpinia* grown in the light (Figure 3-2c). Overall, $\delta^{13}C$ was higher in seedlings grown in the high light treatment ($P < 0.0001$). Among the three N fixation estimates, the ^{15}N natural abundance method was the only one that was significantly different in the two light environments (Figure 3-9). According to this method, N fixation was higher in high light ($P < 0.0001$) and this factor alone explained almost half of the variation in this model (43 %, Table 3-2).

Fertilization effects

In general, fertilization had no or very small significant effects on most response variables with the exception of Ndfa, but did show significant interactions among species with regards to total biomass and its partitioning (Table 3-2, Figure 3-5). When phosphorus was added alone or with nitrogen, plants averaged 21 % higher total biomass compared to plants in the control treatment ($P > 0.001$). Seedlings in any fertilization treatment had higher stem mass fraction and higher final stem height compared to the control plants ($P < 0.01$ and $P < 0.001$ respectively). Across nutrient additions, relative height growth rates, rates of photosynthesis, and leaf nitrogen concentrations remained constant. I found that nodule mass fraction (NMF) and N fixation as measured by the ^{15}N natural abundance method both differed among fertilization treatments. Both NMF and

Ndfa were higher when phosphorus was added ($P < 0.01$ in both cases, Figure 3-4). However nutrient addition only explained 17% of the variation in Ndfa and 1% of the variation in NMF. The rates of acetylene reduction were not affected by nitrogen or phosphorus additions.

In the mixed linear model including time as a random effect, species identity had a significant influence on relative height growth rates but neither nutrient additions nor light environments had an effect on growth (Table 3-3). Relative height growth rates (RGR_h) remained constant during the first four months of seedling growth after which they declined considerably each month ($P < 0.0001$, Figure 3-6). This, in part, might explain the absence of effects of fertilization treatments, which were applied from month 3 onwards. The decline in stem height growth between the months of August and September coincided with the period of highest precipitation in the region and lowest light availability due increased cloud cover.

Comparison of N fixation estimates

I compared three methods of estimating N fixation: absolute nodule dry mass (NDM) or relative nodule mass fraction (NMF), the ^{15}N natural abundance method (Ndfa) and nitrogenase activity using acetylene reduction assay (ARA). NDM and NMF were highly correlated to each other and also somewhat to ARA (Figure 3-11). The two species with the highest biomass investment in nodules (*Acosmium* and *Enterolobium*)

also had higher rates of nitrogenase activity ($P < 0.0001$, Figure 3-3c). Ndfa was strongly correlated to total nodule dry mass but only for *Lysiloma* ($r = 0.60$, $P < 0.01$).

Trait correlations and nitrogen fixation strategies

Significant correlations among traits provide evidence for the "plant economics spectrum" and coordinated physiology and morphology (Reich, 2014), while differences among species provide evidence for a diversity of strategies among the legumes. When I considered pairwise correlations among some of these traits (Figure 3-12), 24 out of 28 correlations were significant ($P < 0.05$). There was a strong, positive correlation between Ndfa and total dry mass ($r = 0.55$, $P < 0.0001$). Nitrogen fixation indexed by Ndfa was also positively correlated to root mass fraction, photosynthesis rates, $\delta^{13}C$ and negatively correlated to leaf mass fraction and leaf nitrogen. Leaf nitrogen and $\delta^{13}C$ were strongly, negatively correlated ($r = -0.66$, $P < 0.0001$). Not surprisingly, root mass fraction and leaf mass fraction were negatively correlated ($r = -0.52$, $P < 0.0001$).

Species differences were significant for most of the variables measured, and in most cases explained more variation in seedling performance and N fixation than did resource treatments (Figure 3-5). Among these differences, *Acosmium* and *Enterolobium* stood out by having extreme values in several categories of biomass partitioning, physiological traits and in their photosynthesis and N fixation response to treatments. In terms of biomass allocation, *Acosmium* had higher leaf mass fraction compared to the other species and also had the lowest average SLA value (Table 3-1). On the other hand, *Enterolobium*, which had the highest SLA of all five species, had the highest total

biomass, and high final stem height and root mass fraction. Among the four N fixing species, *Acosmium* had the lowest leaf N content and the lowest photosynthetic rates while *Enterolobium* was at the top of the group for both traits. In parallel, *Acosmium* had the lowest average photosynthesis response to light and *Enterolobium* the highest ($P < 0.001$). In terms of water use efficiency, while *Enterolobium* had the lowest $\delta^{13}C$ values, this trait in *Acosmium* was on average 2.4 ‰ higher. Finally, the response of N fixation (using the ^{15}N natural abundance) to phosphorus additions showed different degrees of variation among the four legume species (Figure 3-9). Seedlings of *Enterolobium* showed the highest degree of fine tuning N fixation to P and light availability and seedlings of Lysiloma showed strong light limitation of N fixation.

A multivariate discriminant analysis successfully separated the four N fixing legume species and the non-fixing legume along the first canonical axis and explained 73.71 % of the variation in total dry mass, leaf and root mass fractions, stem height, leaf N and $\delta^{13}C$ (Figure 3-13a). When I only considered the variation of those traits plus photosynthetic capacity and Ndfa among the four N fixing species, the first canonical axis summarized and explained 70.62 % of the variation in the data and successfully separated *Acosmium* and *Enterolobium* (Figure 3-13b). In this axis, *Gliricidia* and *Lysiloma* overlapped with each other.

Discussion

In this experiment, I grew four dry forest N fixing legume species and one non-

fixing legume under different light environments and nutrient fertilization regimes. Our data suggest that seedlings switched nitrogen acquisition strategies when I added N, that N fixation was constrained by phosphorus and light availability, and that the cost of fixing N was not reflected in plant growth, biomass or photosynthesis. The different methods of estimating N fixation were not correlated to each other, but both the ^{15}N natural abundance method and absolute or relative nodule mass provided insights into how much and under which conditions the seedlings were fixing N. I saw a diversity of nutrient acquisition strategies within this sample of tropical dry forest legume tree species i.e. there are a number of ways to "be a legume". I discuss each of these points below.

Legume responses to above- and belowground resources

Tropical N fixing legume species are usually assumed to have a facultative strategy of fixation subject to environmental factors, in which case plants rely on different sources of N (fixed N or soil N). In previous studies, the response of N fixing species to fertilization with N or P was a decrease and increase of N fixation respectively (Batterman *et al*., 2013a; Pons et al., 2007). In our study, the reliance of seedlings of four legume species on N fixation changed under different nutrient fertilization treatments as well. The ^{15}N natural abundance method showed higher N fixation in plants that were fertilized with phosphorus (Figure 3-8). Like most responses I measured, the magnitude of the increase in Ndfa with added P varied among species, implying that different legume species varied in their P demand. The relatively small but positive effects of P addition on total plant biomass show that both N fixation and growth were constrained by

P. In our study, I did not detect down-regulation of N fixation with added N fertilizer in the isotopic data, i.e. Ndfa between plants fertilized with N and control plants was similar for all species. However, the $\delta^{15}N$ signature was measured on a subsample of all the leaves of each seedling and thus reflects the integrated isotopic composition of N in leaves that were produced before and after the fertilization.

Early in development, a legume seedling faces the challenge to build a photosynthetic apparatus with very high N concentrations (McKey, 1994). After seedlings exhaust seed-derived N, which can happen within a few weeks of germination (Slot *et al*., 2013), N demand must be satisfied with soil N or by fixed N, which in turn requires a large initial investment of P for the construction of nodules. In my experiment, the variation of leaf N was higher among species than among treatments, which suggests that plants have a "target" or species-specific foliar N that varies only slightly with resource availability. In order to maintain this target foliar N, the sources of N utilized by these seedlings likely varied over the course of our study and as the fertilizers were applied, from seed N reserves, to mineral N, to N fixed. During the first three months, soil N was likely an important source of N for seedlings since plants across all species and treatments had higher values of $\delta^{15}N$ than when they were grown in pure sand (Figure 3-8). In the third month, fertilizing with P contributed to the nutrient balance of the seedlings since I observed an increase in leaf production (Figure 3-7); however, different species varied in how they responded to added P in terms of N fixation. While some species (*Acosmium*, *Enterolobium*) doubled the levels of Ndfa with the extra P, in others fixation did not increase at all (Figure 3-9). Fertilizing with N reduced N fixation

at least for seedlings of one species (*Acosmium*) where relative nodulation was lower than in control plants (Figure 3-4b).

Plants in this experiment followed the model of 'functional equilibrium' in relation to above- and belowground biomass allocation in different light environments (Brouwer, 1962). According to this model, plants respond to a decrease in aboveground resources with increased allocation to shoots (leaves), whereas they respond to a decrease in belowground resources with increased allocation to roots. In the high light treatment, seedlings increased relative allocation to root mass which denotes a higher need for nutrient uptake, while under low irradiance plants allocated proportionately more biomass to leaves and stems, likely to maximize light capture (Poorter and Nagel, 2000). The increase in leaf N concentrations in plants grown in low light could mean greater fractional investments of N in chlorophyll and light-harvesting pigment-binding complexes (Evans and Poorter, 2001; Niinemets, 2007). I also recorded higher rates of maximum photosynthesis with increased irradiance probably because of increased transpiration.

It is known that N fixation imposes a major energetic cost for the plant, in terms of construction and maintenance of nodules, and of fixing N (Phillips, 1980; Gutschick, 1981), with an estimated carbon cost of approximately 6.0 g C g^{-1} N (Vance and Heichel, 1991). According to the resource optimization paradigm (Bloom *et al*., 1995, Vitousek *et al*., 2001), resources within plants should be allocated so that growth is co-limited by all external resources; in other words resources allocated towards C and N uptake should adjust so that growth is equally limited by C and N. In our shade house experiment, the

light treatments provide evidence for energy limitation of both plant performance and N fixation. Under high light conditions, plants accumulated more biomass and had higher levels of N fixation, which in turn provided N needed to support high maximum carbon assimilation rates; high amounts of C were then fixed and were sufficient to allocate to both plant biomass and the maintenance of active N fixing symbionts. Under low light conditions, photosynthetic rates were light limited, as shown by lower rates of carbon assimilation. Plants in this light environment allocated more resources towards maximizing C acquisition (higher relative leaf and stem mass) instead of N uptake (lower relative nodule mass). N fixing bacteria were less compensated by the host plant and therefore fixing N less actively. Under conditions that promoted N fixation i.e. under high light and when phosphorus was added, I observed a slight increase in total plant biomass, despite large increases in Ndfa for some species. This means that for these seedlings, the absolute costs of fixing additional N were not as high as expected.

Comparison of nitrogen fixation methods

Studies of symbiotic nitrogen fixation have rarely, if ever assessed this process using several techniques simultaneously. Here I compare estimates of N fixation through relative nodule mass, the ^{15}N natural abundance method and nitrogenase activity using acetylene reduction assay. First, the nodulation data revealed interesting aspects of how different legumes species utilize diverse strategies to fix N. For example, *Acosmium* had a disproportionately higher biomass investment into nodules compared to the other species but *Enterolobium* had higher average rates of N fixation. Possibly, differences in

the quality or compatibility with the bacterial symbiont could influence the outcome of the symbiosis (Burdon *et al*., 1999). Further information is needed to completely understand the relationship between these species and the bacterial symbiont.

The Ndfa method has assumptions and caveats too. In the field, the use of the ^{15}N natural abundance technique is often problematic because of the lack of a distinct soil end member (Boddey *et al*., 2000). Using this approach with our data was appropriate because our experimental setup resolved for distinct reference values and because I calculated species-specific B values (equation 2). Moreover, the values of $\delta^{15}N$ in the non-fixing legume *Caesalpinia* were significantly different under different nutrient fertilizations and light environments, which suggest that experiments using the ^{15}N natural abundance method benefit from comparing to reference species grown under the same conditions as the N fixing plants. This method allowed me to detect changes in N fixation between treatments. For example in *Enterolobium* N fixation indexed by Ndfa increased in plants that received P while changes in nodule mass fraction were not detectable.

Our measurements of ARA indicated rates of N fixation (average 29.94 ± 22.82 μmol C_2H_4 g^{-1} h^{-1}; range 1–85 μmol C_2H_4 g^{-1}) somewhat higher than most other studies of tropical legumes (2–28 μmol C_2H_4 g^{-1} h^{-1}; Roskoski and Van Kessel, 1985; Walter and Bien, 1989; Pearson and Vitousek, 2001; Barron *et al*., 2010). However, a few other studies reported higher rates: 30–80 μmol C_2H_4 g^{-1} h^{-1} in Thomas *et al*. (2000) measured in three-month old seedlings of *Gliricidia sepium*, and Tilki and Fisher (1998) measured

rates of 17–130 μmol C_2H_4 g^{-1} h^{-1} in adult trees of several legume species. One possible explanation for the high ARA rates is that nodule activity is much higher under our experimental "controlled" conditions in the shade house than in the forest, with rapidly growing seedlings and where ARA rates were measured in nodules in intact condition still attached to the stem.

In general, the correlations among different methods used to quantify N fixation were low. Although our data do not allow us to suggest which method is “best”, I speculate that different methods address slightly different aspects of N fixation. One potential explanation for the lack of correlation between different methods is that each approach operates at different timescales. While our $\delta^{15}N$ data summarized the N acquisition history throughout the lifespan of leaves of different age, the relative nodule mass of seedlings quantifies potential N fixing activity of the plant based on all nodules regardless of their N fixing status at the time of sampling, and ARA reflects the nitrogenase activity at one point in time of one or a few nodules from each plant. It is possible that nodule mass and Ndfa are not well correlated because nodule activity does not scale with nodule mass; many nodules in our final weights might have been old or inactive. The only instance where these two methods were correlated was with *Lysiloma*; however I interpret this correlation with caution since for this species Ndfa values were calculated using $\delta^{15}N$ from adult trees as a reference, instead of $\delta^{15}N$ from seedlings of the non-fixing legume grown in this experiment.

How to "be a legume"

Traditionally, N fixing plants are divided into obligate or facultative functional groups and it has been assumed that in tropical forests N fixation in most legume trees is facultative (Hedin *et al*.; 2009; Menge *et al*., 2009). Among the four legume species that I studied, I observed a variety of fixation strategies that cannot be classified as either perfectly facultative or obligate. First, the proportional investment in nodulation varied among species: the relative biomass allocated to nodules in *Acosmium* was at least twice that in the other three species. Even though *Acosmium* was the only species where I observed nodulation responses to nutrient treatments, its high biomass allocation to nodules in plants grown in soil across all nutrient treatments was not statistically different from plants grown in sand with no N source other than atmospheric N_2 (Figure 3-9). In contrast, for both *Enterolobium* and *Lysiloma*, relative nodule mass in plants grown in sand was higher. This implies that different legume species vary in the degree to which they switch between diverse sources of N during early seedling growth. Second, the degree to which Ndfa increased under high irradiance varied among species: *Enterolobium* and *Lysiloma* fixed significantly more N in high light compared to low light but in *Acosmium* and *Gliricidia* that was not the case. I propose that *Acosmium* has a less versatile strategy of N fixation than *Enterolobium* or *Lysiloma*. In a separate study of roots and nodulation in 20 year-old plantations of three of the same legume species used in this shade house experiment, I found that trees of *Acosmium* allocated resources to nodules continuously but I did not find any nodules under trees of *Enterolobium*. This

suggests that the N fixation strategies that I observed in our shade house experiment persist beyond the seedling ontogenetic stage and may be characteristic of each species.

Most importantly, the way that these legume species differentiate themselves vis-à-vis N fixation is correlated to an axis of traits that typically defines whole-plant nutrient economies, and sorts species' strategies along the continuum of nutrient conservation and nutrient acquisition. For several traits, *Acosmium* and *Enterolobium* had extreme and opposite trait values and at the same time, these two species show high and low levels of fine-tuning N fixation to environmental resources. On one end, *Acosmium* possessed traits that denote high nutrient conservation; for example, high wood density, low SLA, low leaf N, low maximum photosynthetic capacity, high water use efficiency and high leaf mass fraction. On the other hand, *Enterolobium* had traits that maximize nutrient acquisition: low wood density, high SLA, high leaf N, high maximum photosynthetic capacity, low water use efficiency, high root mass fraction and as a result high total plant biomass, high final stem height. This trait correlation underlines great variation in water acquisition as well as nutrient use strategies in seedlings of dry forest tree species.

Even though our study included a limited number of species, it highlights the fact that there are different ways to "be a legume". Two species clearly featured opposite trait values while trait variation in the other two species was found in between. I modified definitions from Reich (2014) to describe "fast legumes" as those species capable of moving water rapidly, and with low tissue density (high SLA), short tissue life span (including nodules) and high rates of resource acquisition and flux (including

photosynthesis and N fixation). “Slow legumes” are less able to move and lose water, have high tissue density (low SLA and high nodule mass fraction), longer tissue life span (constant nodule biomass) and lower rates of resource acquisition and processing (lower photosynthesis and less fine-tuning of N fixation).

The group of legumes features great morphological and ecological diversity, with large ranges of character variation, life forms, life histories and geographical distributions (McKey, 1994). I propose that the versatility of legumes equally extends to their strategy of fixing N. It is possible that the legume lineage has an influence on the degree to which the plant regulates N fixation. Furthermore, slow species are drought tolerant and have low nutrient requirements but on the other hand, fast species grow best and dominate in higher resource conditions (Reich, 2014). Lopez-Iglesias and others (2014) also found species sorting along a drought performance continuum defined by extremes of acquisitive fast-growing species, achieved by soft, thin and physiologically active leaves but with low drought survival, and conservative species, with slow growth, C rich and sclerophyllous leaves, but high drought survival (Lopez-Iglesias *et al*., 2014). In tropical dry forests, the slow and fast legume strategies may represent different ways of dealing with the transient and seasonal water availability of this ecosystem where slow legumes could be more adapted to survive long dry seasons but fast legumes able to fix N and recover faster as soon as conditions become favorable. Our results underscore the importance of trait variation among legume species; with this new perspective, further research should focus on the resilience of slow and fast legumes to expected reduction in precipitation for this region (Christensen *et al*., 2013).

Conclusions

Seedlings of different nitrogen (N) fixing legume species adjusted fixation to environmental resources to varying degrees following a continuous range of phenotypes between obligate and facultative. These species possibly relied on a combination of sources of N (seed N, soil N or fixed N). Phosphorus (P) fertilizer increased N fixation but species differed in their response to added P. I observed similar energy limitation of plant performance and N fixation since high light conditions promoted higher biomass accumulation, N fixation, and maximum carbon assimilation rates. These legume species are arrayed along a continuum defined by nutrient conservation (high wood density, low SLA, low leaf N, low maximum photosynthetic capacity, high water use efficiency, high leaf mass fraction, lower growth rates) and nutrient acquisition (low wood density, high SLA, high leaf N, high maximum photosynthetic capacity, low water use efficiency, high root mass fraction and as a result high total plant biomass, high final stem height, high growth rates). These slow and fast strategies coincided with degrees of regulation of N fixation: obligate and facultative respectively. Our results show great functional trait variation within what is considered a single, well-defined functional group (the legumes) and certainly part of the great evolutionary success of the legume family could be attributed to this diversity of strategies.

Acknowledgements

This study was supported by a L'Oréal-UNESCO Co-Sponsored Fellowship for Young Women in Life Sciences and a research fellowship from the Ministry of Science and Technology of Costa Rica to M.G.G., and NSF CAREER Grant (DEB-1053237) and a UMN Institute of the Environment Resident Fellowship to my advisor, Jennifer S. Powers. I thank German Vargas and Jairo Hidalgo for help with the photosynthesis measurements and Roberto Cordero for generous loan of his LiCOR. Daniel Pérez, Christina Smith, Tomás Gutiérrez, Dinia Ruiz, Geohanny Hernández provided excellent help to set up and harvest this experiment. I also thank María Marta Chavarría and Roger Blanco of Área de Conservación Guanacaste for logistical support.

Figure legends

Figure 3-1. Variation in total plant biomass (a) and partitioning among leaf (b), stem (c) and root fractions (d) among seedlings of four dry forest legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and one non-fixing legume (*Caesalpinia eriostachys*) grown under high (gray boxes) and low irradiance (black boxes). The line within the box shows the median of the data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points

Figure 3-2. Variation in (a) leaf nitrogen, (b) C:N, (c) water use efficiency, and (d) soil available ammonium among seedlings of four dry forest legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) grown under high (gray boxes) and low irradiance (black boxes). The line within the box shows the median of the data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points

Figure 3-3. Variation in (a) maximum carbon assimilation, (b) relative nodule biomass, (c) nitrogenase activity in nodules of seedlings of four dry forest legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) grown under high (gray boxes) and low irradiance (black boxes). The line

within the box shows the median of the data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points

Figure 3-4. Variation in (a) relative nodule mass of seedlings of four dry forest legume species between different nutrient treatments, (b) relative nodule mass of seedlings of *Acosmium panamense* between different nutrient treatments, and (c) the proportional reliance of seedlings of four legume species on N fixation using the ^{15}N natural abundance indirect approach. The line within the box shows the median of the data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points

Figure 3-5. Proportion of the total variance explained by factors with significant contribution ($P > 0.05$) in full factorial analyses of variance. Models were run for each response variable (X axis). Covariate interactions were not included. TDM = total dry mass, LMF = leaf mass fraction, SMF = stem mass fraction, RMF = root mass fraction, NMF = nodule mass fraction, A_{max} = maximum photosynthetic capacity, RGR_h = relative growth rate of the seedling height, Ndfa = the reliance of the N fixing legumes on atmospheric nitrogen

Figure 3-6. Relative growth rate of the height of four N fixing and one non-fixing legume species grown in a shade house and monthly precipitation values for the period of the experiment in Área de Conservación Guanacaste, Costa Rica

Figure 3-7. Average number of leaves in seedlings of four N fixing and one non-fixing legume species. Bars represent standard error

Figure 3-8. Average ^{15}N natural abundance measured in the leaves of seedlings of four N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) grown in soil with different nutrient additions (gray bars) and grown in sand with no nitrogen added ("Sand", white bars). The black bars ("Ref") represent the average ^{15}N of one reference non-fixing reference legume (*Caesalpinia eriostachys*)

Figure 3-9. Proportional reliance of four legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) on N fixation using the ^{15}N natural abundance indirect approach. The bars represent the percentage of N derived from the atmosphere in 6-month old seedlings grown in shade (black) or light conditions (white)

Figure 3-10. Variation in biomass allocation to nodules (a) and nitrogenase activity in nodules (b) of seedlings of four legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) grown in pure sand with no

nutrient additions (gray boxes) or in soil with nitrogen and phosphorus fertilizations (black boxes). The line within the box shows the median of the data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points

Figure 3-11. Comparison of three indirect approaches to estimate N fixation in seedlings of four tropical legume species: reliance of plants on fixation using the ^{15}N natural abundance method (% Ndfa), total dry nodule mass of each seedling (TNM, g) or nodule mass fraction (NMF, mg g^{-1}) and nitrogenase activity measured as acetylene reduction assay (ARA, μmol C_2H_4 g^{-1} h^{-1})

Figure 3-12. Scatterplots showing bivariate relationships between total dry mass (TDM), leaf mass fraction (LMF), root mass fraction (RMF), final stem height, maximum photosynthetic capacity (Amax), leaf nitrogen (N%), water use efficiency ($\delta^{13}C$) and the reliance of the N fixing legumes on atmospheric nitrogen (Ndfa). Statistically significant Pearson's correlation coefficients ($P < 0.05$) are indicated in blue

Figure 3-13. (a) Discriminant analysis including total dry mass (TDM), leaf mass fraction (LMF), root mass fraction (RMF), final stem height, leaf nitrogen (N%), and water use efficiency ($\delta^{13}C$) between four N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and one non-fixing legume (*Caesalpinia eriostachys*). (b) Discriminant analysis including same traits as in (a) plus maximum photosynthetic capacity (Amax), and the reliance of the N fixing

legumes on atmospheric nitrogen (Ndfa) between four N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*)

Figure 3-1

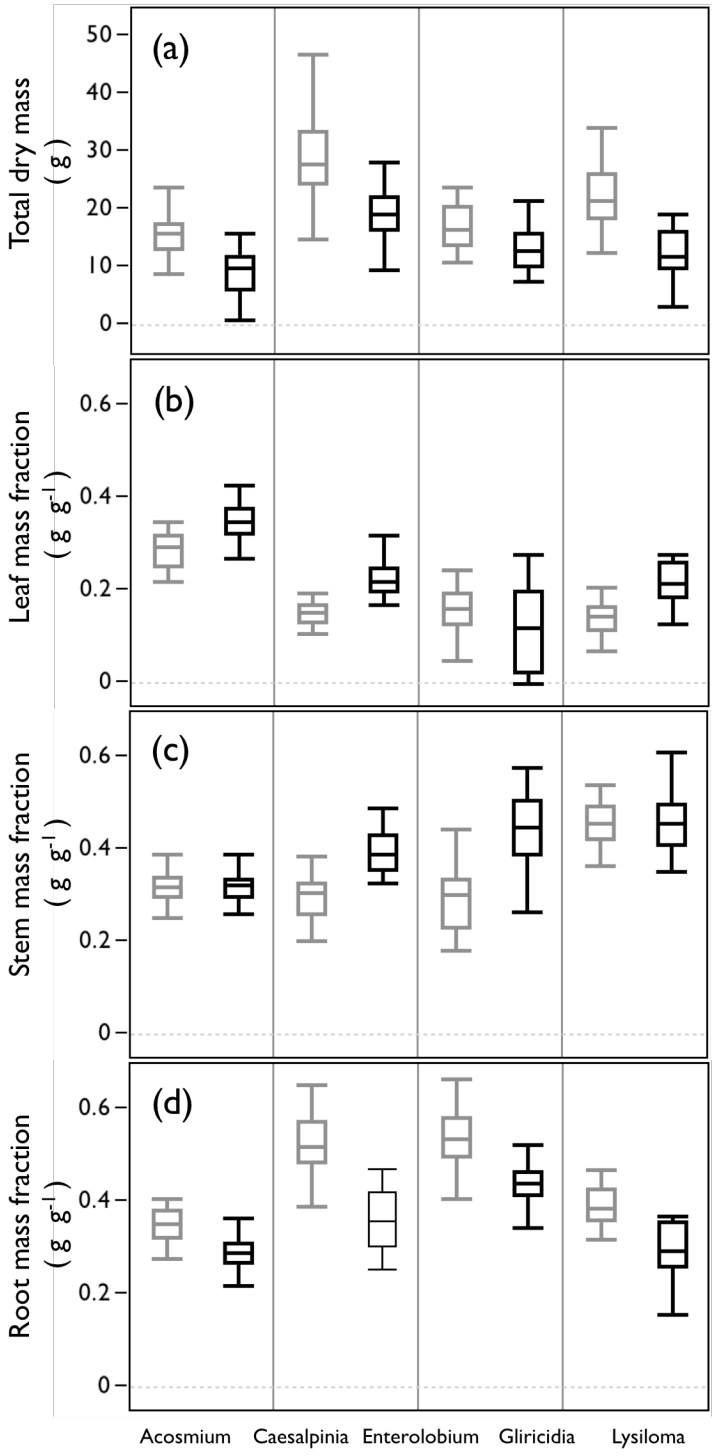

(a)
Total dry mass (g)
50
40
30
20
10
0
(b)
Leaf mass fraction (g g-1)
0.6
0.4
0.2
0
(c)
Stem mass fraction (g g-1)
0.6
0.4
0.2
0
(d)
Root mass fraction (g g-1)
0.6
0.4
0.2
0
Acosmium
Caesalpinia
Enterolobium
Gliricidia
Lysiloma

Figure 3-2

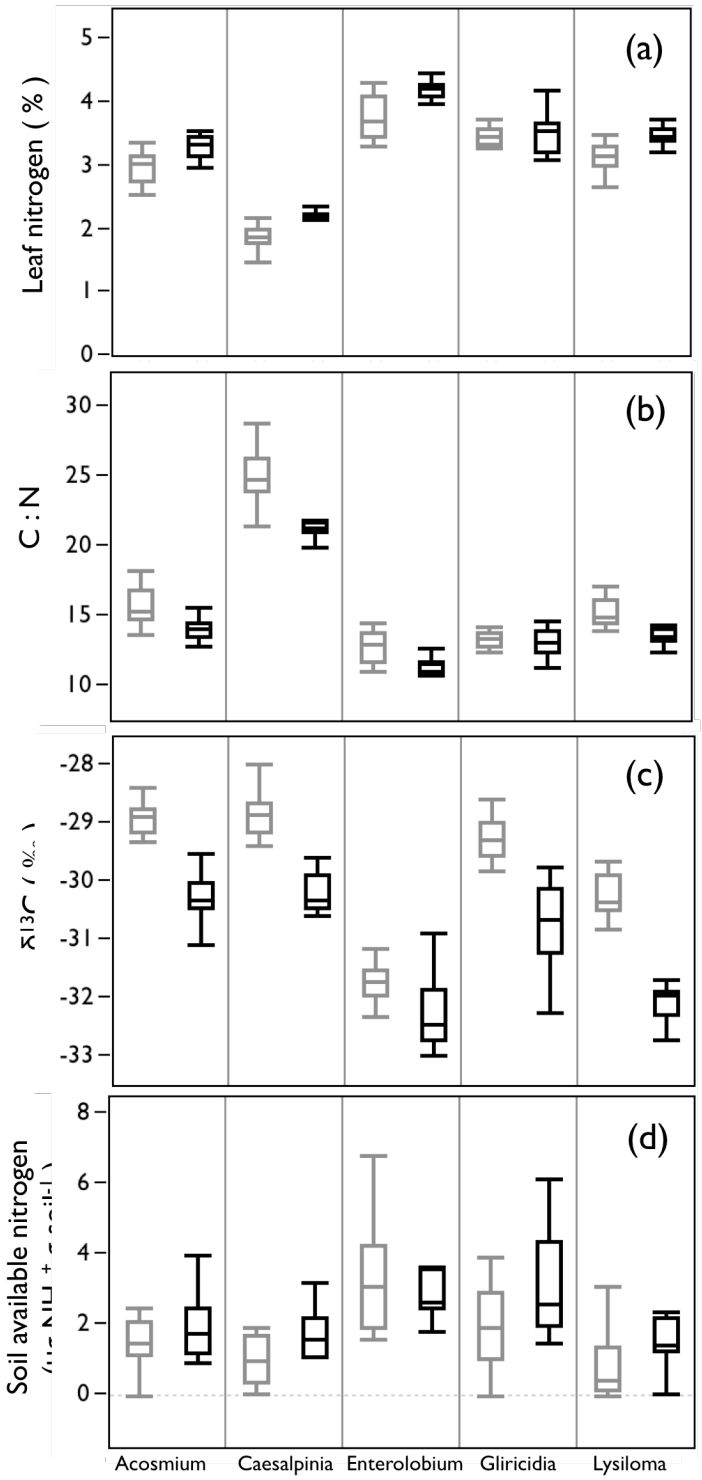

Leaf nitrogen (%)
(a)
C : N
(b)
(c)
Soil available nitrogen
(d)
Acosmium
Caesalpinia
Enterolobium
Gliricidia
Lysiloma

Figure 3-3

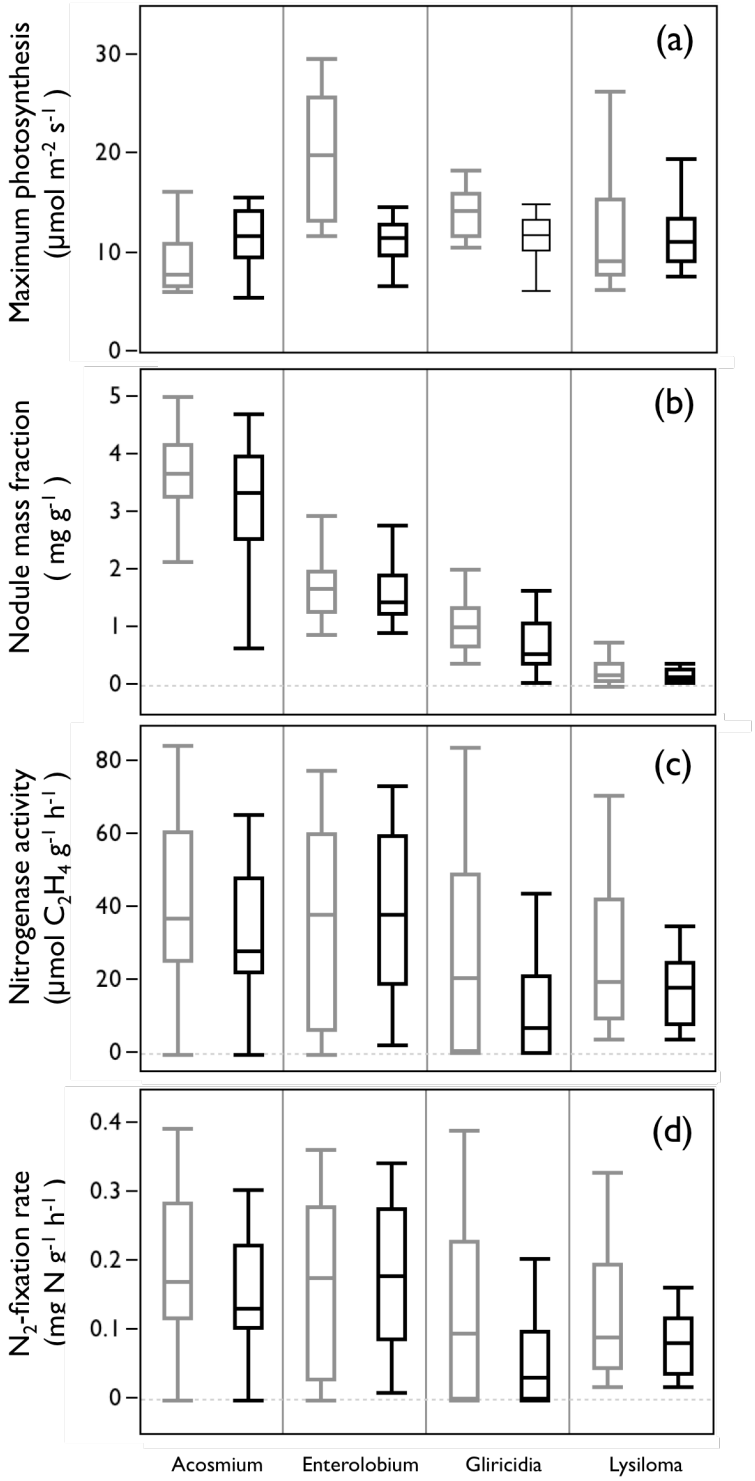

Maximum photosynthesis (μmol m-2 s-1)
(a)
30
20
10
0
Nodule mass fraction (mg g-1)
(b)
5
4
3
2
1
0
Nitrogenase activity (μmol C2H4 g-1 h-1)
(c)
80
60
40
20
0
N2-fixation rate (mg N g-1 h-1)
(d)
0.4
0.3
0.2
0.1
0
Acosmium
Enterolobium
Gliricidia
Lysiloma

Figure 3-4

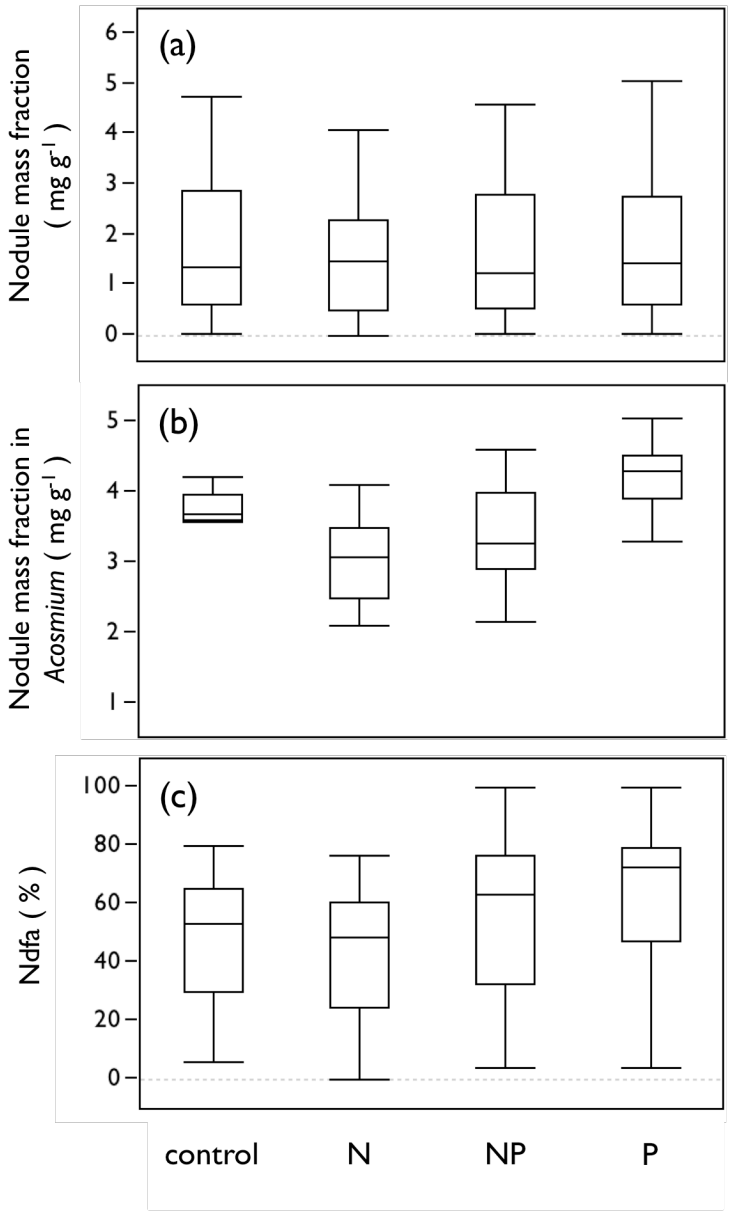

(a)
Nodule mass fraction (mg g-1)
6
5
4
3
2
1
0
(b)
Nodule mass fraction in Acosmium (mg g-1)
5
4
3
2
1
(c)
Ndfa (%)
100
80
60
40
20
0
control
N
NP
P

Figure 3-5

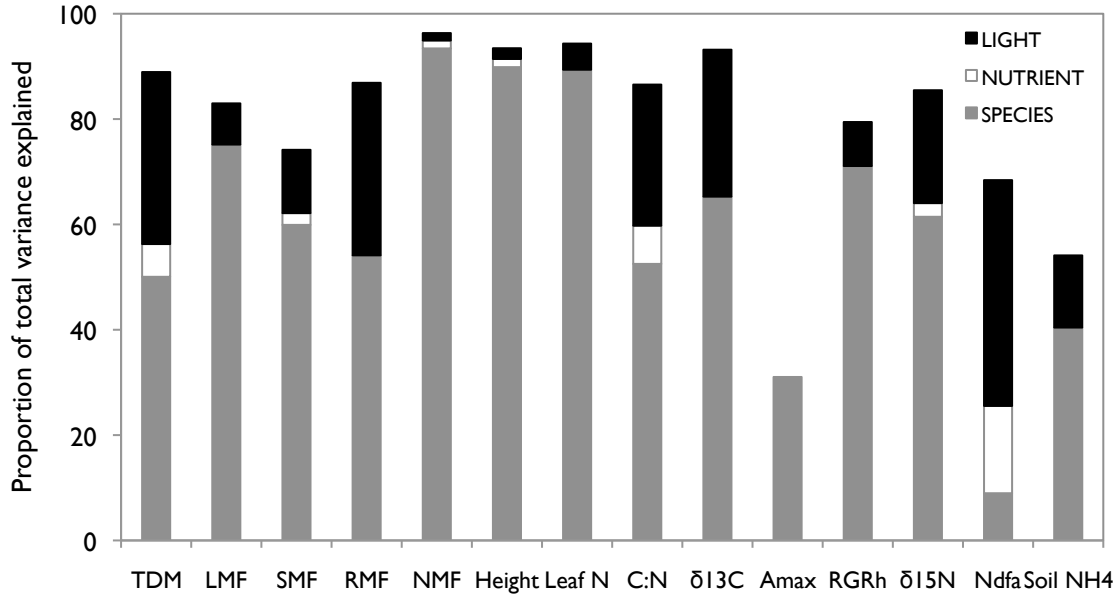


Figure 3-6

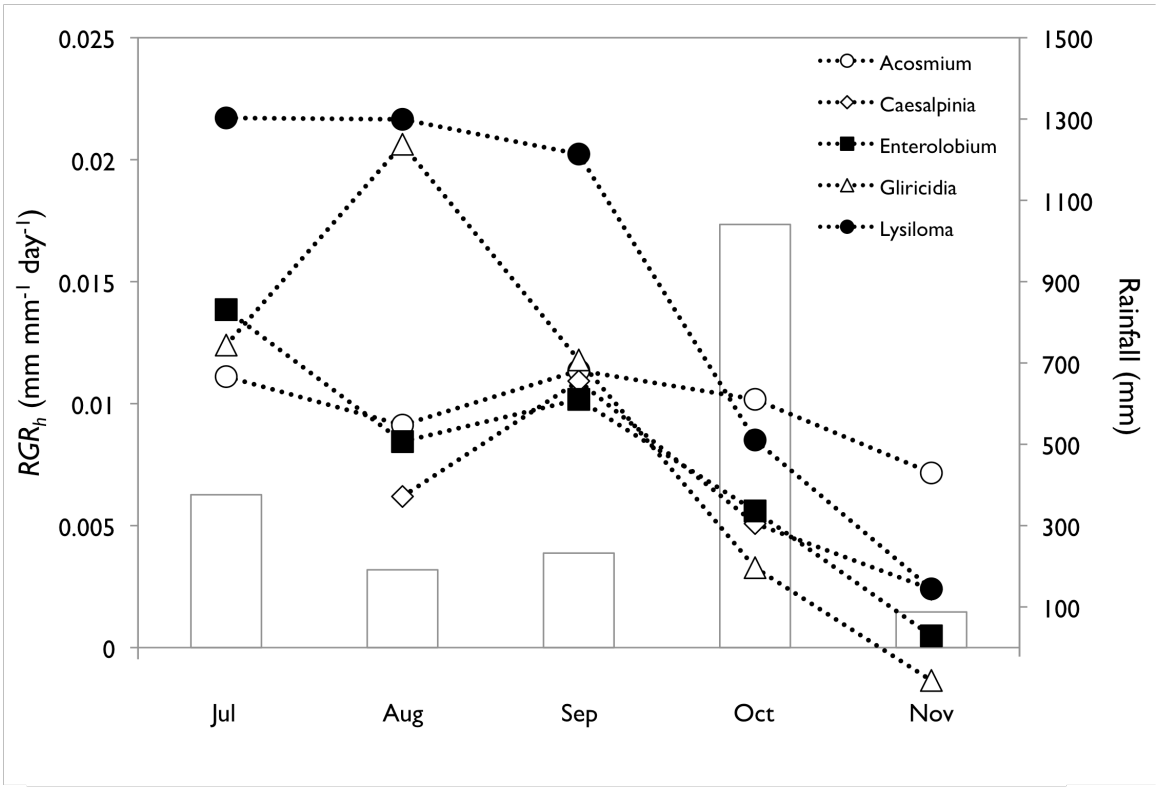


Figure 3-7

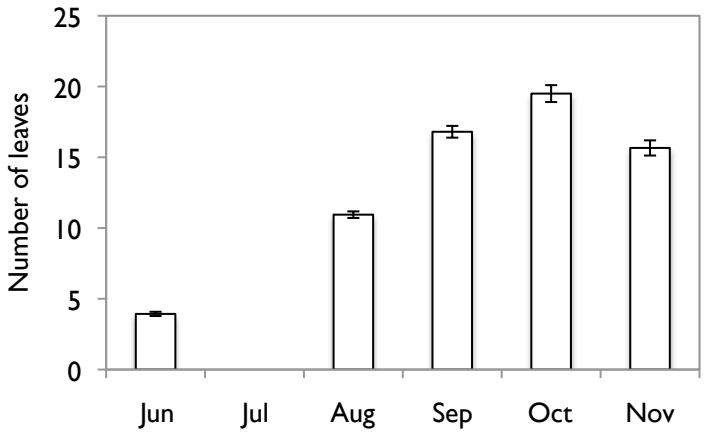


Figure 3-8

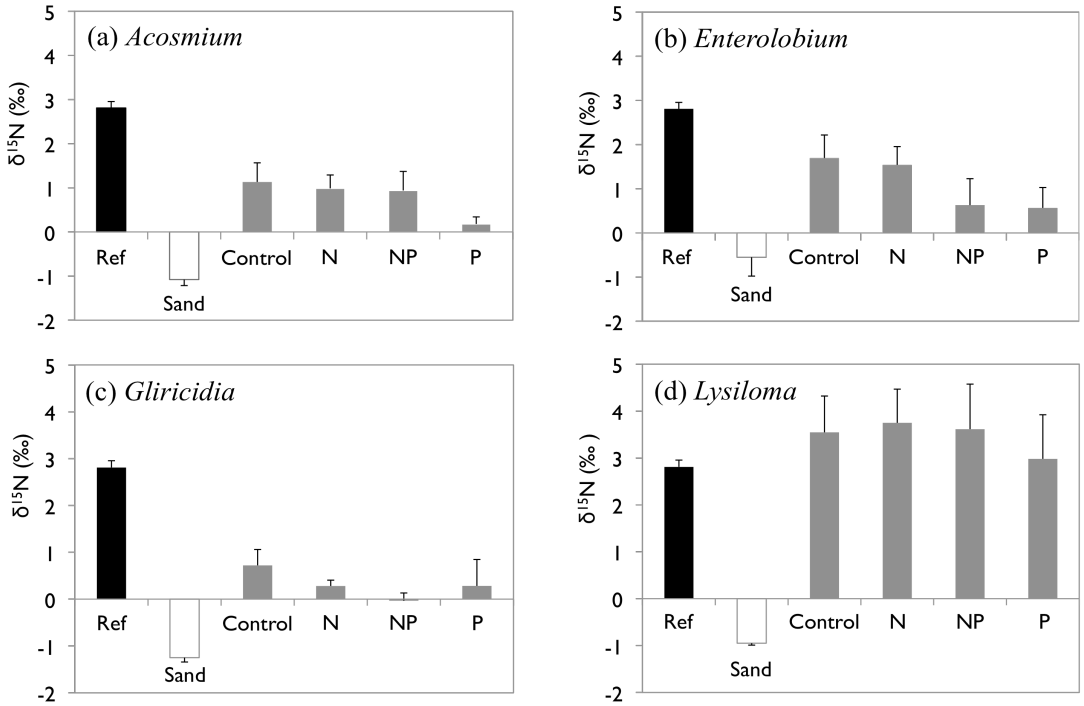
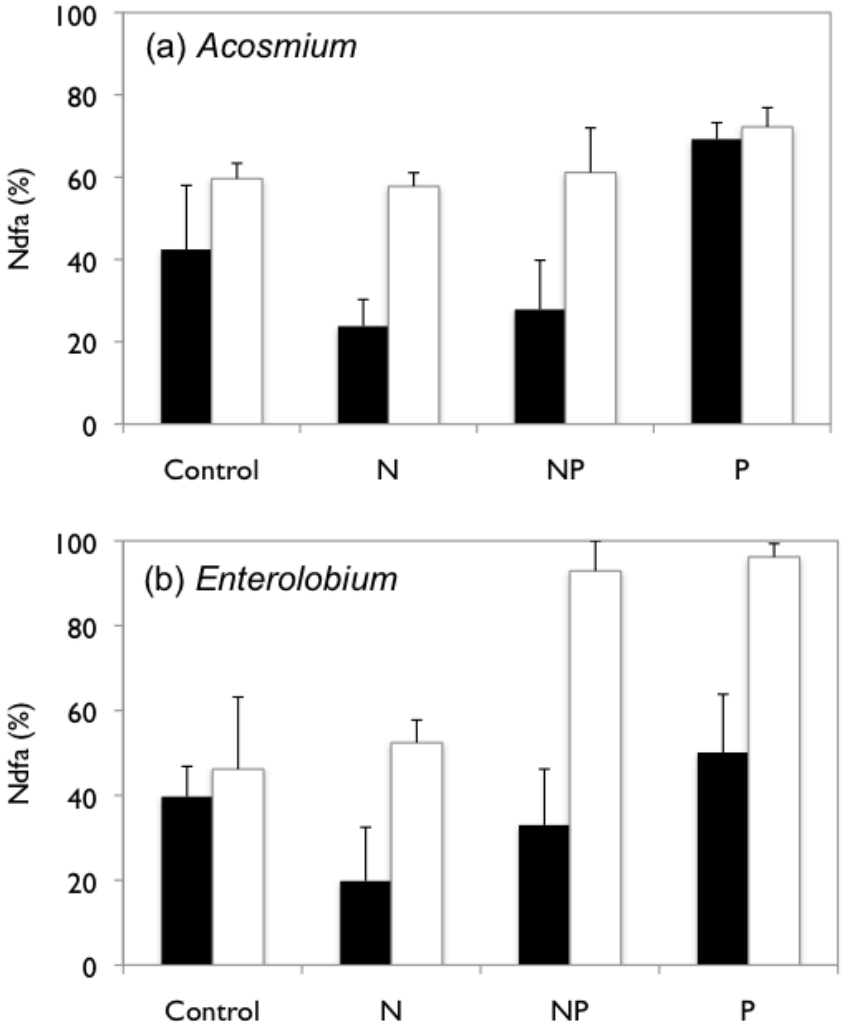


Figure 3-9


(a) Acosmium
Ndfa (%)
100
80
60
40
20
0
Control
N
NP
P
(b) Enterolobium
Ndfa (%)
100
80
60
40
20
0
Control
N
NP
P

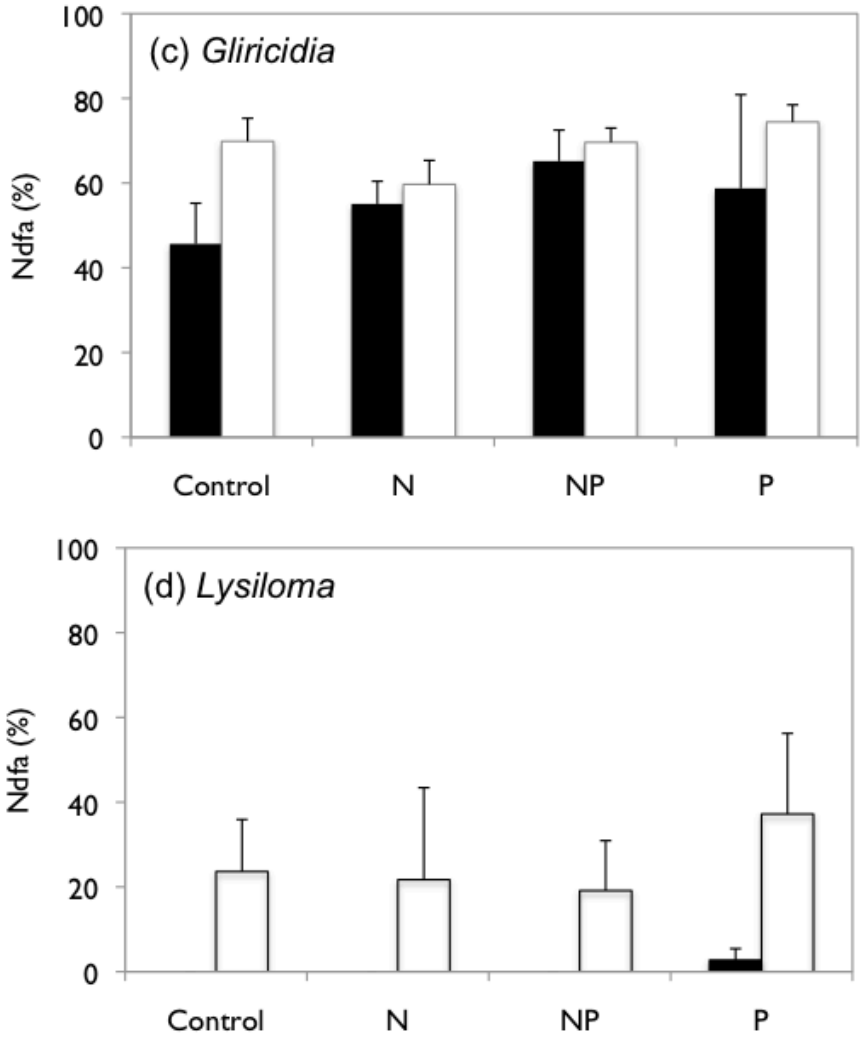

(c) Gliricidia
Ndfa (%)
100
80
60
40
20
0
Control
N
NP
P
(d) Lysiloma
Ndfa (%)
100
80
60
40
20
0
Control
N
NP
P

Figure 3-10

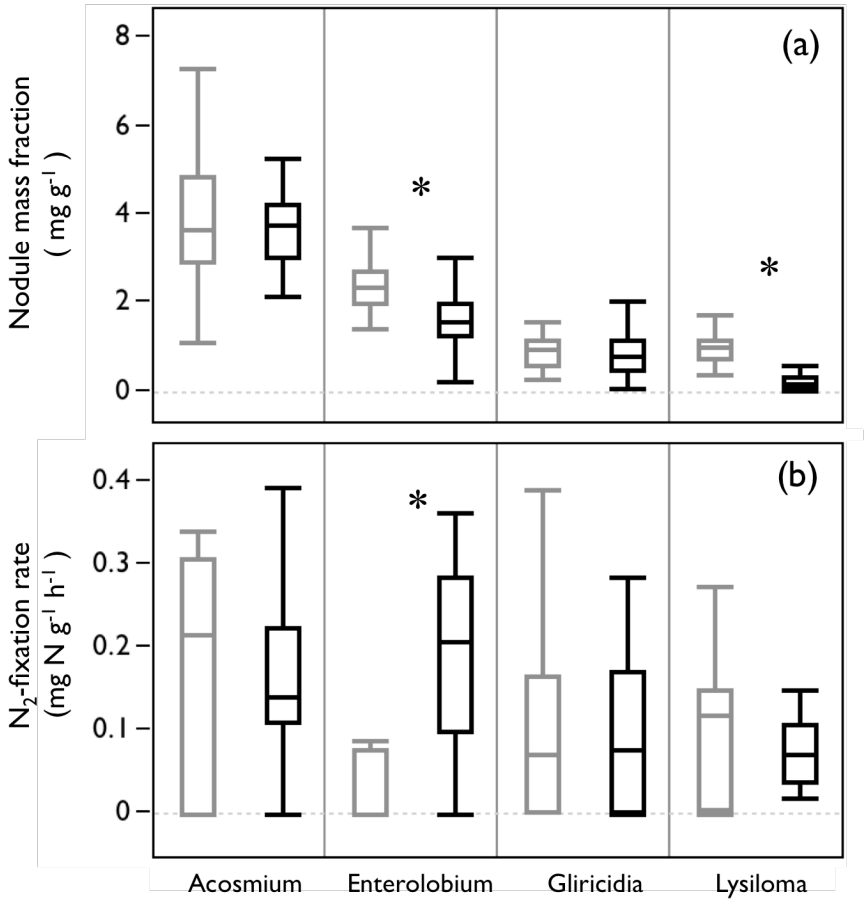

Nodule mass fraction
(mg g-1)
8
6
4
2
0
(a)
*
*
N2-fixation rate
(mg N g-1 h-1)
0.4
0.3
0.2
0.1
0
(b)
*
Acosmium
Enterolobium
Gliricidia
Lysiloma

Figure 3-11

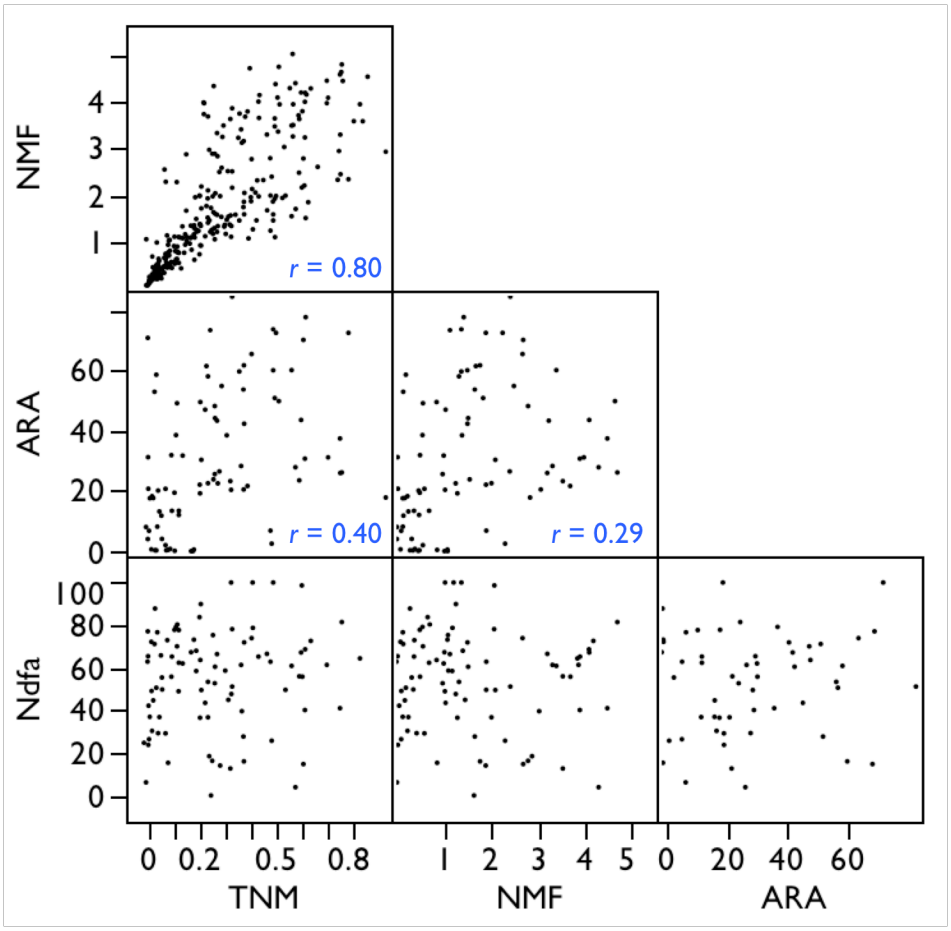

NMF
ARA
Ndfa
r = 0.80
r = 0.40
r = 0.29
0 0.2 0.5 0.8
TNM
1 2 3 4 5
NMF
0 20 40 60
ARA

Figure 3-12

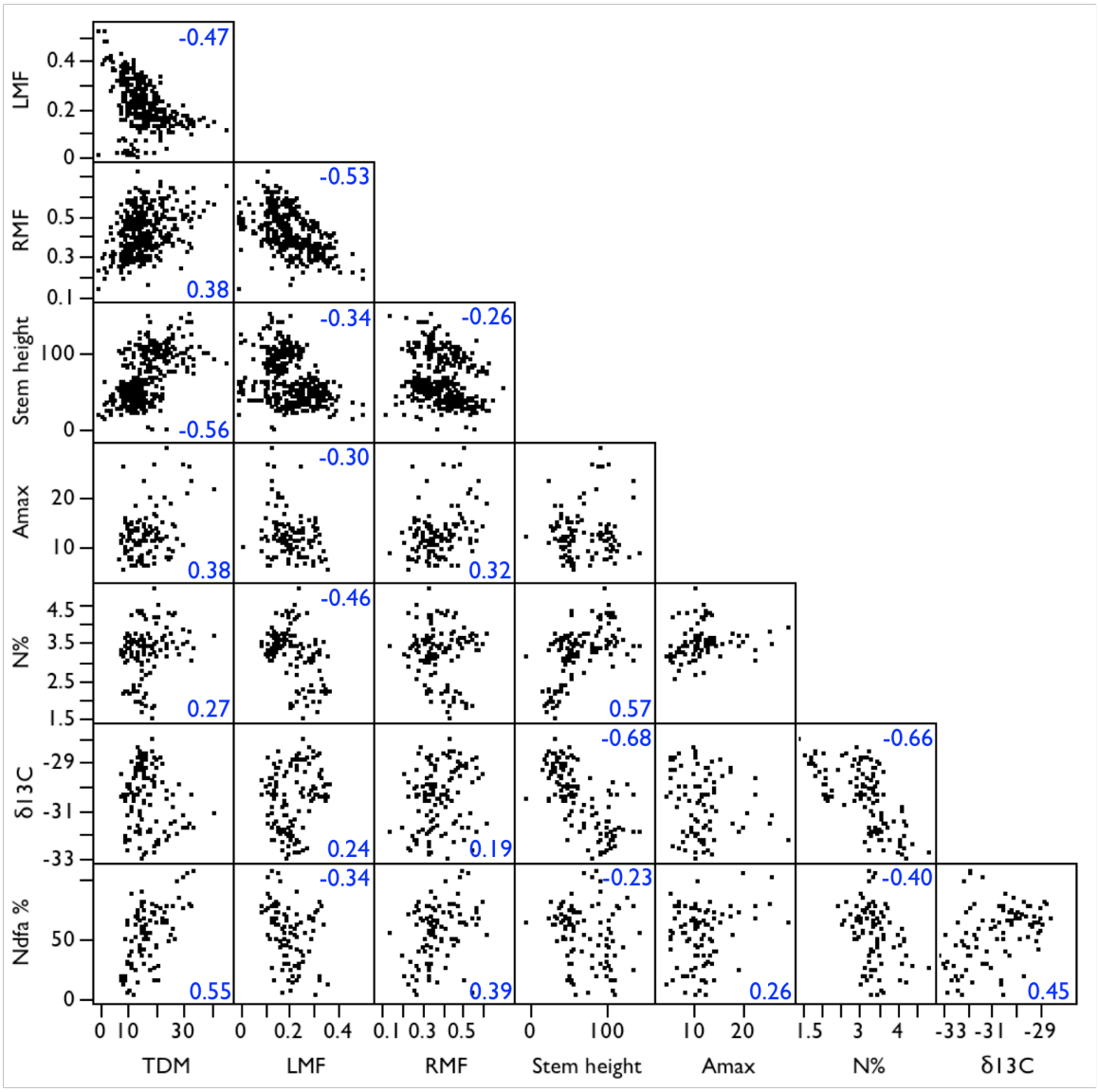

LMF
RMF
Stem height
Amax
N%
δ13C
Ndfa %
TDM
LMF
RMF
Stem height
Amax
N%
δ13C
-0.47
-0.53
0.38
-0.34
-0.26
-0.56
-0.30
0.38
0.32
-0.46
0.27
0.57
-0.68
-0.66
0.24
0.19
-0.34
-0.23
-0.40
0.55
0.39
0.26
0.45

Figure 3-13

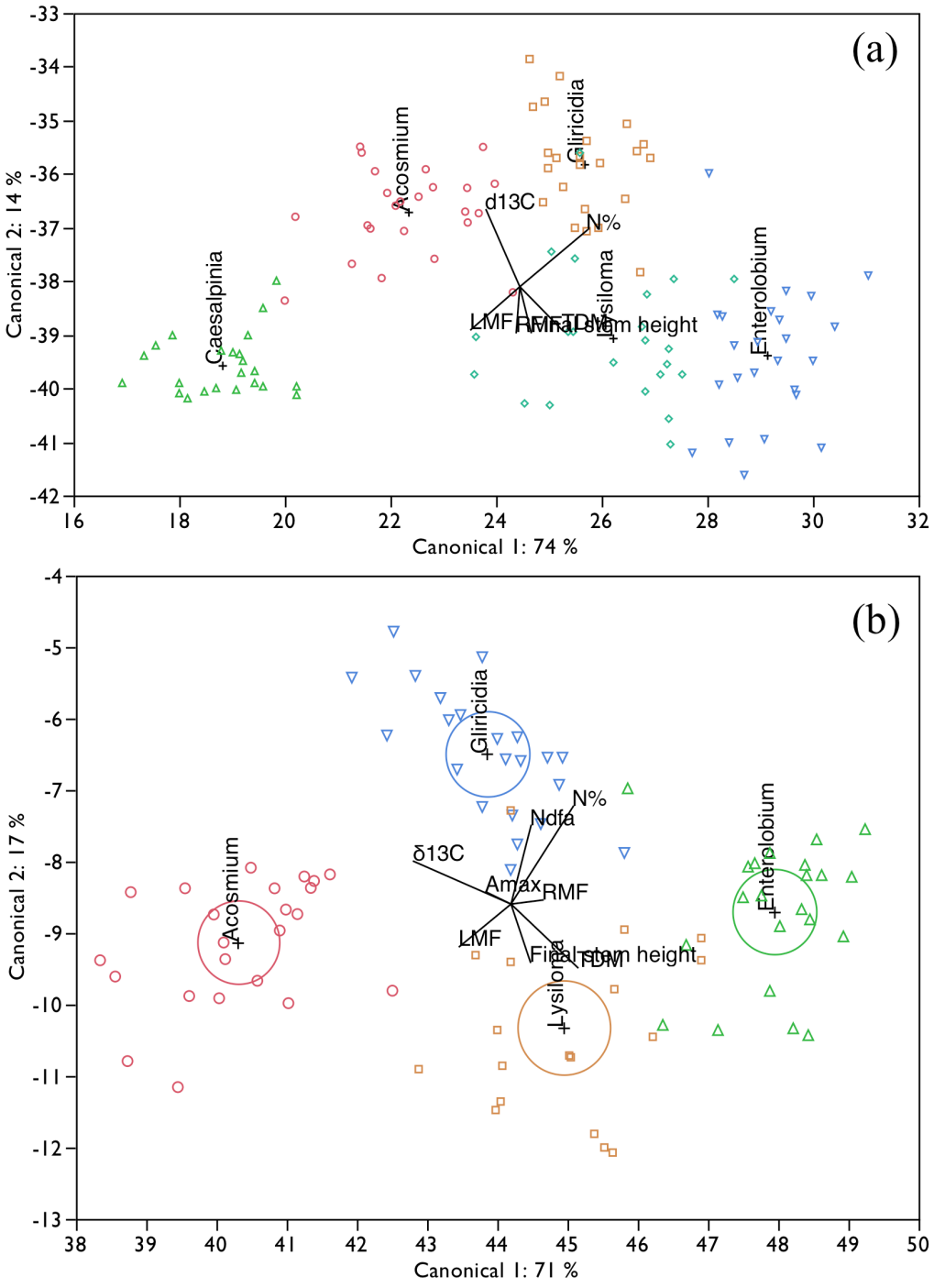

(a)
Canonical 2: 14 %
Canonical 1: 74 %
Acosmium
Gliricidia
d13C
N%
Caesalpinia
Lysiloma
Enterolobium
LMF
Final stem height
(b)
Canonical 2: 17 %
Canonical 1: 71 %
Gliricidia
N%
Ndfa
δ13C
Amax
RMF
Acosmium
LMF
Final stem height
Enterolobium
Lysiloma

Table 3-1. Species taxonomy and ecological features of four N fixing and one non-fixing legume species common in the dry tropical forests of Área de Conservación Guanacaste, Costa Rica

Species	Subfamily	Tribe	Nodulation	Nodule type[1]	Height at maturity[2]	Local abundance[3]	Leaf P (%)[4]	SLA ($cm^2\ g^{-1}$)[4]	Wood density ($g\ cm^{-3}$)[4]
Acosmium panamense	Faboideae	Sophoreae	N fixer	Determinate	20 m	2.53	0.068	92.19	0.768
Caesalpinia eriostachys	Caesalpinioideae	Caesalpinieae	non-fixer	-	10 – 15 m	1.41	0.178	85.44	0.798
Enterolobium cyclocarpum	Mimosoideae	Ingeae	N fixer	Indeterminate	25 – 35 m	1.62	0.101	145.51	0.377
Gliricidia sepium	Faboideae	Robinieae	N fixer	Determinate	10 – 12 m	4.82	0.125	137.82	0.776
Lysiloma divaricatum	Mimosoideae	Ingeae	N fixer	Indeterminate	15 m	3.81	0.069	113. 83	0.733

[1]Frioni et al. (1995), Parveen et al. (1997), González-Ruiz et al. (2008)

[2] From Cordero and Boshier (2003)

[3] Importance values (relative frequency + relative density + relative dominance) from Powers and Becknell, unpublished

[4]Data collected in 2008 from individuals in Área de Conservación Guanacaste. Values represent means of 1-7 trees, adapted from Powers and Tiffin (2010)

Table 3-2. *F* values from full factorial models with species, light, nutrients as fixed effects on biomass partitioning and other traits measured in seedlings of four N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and one non-fixing legume (*Caesalpinia eriostachys*). Degrees of freedom are shown in parenthesis

Trait	R^2	*P* full model	Species	Light	Nutrients	Species x Light	Species x Nutrients	Species x Light x Nutrients
TDM	0.56	< 0.0001	50.66 (4)	132.19 (1)	8.40 (3)	2.56 (4)	-	2.23 (12)
LMF	0.74	< 0.0001	166.70 (4)	69.35 (1)	-	16.93 (4)	4.62 (12)	-
SMF	0.68	< 0.0001	88.01 (4)	7.61 (1)	4.38 (3)	25.17 (4)	2.01 (12)	2.05 (12)
RMF	0.67	< 0.0001	85.31 (4)	206.53 (1)	-	7.69 (4)	-	2.45 (12)
NMF	0.81	< 0.0001	277.03 (3)	12.51 (1)	4.47 (3)	2.76 (3)	-	-
Stem height	0.82	< 0.0001	323.88 (4)	29.38 (1)	7.44 (3)	15.14 (4)	-	1.89 (12)
Leaf N	0.94	< 0.0001	290.53 (4)	64.55 (1)	-	3.50 (4)	-	2.90 (12)
C:N	0.68	< 0.0001	52.50 (4)	26.79 (1)	7.26 (3)	4.09 (4)	4.88 (12)	3.56 (12)
$\delta^{13}C$	0.93	< 0.0001	166.24 (4)	284.20 (1)	-	7.12 (4)	-	-
Amax	0.46	< 0.01	7.35 (3)	-	-	8.14 (3)	-	-
$\delta^{15}N$	0.83	< 0.0001	57.81 (4)	80.65 (1)	3.26 (3)	6.76 (4)	-	-
Ndfa	0.63	< 0.0001	3.28 (3)	47.05 (1)	6.6 (3)	-	-	-
ARA	0.43	0.001	1.36 (3)	-	-	-	-	-
Soil NH_4^+	0.51	< 0.01	40.42 (4)	13.69 (1)	-	-	-	-

where TDM = total dry mass, LMF = leaf mass fraction, SMF = stem mass fraction, RMF = root mass fraction, NMF = nodule mass fraction, A_{max} = maximum photosynthetic rate, Ndfa = the reliance of the N fixing legumes on atmospheric nitrogen, ARA = nitrogense activity as acetylene reduction assay

Table 3-3. Results of non-linear mixed models exploring differences in monthly relative growth rates of height in seedlings of four N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and one non-fixing legume (*Caesalpinia eriostachys*) over the fist six months after germination

	Value	Standard error	*P*-value
Intercept	0.00983	0.00248	0.0001
Caesalpinia eriostachys	-0.00249	0.00084	0.0030
Enterolobium cyclocarpum	-0.00214	0.00077	0.0053
Gliricidia sepium	-0.00191	0.00081	0.0183
Lysiloma divaricatum	0.00377	0.00082	0.0000

Supplementary information

Table S3-1. ICP multi-elements in forest soil prior to the experiment.

Element	Concentration (mg kg^{-1})
Al	28813.67
B	< 0.22
Ca	2544.10
Cd	< 0.22
Cr	2.874
Cu	29.59
Fe	26120.00
K	862.53
Mg	1170.50
Mn	1032.59
Na	217.88
Ni	3.767
P	32.28
Pb	< 3.52
Si	541.33
Zn	56.62

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CHAPTER 4

Nitrogen fixation by legume trees and contributions to ecosystem-level nitrogen cycling in regenerating tropical dry forest

Summary

Biological nitrogen (N) fixation is the major source of new N that fuels net primary productivity in tropical forests. Symbiotic fixation by legumes is thought to be a facultative process that decreases over successional time. However empirical evidence is scarce particularly from tropical dry forests, where legume trees are especially abundant. I assessed N fixation by legumes using two methods (nodule counts and the ^{15}N natural abundance method) and measured other N inputs to the ecosystem from free-living fixation in the leaf litter and soil, wet deposition, and internal recycling through litterfall and decomposition, to constrain the contribution of symbiotic N fixation rates to overall ecosystem N cycling in regeneration tropical dry forest in Costa Rica. Nodulation was highly seasonal and ranged from 1.42 ± 3.73 to 6.07 ± 9.10 g m^{-2}. The ^{15}N natural abundance method did not accurately reflect rates of N fixation perhaps because of high percentages of N resorption. Neither of the two indices of N fixation was related to soil N or phosphorus availability. Free-living N fixation in leaf litter and soil had high temporal variation among seasons and high spatial variation among plots that varied in forest age and species composition, but on average this flux was low, accounting for an input of 0.99 kg N ha^{-1} yr^{-1}. N fluxes from wet N deposition were estimated at 2.18 kg N ha^{-1} yr^{-1}.

My estimates of N fixation rates by legumes in a dry forest chronosequence were between 5 and 20 kg N ha^{-1} yr^{-1} and represented the largest N input compared to free-living fixation or N wet deposition, but accounted for a small proportion (3.4 – 13.5 %) of total N demand suggesting that decomposition provides the majority of N needed for plant growth. However, because the percentage of legume trees did not vary systematically with forest age, I saw no directional trends in the contribution of N fixation to ecosystem N budgets with succession, which suggests that tropical dry forests may differ from wet forests in this respect.

Key words: symbiotic nitrogen fixation – legumes – free-living fixation – nitrogen wet deposition – tropical dry forests

Introduction

Biological nitrogen (N) fixation is considered the major natural source of new N to forest ecosystems that fuels a large proportion of net primary productivity in tropical forests (Cleveland *et al*., 2013). In the last half century, humans have increased artificial N inputs to terrestrial ecosystems (Galloway *et al*., 2008) and more recently tropical forests became hotspots of increasing anthropogenic N inputs (Austin *et al*., 2013). To evaluate how much we are altering the global nitrogen (N) cycle in this region and to model global net primary productivity, it is essential to resolve major uncertainties

around the N budgets from terrestrial ecosystems. In 1999, Cleveland and others pointed out that understanding the primary controls over N fixation is a major gap in knowledge.

Conceptual frameworks that explain the emergence and maintenance of N fixation by legume trees in tropical forests have been recently developed (Hedin *et al*., 2009; Menge *et al*., 2014). Empirical support validating those theories shows that the process of N fixation is a facultative strategy for tropical legumes, and that N fixation activity progressively decreases over forest succession as N capital builds up and the proportion of legume trees in the forests declines (Barron *et al*., 2010; Batterman *et al*., 2013; Sullivan *et al*., 2014). With one exception (Pearson and Vitousek, 2001), most of this evidence was gathered in tropical rainforests and it is possible that in other tropical biomes, such as dry forests, controls on N fixation may not act in the same way. In tropical dry forests, legumes trees are especially abundant and species-rich, (Gentry, 1995; Gillespie *et al*., 2000), and it is possible that seasonal water shortages exert large control on fixation rates and nodulation (Teixeira *et al*., 2006; González-Ruiz *et al*., 2008).

One way to determine the importance of symbiotic N fixation by leguminous trees is to compare the magnitude of this flux to other N inputs to this ecosystem such as free-living fixation or atmospheric deposition, relative to N demand and internal N cycling pathways (e.g. N return to the soil from litter fall; Figure 4-1). Free-living or asymbiotic N fixation can occur on a variety of substrates including soils, leaves, leaf litter, and decaying wood (Reed *et al*., 2011). The presence of N fixing bacteria is ubiquitous in terrestrial ecosystems, especially on substrates such as leaf litter with high C:N as

potential energy source (Vitousek and Hobbie, 2000). Previous studies that have measured free-living fixation in tropical forests suggest that they contribute significantly to the N balance of tropical ecosystems (Reed *et al*., 2008; Barron *et al*., 2009; Cusack *et al*., 2009). There exist multiple factors that may limit N-fixation at a stand-level, including temperature, and labile carbon and phosphorus availability (Reed *et al*., 2011), but the highly pulsed seasonal availability of water that characterizes tropical dry forests is likely to be a strong control on both symbiotic and asymbiotic fixation.

Here I report rates of symbiotic N fixation measured in individual legume trees over a 2-year period in tropical dry forests of northwestern Costa Rica. I simultaneously measured rates of free-living N fixation in leaf litter and soils as well as rates of N deposition to this ecosystem, and compared these rates to other metrics of N cycling measured in plots secondary that varied in age and species composition. My goals were three-fold: first, to estimate two indices of symbiotic N fixation (nodule mass and the N isotopic composition of legume trees) in relation to environmental controls in five species of dry forest legumes present in regenerating tropical dry forest; second, to quantify free-living N fixation in litter and soils seasonally in a chrononsequence of forest plots; and last, to estimate the potential contribution of N fixation legume trees to ecosystem-level N cycling by constructing N budgets of inputs and internal N transformations (Figure 4-1). To estimate N fixation by legumes I simultaneously assessed nodulation and used the ^{15}N natural abundance method (Shearer and Kohl, 1986). To determine the total contribution of N fixed by legumes to leaf litter I measured N resorption in N fixing legumes. Because N fixers have higher requirements of phosphorus to support the

metabolic and energetic costs of N fixation (Vitousek *et al*., 2002) I predicted that N fixation would increase with soil P availability. I further predicted that in soils with high N availability, legume trees would down-regulate fixation. In an environment where water availability is an important driver of nutrient dynamics, I expect that N fixation would also respond to soil moisture.

Methods

Site and Species

In this study, I quantified nitrogen fixation in focal N fixing legume trees that were widely distributed throughout the lowland region Área de Conservación Guanacaste (ACG), in eighteen 0.1 ha plots in tropical dry forests of two national parks in the province of Guanacaste, Costa Rica: Parque Nacional Santa Rosa in the Área de Conservación Guanacaste (ACG) and Parque Nacional Palo Verde in Área de Conservación Tempisque (about 60 km southeast of Santa Rosa). Santa Rosa has a 30-year mean annual precipitation of 1765 mm (www.investigadoresACG.org), and Palo Verde has a 30-year mean annual precipitation of 1444 mm (www.ots.ac.cr) with a 6 month dry season in both parks (Gillespie *et al*., 2000). Tree species richness (stems > 10 cm diameter at breast height) in 0.1 ha forest inventory plots in the region ranges from 1 to 21 species (Powers, unpublished). Soils in this region are dominated by Entisols and Vertisols, but Mollisols, Alfisols, Inceptisols and Ultisols are also present (Leiva *et al*., 2009). In Santa Rosa, soils derive from an eroding plateau made up of volcanic lava

flows and ash deposits (Hartshorn, 1983). In Palo Verde, soils developed from alluvial areas along the floodplain and wetlands of the Tempisque River and the eroding limestone hills (Hartshorn, 1983). Both parks were established in the 1970s, mostly on areas previously used for agriculture or pastureland but also including some older forests. Today, species-rich secondary forests dominate the majority of this landscape. Plant community composition includes evergreen and deciduous tree species, and varies across successional and soil gradients (Powers *et al*., 2009). Less fertile soils in Santa Rosa are dominated by an evergreen species of live oak (*Quercus oleoides*), while the more fertile soils of Santa Rosa and Palo Verde support more species-rich forests with more deciduous canopies (Powers *et al*., 2009).

I studied individual N fixing trees that were distributed across Parque Nacional Santa Rosa within ACG. I selected trees of the following species from the Fabaceae family: *Acosmium panamense* (Benth.) Yakovlev, *Dalbergia retusa* Hemsl., *Enterolobium cyclocarpum* (Jacq.) Griseb. and *Gliricidia sepium* (Jacq.) Kunth exWalp and *Lysiloma divaricatum* (Jacq.) J.F. Macbr. All species are henceforth referred to by genus name only. These species are known for their nodulation capacity and high local abundance in Guanacaste and other dry forests of the Mesoamerican region (Table 1). I identified twenty individuals of each species that were > 10 cm in diameter at breast height. Focal trees were distributed across an area of ~30 km^2 of Santa Rosa.

Estimates of symbiotic nitrogen fixation

I used two indirect approaches to calculate the reliance of trees on fixation: dry mass of nodules per m^2 and the ^{15}N natural abundance isotopic method. I assessed nodulation four times over a two-year period: May 2011 (late dry-early wet season), August 2011 (wet season), March 2012 (dry season), and June 2012 (wet season). During each sampling time and under each tree (N = 100), I collected eight soil samples from the top 15 cm using a 8 cm diameter root auger. Data from a previous study (chapter 3) shows that in these species most nodules occur in the top 15 cm. Cores were taken in each of eight cardinal directions from the bole and at 100 cm before the crown drip line. In the laboratory, nodules were carefully removed from each soil sample, and rinsed with distilled water. After drying at 65˚C for 48 h, nodule dry weight was determined and converted to an area basis (nodule mass per m^2). In total, I sampled and analyzed 2,800 soil cores. N fixation rates were calculated by multiplying nodule mass (g m^{-2}) by nodule activity (g N g $nodule^{-1}$ h^{-1}) that was previously determined on seedling of the same species (Chapter 3).

I used the ^{15}N natural abundance method once during each wet season (September 2011 and July 2012). This approach takes advantage of the difference in nitrogen isotopic composition of fixed N coming from air and the nitrogen isotopic composition of soil mineral N. N fixing plants relying solely on atmospheric N_2 as an N source should have $d^{15}N$ signatures similar to air, and plants relying solely on mineral N sources from the soil will have ^{15}N values close to the mineral N pool. I used the following mixing model after Shearer and Kohl (1986):

$$\%Ndfa = (\delta^{15}N_{reference} - \delta^{15}N_{N\text{-}fixing}) / (\delta^{15}N_{reference} - B) \times 100$$

where Ndfa is the percentage of nitrogen derived from the atmosphere, $\delta^{15}N_{reference}$ is the average $\delta^{15}N$ of several non-legume species growing in the vicinity of the N fixing species; $\delta^{15}N_{N\text{-}fixing}$ is the average ^{15}N abundance of the N fixing legume species, and B is the average $\delta^{15}N$ for each species grown in sand, i.e. when plants are relying on N fixation only to meet all of their N requirements (Chapter 4). This model require that the ^{15}N abundance of non-N fixing reference species deviates significantly from the atmospheric abundance, and that reference and tested species extract N from the same soil sources and have similar phenology (Shearer and Kohl, 1986). In my data, as reference I averaged the ^{15}N abundance of those species with ^{15}N values $\geq$ 3 ‰ (Figure 4-2). Any negative estimated Ndfa values were set to zero, and any higher than 100% were set to 100.

Nitrogen resorption

During the dry season of 2013, I collected recently senesced leaf litter beneath the canopy of each focal tree. Litter samples were analyzed for total N. To calculate the percentage of N resorption, I used the following equation after Reich *et al*. (1995):

(%N green leaves - %N leaf litter) / (%N green leaves) x 100 %

Soil nitrogen and phosphorus

During each nodulation survey, I monitored soil nitrogen and phosphorus availability under each tree using the "buried core" method for N and Bray's extraction for P. Beneath the canopy of each focal tree, I collected four samples from the top 10 cm of mineral soil using a 2.5 cm diameter corer after removing the litter layer. Cores were taken in each of eight cardinal directions from the bole and at different random distances between the bole and 100 cm before the crown drip line ("t_0"). At 5 cm distance from where each sample was taken, I installed a 2.5 cm by 15 cm tall PVC core with a rubber stopper and retrieved it four weeks later ("t_1"). Soil from the four individual cores was homogenized into one polyethylene bag per individual sampled and returned to the laboratory. One subsample was removed from bag t_0 and was air-dried for phosphorus analysis. Within the next five hours, each 10 g soil sample was shaken for 1 h in 50 mL of 2 M KCl solution. All soil extracts were analyzed for NH_4^+ and $NO3^-$ with spectrophotometry following Doane and Howarth (2003). Gravimetric moisture was determined by oven-drying subsamples at 110 ºC for 48 h. *In situ* net N mineralization was calculated from the net change in the NH_4^+ and $NO3^-$ concentrations. Subsamples of 3 g of air-dried soil were extracted in 25 mL of a 0.03N NH_4F and 0.025N HCL solution and analyzed for PO_4^{3-} with spectrophotometry following Lajtha *et al*. (1999). A subsample (N = 10 trees per species) of air-dried soils was analyzed for total phosphorus by quantification with ICP-AES (Perkin Elmer Optima 3000DV) at the Research Analytical Laboratory at the University of Minnesota.

Estimates of free-living nitrogen fixation

I used the acetylene reduction assay (ARA) method to measure dry and wet season leaf litter and soil N fixation rates in fourteen 0.1 ha dry forest plots. I also measured wet season free-living N fixation in leaf litter and soil collected under a subsample of my focal trees (N = 10 trees per species). The ARA method quantifies activity of the enzyme responsible for nitrogen fixation, nitrogenase, and takes advantage of the fact that this enzyme also reduces ethylene to acetylene (methods for ARA follow Reed *et al*., 2007 and 2008). In each plot, I randomly collected six leaf litter and six surface soil samples. Under each focal tree, my sample size was four samples of leaf litter and four soil samples. Leaf litter samples of ~1g were collected from the surface of the soil and then placed into 45-mL clear acrylic tubes. After gently removing all litter from the soil surface, I took soil samples as intact cores using 45-mL, 2.54 cm in diameter clear acrylic tubes and inserted them to a depth of 1cm. Tubes were injected with enough acetylene to create a 10% headspace concentration by volume (through a lid fitted with a septum), and vented to the atmosphere to equilibrate pressure in the tubes. All samples were left overnight to incubate in a rain-sheltered space close to the forest for about 20 hours. Because I were interested in *in situ* rates of N fixation, soil moisture content was not manipulated. Sample headspaces were mixed by plunging with a syringe, subsampled, injected into pre-purged 10 ml Vacutainer tubes. Ethylene concentrations were measured using a Shimadzu 14-A Gas Chromatograph equipped with a flame ionization detector (330 ºC) and a Poropak N column (110 ºC; Supelco, Bellefonte, Pennsylvania, USA).

Rates of acetylene reduction were calculated using the unit nanomoles (10^{-9} mol) of acetylene reduced per gram dry mass of sample per hour of incubation. I subtracted the ethylene produced from litter and soil in absence of acetylene as well as the concentration of ethylene in the acetylene. The relationship between acetylene reduction rates and N fixation rates has been determined near 3 moles of acetylene reduced for every one mole of N fixed (Hardy *et al*., 1968; Vitousek, 1994; Vitousek and Hobbie, 2000). Thus, I applied the theoretical conversion ratio of 3:1 for all C_2H_4:N_2 rate conversions.

Wet deposition

I measured nitrogen wet deposition in gross rainfall monthly from April 2011 until July 2012. In an open area within ACG, I placed eight 3.78 L collectors at a height of 2 m. Once during each month, after a rainfall event, I combined water in all collectors, and kept a 30 mL subsample. Each sample was acidified with 0.06 mL of H_2SO_4 and stored frozen until chemical analysis. Each sample was analyzed for NH_4^+ and NO_3^- with spectrophotometry following Doane and Howarth (2003). To calculate rates of N wet deposition on a year and area basis, I used monthly ACG rainfall data for 2011 (M.M. Chavarría, pers. comm.) and added all NH_4^+ and NO_3^- measured in rainfall during 2011.

Ecosystem N Budgets

To constrain the relative contribution of N inputs to the magnitude of internal N transformations within forests, I compared my data on N inputs via wet deposition,

symbiotic N fixation, and free-living N fixation to the quantities of N cycled through litterfall and fine root production. I used data on annual leaf litterfall quantities and N concentrations for 2012 reported in a previous paper (for details see Waring *et al.*, in review). These data come from 18 0.1 ha plots that are distributed across gradients of forest age and composition (Waring et al in review; Kissing and Powers, 2011; Powers and Peréz-Aviles, 2013). I assumed that the N in leaf litter fall and fine root production turns over every year and that these fluxes represent total ecosystem N internal transformations.

In order to obtain annual rates of symbiotic N fixation by legume trees, I extrapolated average rates of N fixation indexed by the acetylene reduction assay in nodules of seedlings of the same legume species (Chapter 4). These rates were similar to rates measured in adult legume trees in field conditions in other tropical forests (Barron *et al*., 2010). Rates were then converted to an area basis by multiplying the average nodulation density reported here by the root zone of legume trees in each of the 18 0.1 ha plots. The root zone radius was defined as 18 cm for each cm of DBH (University of Minnesota Extension, www.extension.umn.edu). In these plots, the percentage of basal area of legume trees ≥ 10 cm diameter at breast height ranged from 0 to 40% and averaged 15% (Powers, unpublished data). I determined a low and a high estimate of ecosystem N fixation by using dry and wet season averages of nodule density for each of my five focal species (between 0.51 and 6.37 g nodules m^{-2}). For those species where I did not measure nodulation, I assigned nodule density values of the closest taxonomically related focal species. I used lowest and highest species average of nodule N fixation rates

(0.09 and 0.17 mg N g^{-1} h^{-1}). For these estimates, I assumed that nodule activity in seedlings and mature trees do not differ, and that the relationships between environmental factors like nutrient availability (which varies among plots) does not affect nodule activity, an assumption supported by my data.

I also quantified ARA rates in litter and soil in fourteen of the 18 0.1 ha dry forest plots (described below) during each dry and wet seasons of 2012. In each plot, I collected six leaf litter and six soil samples from each plot. To calculate soil N fixation estimates on an area basis, I extrapolated values from soil samples (measured on a tube 2.54 cm in diameter) to area-based rates (kg N ha^{-1} yr^{-1}). To estimate leaf litter N fixation rates by area, I multiplied sample values by litter production in each plot during 2012 (Waring *et al*., in review).

Data analysis

To determine the relationships between symbiotic N fixation in mature trees and environmental variables (Goal 1), I developed linear regression models for each sampling event using the three indices of N fixation (nodule mass, Nfda and ARA) as response variables, and soil moisture, soil nitrogen and phosphorus as predictor variables. Because I used the ^{15}N natural abundance method once each year, values of Ndfa were only compared to soil properties measured during the rainy season of the corresponding year. To evaluate whether species differed in nodulation over time, I ran mixed linear models with 'species' as fixed factor and time as a random effect, using the nlme package in R (Pinheiro *et al*., 2012). I applied linear regression models to compare the rates of free-

living N fixation in leaf litter and soils in the plots to variables of forest productivity and soil properties measured previously in each plot (Waring *et al.*, in review), and season (Goal 2). When multiple factors explained a response variable, I used stepwise multiple regressions to determine which independent variables explained most of the variability in the dependent variable. To determine the relative contribution of legumes to ecosystem fluxes I calculated estimates of symbiotic N fixation on an annual basis (expressed as kg N ha^{-1} yr^{-1}) and compare them to other N inputs to these forests, mainly free-living N fixation, N wet deposition, and to the main flux of N recycling, through leaf litter fall and fine root production (Goal 3). My goal was to constrain the potential contribution of free-living N fixation relative to other N inputs to the ecosystem, and bracket the proportion of total N from N fixation relative to internal ecosystem transformation (e.g. N fluxes in litter fall and fine root production).

Results

Patterns of nodule mass

Mean nodule mass ranged from 1.42 ± 3.73 g m^{-2} in *Lysiloma* to 6.07 ± 9.10 g m^{-2} in *Acosmium*. Nodulation was highly seasonal (Figure 4-3). In general, nodulation was lower during the dry season of 2011 and higher during the wet season of that year but despite these trends I only found significant differences in nodule mass between sampling events in *Enterolobium* ($P < 0.01$). In the mixed linear model including time as a random effect, species identity had a significant influence on nodulation (Table 4-4), where

Acosmium had the highest densities of nodulation and *Enterolobium* and *Lysiloma*, the lowest.

Patterns of Ndfa

I found that all species resorbed more than 20% of leaf N. Species differences in N resorption were significantly different ($P < 0.0001$). Resorption of N was greater in *Gliricidia* and *Dalbergia* (22 – 26 % on average) than in *Acosmium*, *Enterolobium* and *Lysiloma* (30 – 48 % on average). I found that average N resorption was negatively correlated with tree growth rate measured as tree diameter increment per year ($P < 0.05$, $R^2 = 0.84$, Figure 4-10). I found that $\delta^{15}N$ in legume species was significantly different than reference species both in 2011 and 2012 ($P < 0.0001$; Figure 4-2), substantiating the use of Ndfa in my data. However, because these trees were retranslocating N, I speculate that the Ndfa values might not accurately reflect the proportion of N that trees were recently obtaining from fixation but instead represent a combination of N acquired during more than one growing season. In general, the ^{15}N natural abundance method yielded high estimates of N fixation: in 2011 mean Ndfa was 85 % and in 2012 81 %.

Patterns of soil chemical variables

The soils under my focal trees showed a large range of variation both in terms of total soil P (60 – 400 μg P g soil^{-1}, Figure 4-4) and available P (0.01 – 10 μg P g soil^{-1}, Figure 4-5). There were no significant differences in available P between different

sampling events. However, I found seasonal changes in both soil nitrate and ammonium ($P < 0.0001$; Figure 4-9). On average, soil nitrate was lower during the rainy season (1.39 ± 1.80 and 1.18 ± 1.96 μg N g soil^{-1} for 2011 and 2012) and higher during the dry season (4.53 ± 4.23 and 3.91 ± 4.11 μg N g soil^{-1} for 2011 and 2012). I measured the highest levels of soil ammonium during the dry season (May) of 2011 (7.85 ± 5.15 μg N g soil^{-1}) and the lowest during the dry season (March) of 2012 (3.90 ± 5.82 μg N g soil^{-1}). Nitrogen mineralization ranged from -0.38 to 1.46 μg N g soil^{-1} day^{-1} and did not show species differences or variation across sampling events.

Symbiotic N fixation and environmental drivers

There was no relationship between nodule mass (or Ndfa) and soil moisture. Neither nodule mass nor Ndfa were correlated with total P (Figure 4-4); however, most individuals with densities of nodulation > 10 g m^{-2} were found in soils with total P < 200 ppm. In the same way, there were no significant correlations between nodule mass (or Ndfa) and available P but during the wet season of 2012, I observed nodulation particularly high in individuals found in soils with available P < 1 μg P g soil^{-1} (Figure 4-5). Neither nodule mass nor Ndfa were correlated to soil nitrate or ammonium, or to N mineralization (Figures 4-6, 4-7, 4-8).

Free-living fixation

Under the canopy of N fixing legume trees, rates of free-living N fixation in leaf litter were on average 2.34 ± 3.29 ng N g^{-1} h^{-1}, and ranged from 0.04 to 13.56 ng N g^{-1} h^{-1}. In soils under the crown of legumes, both the average (0.53 ± 0.90 ng N g^{-1} h^{-1}) and the range (0.003 – 3.65 ng N g^{-1} h^{-1}) of rates of N fixation were lower than in the litter layer (Figure 4-11). I found that both leaf litter and soil N fixation under legumes were positively correlated with tree nodule density ($P < 0.01$ and $P < 0.05$, $R^2 = 0.21$ and $R^2 = 0.08$ respectively). In addition, there was a weak, negative correlation between N fixation in leaf litter and soil nitrate ($P < 0.05$, $R^2 = 0.09$) and soil N fixation was weakly positively correlated with soil moisture ($P < 0.05$, $R^2 = 0.10$). There were no differences in leaf litter or soil N fixation among different legume tree species.

The rates of N fixation in the leaf litter layer of fourteen 0.1 ha plots (0.03 – 65.30 ng N g^{-1} h^{-1}) varied among plots and between seasons (Figure 4-12). In general, these rates were higher than rates measured in leaf litter collected underneath the canopy of N fixing trees. The rates of fixation measured in litter during the dry season were positively correlated with leaf litter production ($P < 0.05$, $R^2 = 0.28$) as well as with twig production ($P < 0.01$, $R^2 = 0.47$). Fixation rates in leaf litter measured during the rainy season were positively correlated with soil available phosphorus ($P < 0.01$, $R^2 = 0.52$). In soils, the rates of N fixation measured in the dry forest plots were also higher than under legume trees, and ranged from 0.01 to 6.93 ng N g^{-1} h^{-1}. During the dry season N fixation in soils was related to fruit production ($P < 0.05$, $R^2 = 0.47$) and to a number of soil properties including available phosphorus ($P < 0.05$, $R^2 = 0.56$), cation exchange capacity ($P < 0.05$,

R^2 = 0.51), calcium ($P < 0.05$, R^2 = 0.62), and aluminum ($P < 0.05$, R^2 = 0.41). Using stepwise multiple regression, I found that the model that best explained dry season N fixation in soils included cation exchange capacity and soils exchangeable calcium (R^2 = 0.85).

Ecosystem N fluxes

The concentration of nitrate in rainfall varied throughout the year 2011 from 0.01 to 0.38 mg L^{-1} and ammonium ranged from 0 to 0.24 mg L^{-1} (Table 4-2). Concentrations of both N forms were particularly high in May, at the very beginning of the rainy season and in September, right after a short dry period associated with a reduction in rainfall during August. When I scaled these concentrations to an ecosystem scale, the N inputs from wet deposition of nitrate and ammonium added up to 2.18 kg N ha^{-1} yr^{-1}.

I found that the average estimates of low and high symbiotic N fixation rates ranged between 5.02 and 20.24 kg N ha^{-1} yr^{-1} and had a large range of variation among plots, due to the varying contributions of legume trees to total basal area (Figure 4-13). Nitrogen fixation rates did not decrease with stand age. My estimates of free-living N fixation were much lower than those of symbiotic N fixation (~1 kg N ha^{-1} yr^{-1}). Finally, I found that large amounts of N are recycled in this ecosystem through both leaf litterfall and fine root turnover (101.92 and 47.51 kg N ha^{-1} yr^{-1}). Assuming that N in litterfall and fine root production represent ecosystem N demand, from 3.4 to 13.5 % of total N

demand may come from fixation by legumes, 1.5 % from wet deposition, and 0.7 % from free-living fixation.

Discussion

Mine is one of the first studies to quantify multiple N inputs and fluxes in tropical dry forest ecosystems and constrain the contribution of legumes to ecosystem N budgets. These included simultaneous measurements of symbiotic and free-living N fixation at both individual and plot scales, as well as N wet deposition and N recycling through litter fall and fine root production (Figure 4-1). In my study of symbiotic N fixation in five legume species (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) distributed across the landscape of Área de Conservación Guanacaste, I found that nodulation, in most species, was highly seasonal, but, contrary to expectation, was uncorrelated to soil nutrient availability. In fourteen 0.1 ha plots, free-living fixation showed high spatial and seasonal variability and was positively related to forest litter fall and several soil variables. In this ecosystem, N fixation by legume trees represents the most important N input, but the magnitude of this input varied by legume basal area, which did not vary systematically with forest age.

Seasonal influence on N fixation

As a consequence of the distribution of precipitation and the occurrence of a long seasonal drought, in tropical dry forests nutrient fluxes are also seasonal (Lodge *et al*.,

1994). My results suggest that inputs of nitrogen through symbiotic and free-living N fixation are not an exception. On one hand, I observed that nodulation is highly seasonal in four of the most common legume species in this ecosystem. Other nodulation surveys from seasonally dry forests have observed a similar pattern (Teixeira *et al*., 2006; González-Ruiz *et al*., 2008). During the dry seasons of 2011 and 2012, nodulation in Santa Rosa decreased considerably suggesting that fixation could be water limited. Soil moisture is known to regulate and promote *Rhizobium* (N fixing bacteria) proliferation, survival, and root colonization (Zahran, 2001). In my study, nodulation was not correlated to soil moisture, which might be explained by a time lag between soil moisture conditions and nodule senescence. For example, in some species nodulation was higher during the dry season of 2012 compared to the dry season of 2011 (Figure 4-3). However, precipitation during the last month of the rainy season of 2011 was much higher than average and this could have enhanced nodule survival throughout the following dry season. According to my Ndfa data, N fixation was high even in trees where I did not find any nodules. Because I observed high levels of N resorption, I consider possible that the ^{15}N signal that I measured actually reflects a combination of recently fixed N or recycled from previous growing seasons.

Nutrient controls on nitrogen fixation

My study challenges two assumptions that are frequently made about N fixation in tropical forests. The first one is that this process is responsive to changes in soil nitrogen and phosphorus availability (Hedin *et al*., 2009; Batterman *et al*., 2013). My

results differ from these previous studies in that N fixation, indexed by either nodule density or Ndfa, was not related to either of those soil variables. In fact, I observed high levels of nodulation in soils that are considered P-deficient in lowland Amazonia (< 200 ppm; Quesada *et al*., 2010). This observation is contrary to the assumption that N fixers have high P requirements for building and maintaining the enzymes and proteins necessary for fixation and to balance the high metabolic use of N (Vitousek *et al*., 2002). However, legumes can host arbuscular mycorrhizal fungi (AMF) and less frequently ectomycorrhizae (Frioni *et al.*, 1999; de Varennes and Goss, 2007) and having such P-acquiring symbioses near to nodules can contribute to acquisition of the significant requirement for P of nodulation (Sprent and James, 2007). A deeper understanding of how mycorrhizal associations influence N fixation in legumes could possibly help explain the apparent disconnect between nodulation and soil P.

In tropical forests, N fixation is thought as a facultative process down-regulated by the availability of soil N (Hedin *et al*., 2009). As N accumulates in ecosystems over successional time, I should expect decreased fixation in old-growth forests compared to secondary forests. Recently, several studies have corroborated this hypothesis (Barron *et al*., 2010; Batterman *et al*., 2013). However, all supporting evidence to these ideas was collected in tropical rainforests. In this study, N fixation (or legume abundance) did not decrease with stand age. Also, there was no down-regulation of nodulation with soil nitrate, even though the values of soil nitrate in my study were similar to those of a rainforest in Panama where legume trees decreased fixation substantially (Barron *et al*., 2010). In tropical dry forests, seasonal pulses of nutrient decomposition govern nutrient

availability. Another possible explanation to this lack of regulation of fixation vis-à-vis soil nutrients is that down-regulation only occurs at the very beginning of the rainy season. At the onset of rains, decomposition of all leaf litter that accumulated over the dry season creates a pulse of N mineralization. After this, high losses of N through leaching or gaseous N emissions can happen. I hypothesize that "down-regulation" of N fixation in tropical dry forest legumes occurs only during the dry season (due to water limitation to most physiological processes) and can extend through a period of high soil N availability (Figure 4-14). Then, to support high rates of growth during a 6-month growing season, trees rely on combined sources of N throughout the wet season. In May of 2011, I recorded low levels of nodulation and this period coincided with the first weeks of rain in Guanacaste, when I recorded the highest N in wet deposition (Table 4-2). It is possible that at this time, trees were preferentially relying on sources of N other than fixation.

Finally, it is worth noting that some species differed in the degree to which they regulate N fixation. In particular, nodulation in *Acosmium panamense* showed less seasonal variation than in other species (Figure 4-3), which suggests that the response of N fixation to environmental conditions, mainly water, is different among legumes. This is consistent with data from both shade house experiments on seedlings, and surveys of mature trees in plantations. In a survey of roots and nodulation in 20 year-old plantations of four of the same legume species (Chapter 3), I observed the same contrast in nodulation patterns between trees of *Acosmium,* which allocated resources to nodules continuously, and *Enterolobium* where nodulation was very low. In addition, I found that

6-month old seedlings of *Acosmium* grown in a shade house (Chapter 4) had a disproportionately higher biomass investment into nodules relative to the other legumes while seedlings of *Enterolobium* had a more dynamic strategy of regulating N fixation under different light environments and with the addition of P fertilizer. Together, these observations challenge the assumption that in tropical forests all N fixers are facultative (Menge *et al*., 2014).

Free-living N fixation in a dry forest chronosequence

Water availability is one of the factors known to regulate free-living N fixation (Reed *et al*., 2011). In a previous study in a tropical forest with unusually high rainfall, Reed *et al*. (2007) found increased water availability resulted in increased fixation. My measurements of free-living N fixation in dry forest plots also showed strong seasonal variation. However, fixation in the leaf litter layer was higher during the dry season with the exception of one "hotspot" in one of the plots during the wet season (Figure 4-12). Dry season rates were correlated with components of litterfall production (leaf litter and twigs). Given the high decomposition rates and litterfall turnover in these forests, the low wet season rates of free-living fixation could be constrained by substrate limitation and decrease in labile carbon availability. Hotspots of high fixation activity have been previously reported in a lowland rainforest (Reed *et al*., 2010) and were characterized by N fixer communities with different composition and diversity. The activity of N fixers in the soil layer was low during the rainy season, but during the dry season I observed high

rates in areas where I also measured high leaf litter N fixation. Because free-living fixation in soils was correlated with soil chemical properties (cation exchange capacity, aluminum and calcium) only during the dry season, I hypothesize that seasonal variation in N fixation rates is driven not only by changes in soil moisture and temperature but also by changes in leaf litter chemistry.

Because of the high energetic and metabolic costs of the process of fixation, N fixing organisms have a high demand for adenosine triphosphate (ATP) and thus for phosphorus (Vitousek *et al*., 2002). In our dry forest plot chronosequence, I found that both wet season free-living fixation in leaf litter and dry season N fixation in soils increased with soil P. This reinforces the idea that during the dry season free-living N fixation in leaf litter is in general more constrained. Nitrogen fixation rates in litter collected under legume species were lower than in many plots and were also negatively correlated with soil ammonium. I infer that like in other tropical forests (Reed *et al*., 2008), in this dry forest ecosystem tree species identity exerts an important influence on the spatial variation of free-living fixation. Powers and Tiffin (2010) compared several leaf traits of 87 tree species of this ecosystem and found that legumes had higher leaf carbon and higher leaf N:P ratios. In this case, it is possible that legumes created areas of proportionately low P availability that combined with high availability of N in the soil limited the activity of free-living N fixing organisms.

Nitrogen inputs to a tropical dry forest

A first step towards understanding how much are I altering the global nitrogen cycle is having robust estimates of the magnitude of nitrogen inputs at an ecosystem scale. Seasonally dry forests are the most common tropical biome, yet nitrogen budgets that include more than one flux in or out of this ecosystem, are rare. Based on the notorious abundance of legumes in dry forests compared to other tropical forests, I hypothesized that symbiotic N fixation would account for considerable N inputs to these ecosystems. My estimates of N fixation rates by legumes in a dry forest chronosequence were between 5 and 20 kg N ha^{-1} yr^{-1} and represented the largest N input compared to my measurements of free-living fixation or N wet deposition. Nitrogen inputs from free-living fixation to these forests were much lower than in other dry forests (Ley and D'Antonio, 1998). Based on my data, new N from symbiotic fixation would only support a small percentage (3.4 to 13.5 %) of annual N demand. This means that in this tropical dry forest the origin of the majority of new N needed for plant growth is derived from decomposition of leaf litter, fine roots, and soil organic matter.

My rates of N fixation based on legume abundance of a chronosequence of dry forest plots were bracketed between rates estimated by a model that predicts fixation based on legume abundance (Cleveland *et al*., 1999) and rates measured independently of legume abundance in primary and secondary rainforests in Costa Rica (Sullivan *et al*., 2014). My low estimate of N fixation was similar to rates obtained with simulation modeling and ecosystem N balances methods for an Amazonian rainforest (4.5 – 6.8 kg N ha^{-1} yr^{-1}; Cleveland *et al*., 2009). Because my extrapolations were based on a dry and

wet season nodule density factor measured on several legume species distributed across Santa Rosa, I interpret my low and high estimates of N fixation as two potential magnitudes of the contribution of legumes during each of the two contrasting seasons of dry forests. I acknowledge that this approach of "bottom-up" scaling from N fixation rates in nodules to the stand level has a number of limitations and assumptions, mainly the fact that I observed important differences in N fixation strategies among species and the mechanism that drives this variation is still unknown. Finally, in my study stand-level N fixation rates did not decrease with stand age because the percentage of legume trees did not vary systematically with forest age. A more intensive effort of sampling the flora in this region shows that this pattern is prevalent throughout the landscape: legume abundance is highly variable (0.3 and 58 % of basal area) but unrelated to stand age (Introduction, Figure 1). Given that down-regulation of fixation in these forests is more likely driven by seasonality than by the availability of soil N, a decrease in N fixation with succession could only be driven by legume species replacement. This suggests that the contribution of N fixation to ecosystem N cycling in tropical dry forests is intrinsically different than in tropical wet forests.

Conclusions

In this study, I focused on the process of N fixation by legume trees and measured other N inputs to the forest with the goal of estimating the importance of the contribution of legume trees to ecosystem-level N cycling. In general, rainfall seasonality orchestrated most of the nutrient fluxes in this dry forest. I found that nodulation was highly seasonal

and not related to soil nutrient availability. Other nutrient acquisition strategies like mycorrhizal associations may explain the apparent disconnect between nodulation and soil P (Waring *et al*., in review). Down regulation of symbiotic N fixation only occurred during the dry season and I infer that the costs of wet season fixation in dry forests are low compared to the N demand of the growing season. In this dry forest, free-living N fixation in leaf litter and soil represented seasonal and highly variable N inputs across the landscape, but were low compared to symbiotic fixation by legumes. The patterns in wet N deposition were not an exception to seasonality and this flux was largest at the onset of rains. Nitrogen fixation by legumes was the largest input of new N to this ecosystem but accounted for a small proportion of total N demand suggesting that decomposition provides the majority of N needed for plant growth. My approach that linked individual species to the ecosystem scale highlighted the prevalent role of water availability as a major control on nutrient dynamics of tropical dry forests. Finally, because the abundance of legumes did not change with stand age, the contribution of N fixation by legumes to the N cycle of dry forests does not change throughout succession.

Acknowledgements

This study was supported by a L'Oréal-UNESCO Co-Sponsored Fellowship for Young Women in Life Sciences and a research fellowship from the Ministry of Science and Technology of Costa Rica to M.G.G., and NSF CAREER Grant (DEB-1053237) and a UMN Institute of the Environment Resident Fellowship to my advisor Jennifer S.

Powers. I thank Daniel Pérez, Geohanny Hernández, Randy Ferguson and Saúl Chevez for their excellent help in the field. We also thank María Marta Chavarría and Roger Blanco of Área de Conservación Guanacaste for logistical support.

Figure Legends

Figure 4-1. Conceptual model of key nitrogen inputs and transformations at an individual tree and ecosystem scale in this study. Inputs are in solid lines, while internal fluxes are in dashed lines

Figure 4-2. Nitrogen isotopic composition of five N fixing legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) and reference species used to calculate Ndfa during 2011 and 2012. Reference species were: *Capparis frondosa*, *Crescentia alata*, *Guazuma ulmifolia*, *Pisonia aculeata*, and *Trichillia martiana*

Figure 4-3. Seasonal variation in nodule mass in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) compared to monthly rainfall in Area de Conservación Guanacaste. N = 20 trees per species

Figure 4-4. Two indices of N fixation during 2012 versus soil total phosphorus in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = 10 trees per species

Figure 4-5. Two indices of N fixation, nodule mass and Ndfa versus soil available phosphorus in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = ~20 trees per species

Figure 4-6. Two indices of N fixation, nodule mass and Ndfa versus soil ammonium in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = ~20 trees per species

Figure 4-7. Two indices of N fixation, nodule mass and Ndfa versus soil nitrate in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = ~20 trees per species

Figure 4-8. Two indices of N fixation, nodule mass and Ndfa versus *in situ* nitrogen mineralization rates in five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = ~20 trees per species

Figure 4-9. Seasonal variation in availability of (a) ammonium and (b) nitrate, and nitrogen mineralization rates (c) in soils under trees of five species of legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = 100. The line within the box shows the median of the

data, the ends of the box represent the 75th and 25th quantiles, and the whiskers extend to the minimum and maximum data points. Capital letters indicate significant differences by Tukey's HSD post hoc test ($P < 0.05$)

Figure 4-10. Nitrogen resorption in five species of legumes versus tree diameter increment (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = 20 individuals per species

Figure 4-11. Wet season rates of free-living N fixation in (a) leaf litter and (b) surface soil under the canopy of five species of N fixing legumes (*Acosmium panamense*, *Dalbergia retusa*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*). N = 10 individuals per species

Figure 4-12. Rates of free-living N fixation in (a) leaf litter and (b) surface soil in fourteen 0.1 ha plots in Parque Nacional Santa Rosa (1 – 12) and Palo Verde (13 – 18). N = 6 samples per plot

Figure 4-13. Estimations of symbiotic nitrogen fixation at an ecosystem scale based on (a) legume basal area and (b) stand age of a chronosequence of in 0.1 ha dry forest plots in Área de Conservación Guanacaste and Parque Nacional Palo Verde. In (a), range of rates found by Cleveland *et al*. (1999) and Sullivan *et al*. (2014) are shown.

Figure 4-14. Conceptual model of seasonal regulation of nitrogen fixation by legume trees in tropical dry forests

Figure 4-1

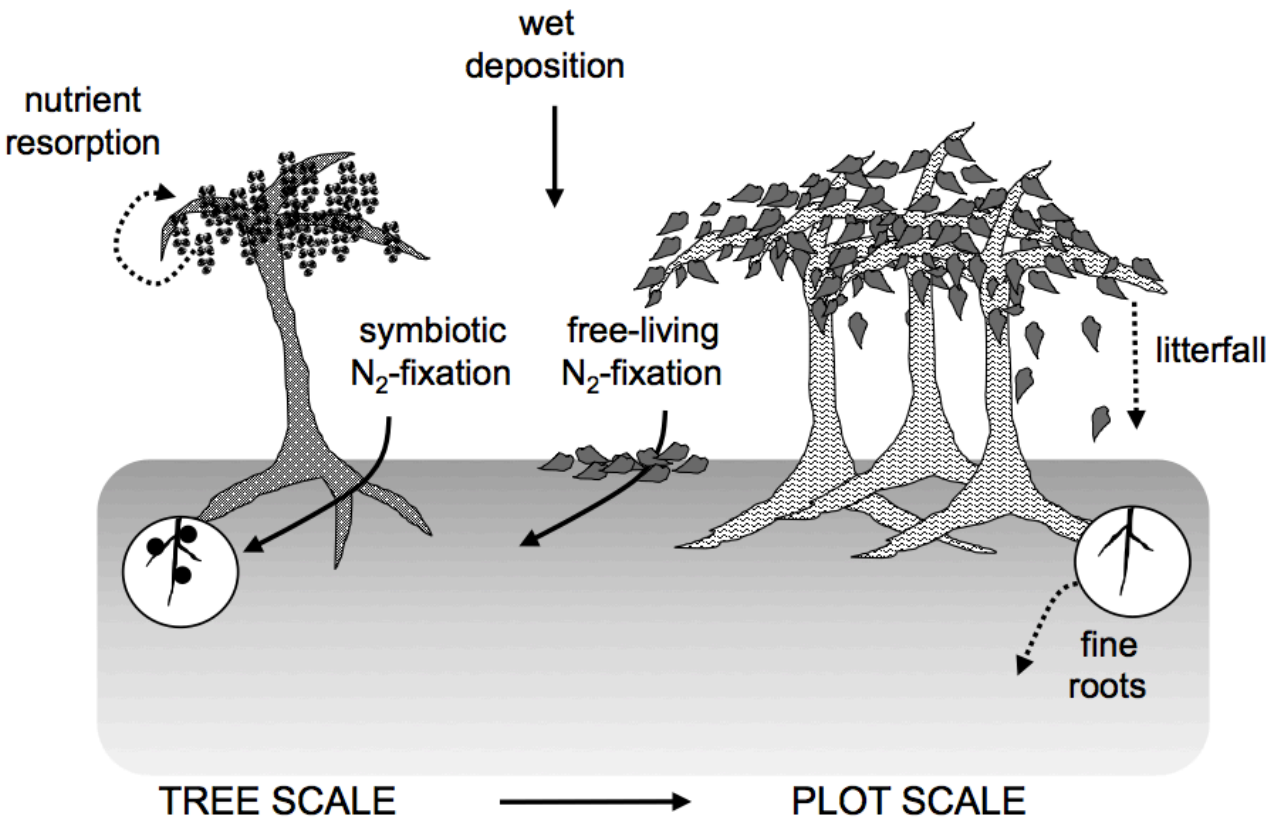

wet
deposition
nutrient
resorption
symbiotic
N_2-fixation
free-living
N_2-fixation
litterfall
fine
roots
TREE SCALE
PLOT SCALE

Figure 4-2

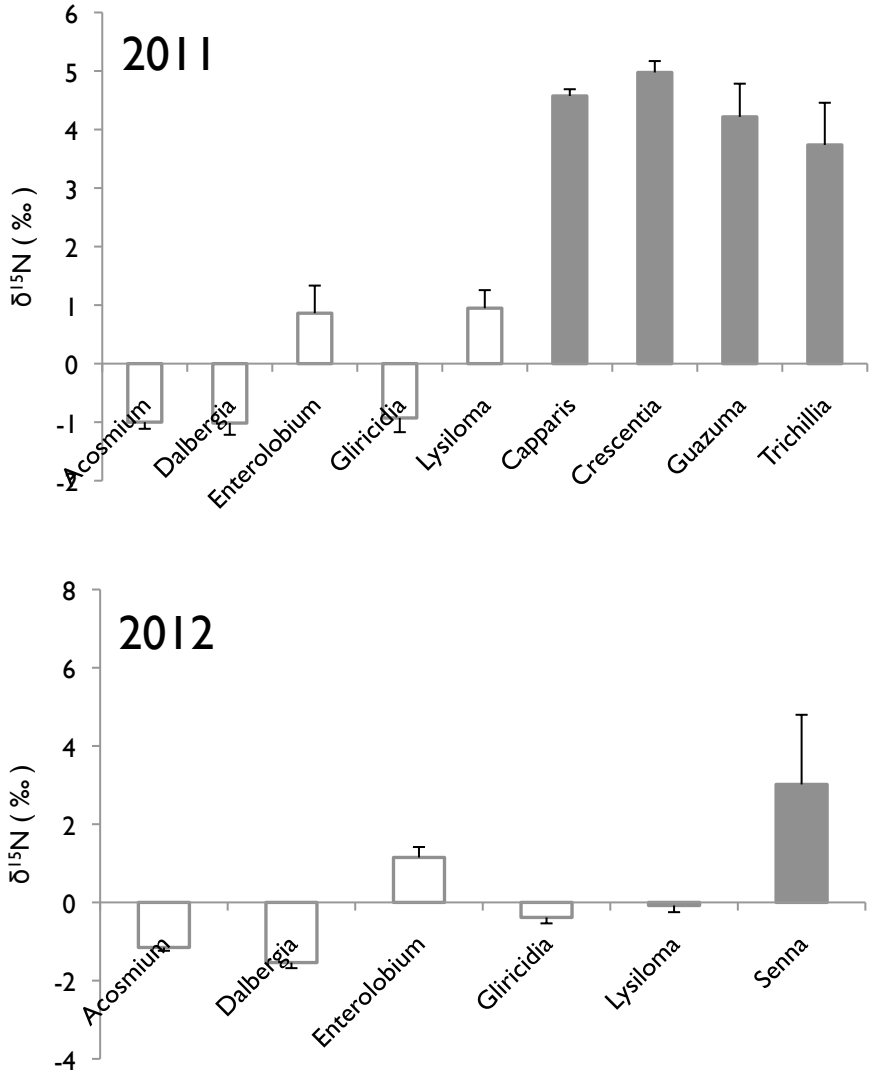

2011
δ15N (‰)
6
5
4
3
2
1
0
-1
-2
Acosmium
Dalbergia
Enterolobium
Gliricidia
Lysiloma
Capparis
Crescentia
Guazuma
Trichillia
2012
δ15N (‰)
8
6
4
2
0
-2
-4
Acosmium
Dalbergia
Enterolobium
Gliricidia
Lysiloma
Senna

Figure 4-3

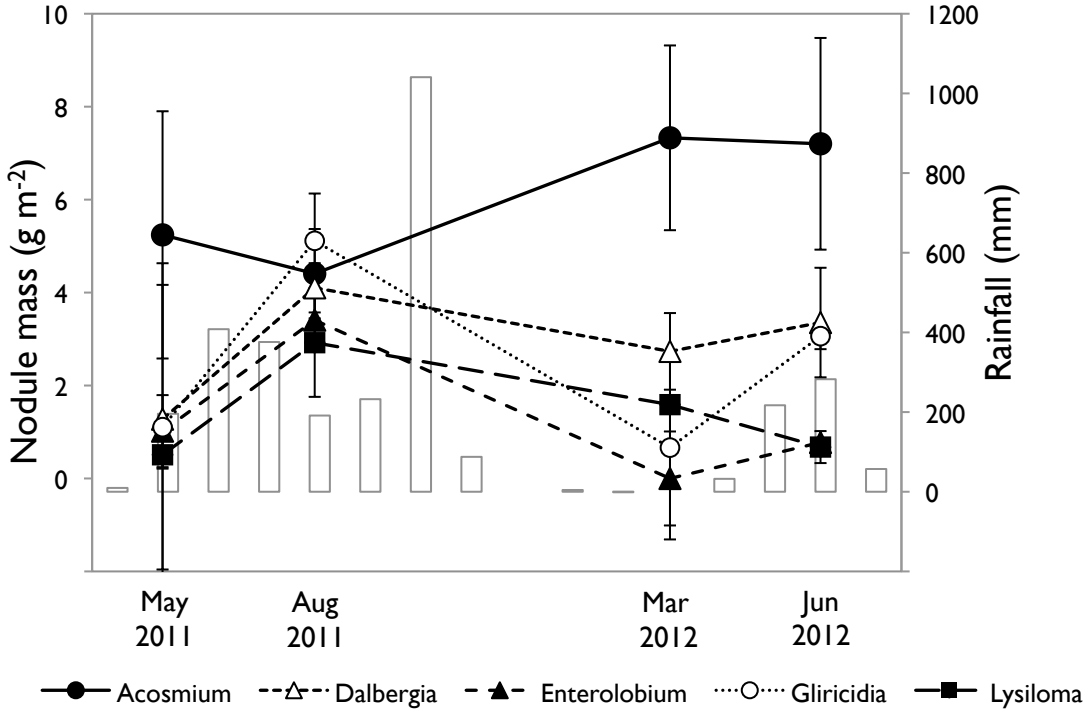

10
8
6
4
2
0
Nodule mass (g m-2)
1200
1000
800
600
400
200
0
Rainfall (mm)
May 2011
Aug 2011
Mar 2012
Jun 2012
Acosmium
Dalbergia
Enterolobium
Gliricidia
Lysiloma

Figure 4-4

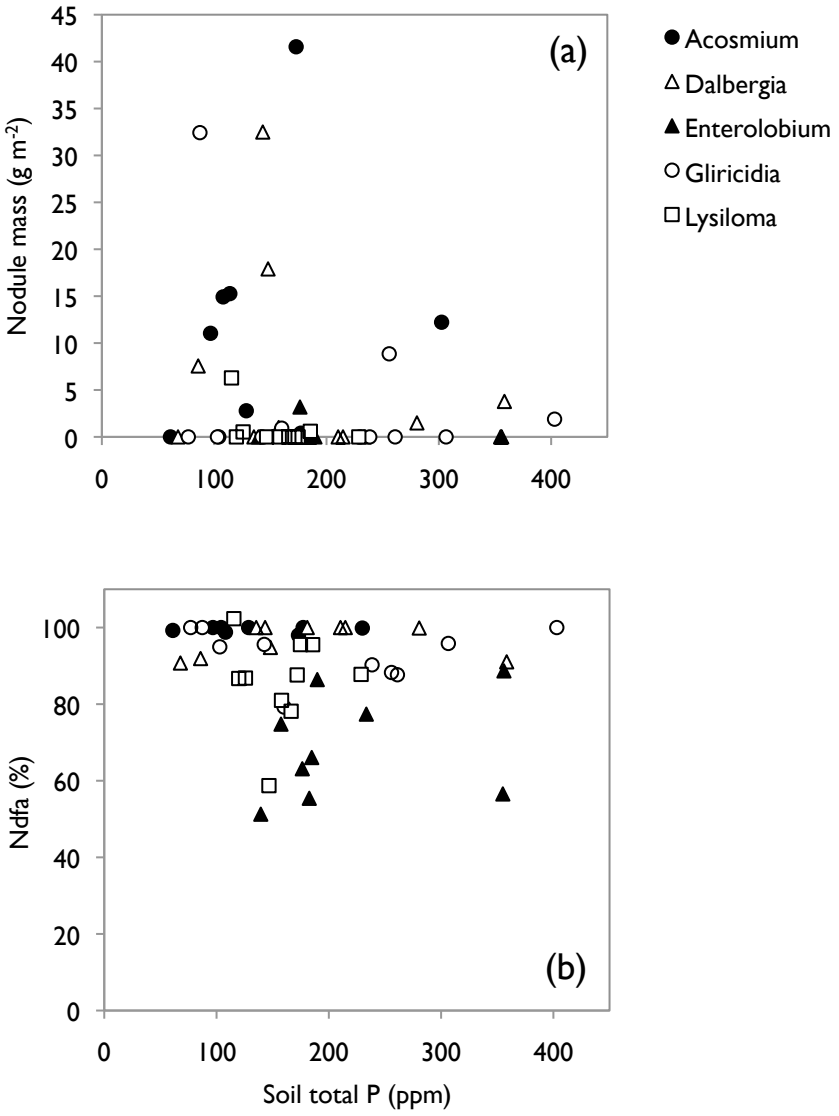
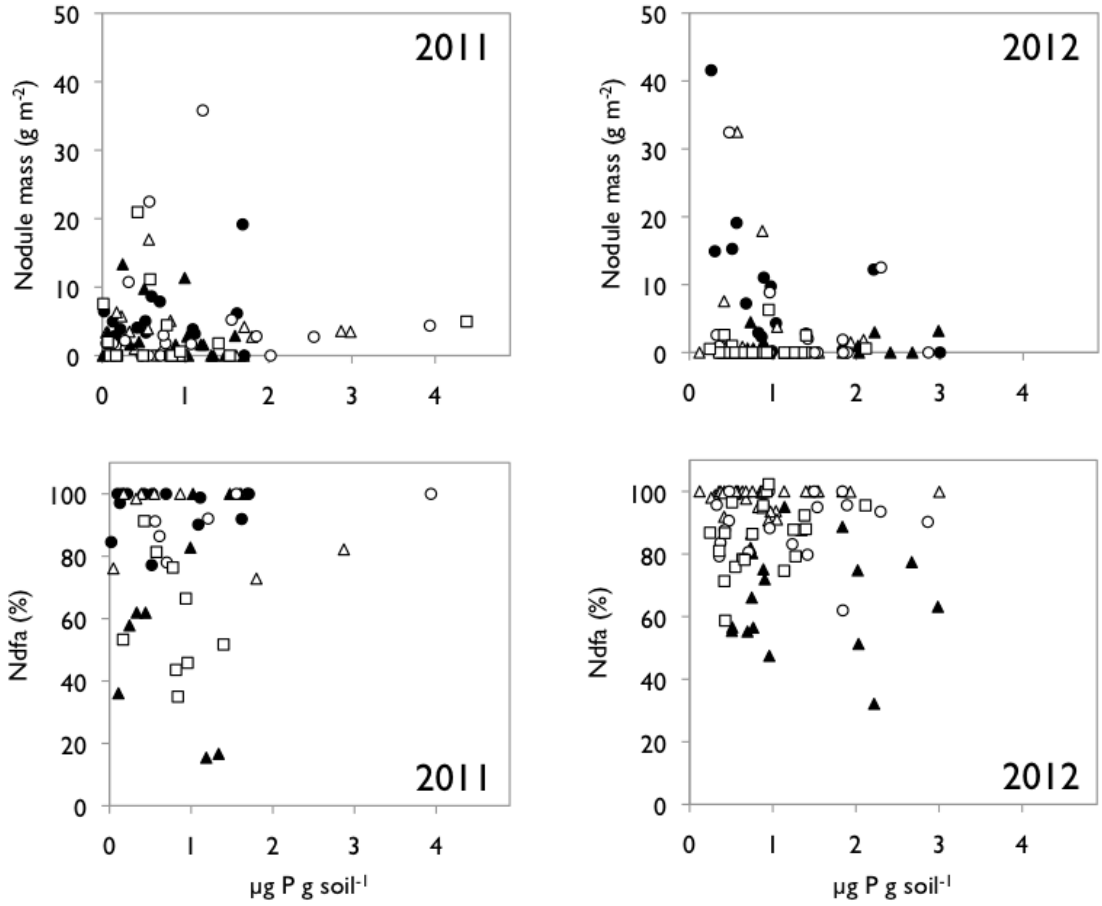

(a)
Nodule mass (g m-2)
0
5
10
15
20
25
30
35
40
45
0
100
200
300
400
Acosmium
Dalbergia
Enterolobium
Gliricidia
Lysiloma
(b)
Ndfa (%)
0
20
40
60
80
100
0
100
200
300
400
Soil total P (ppm)

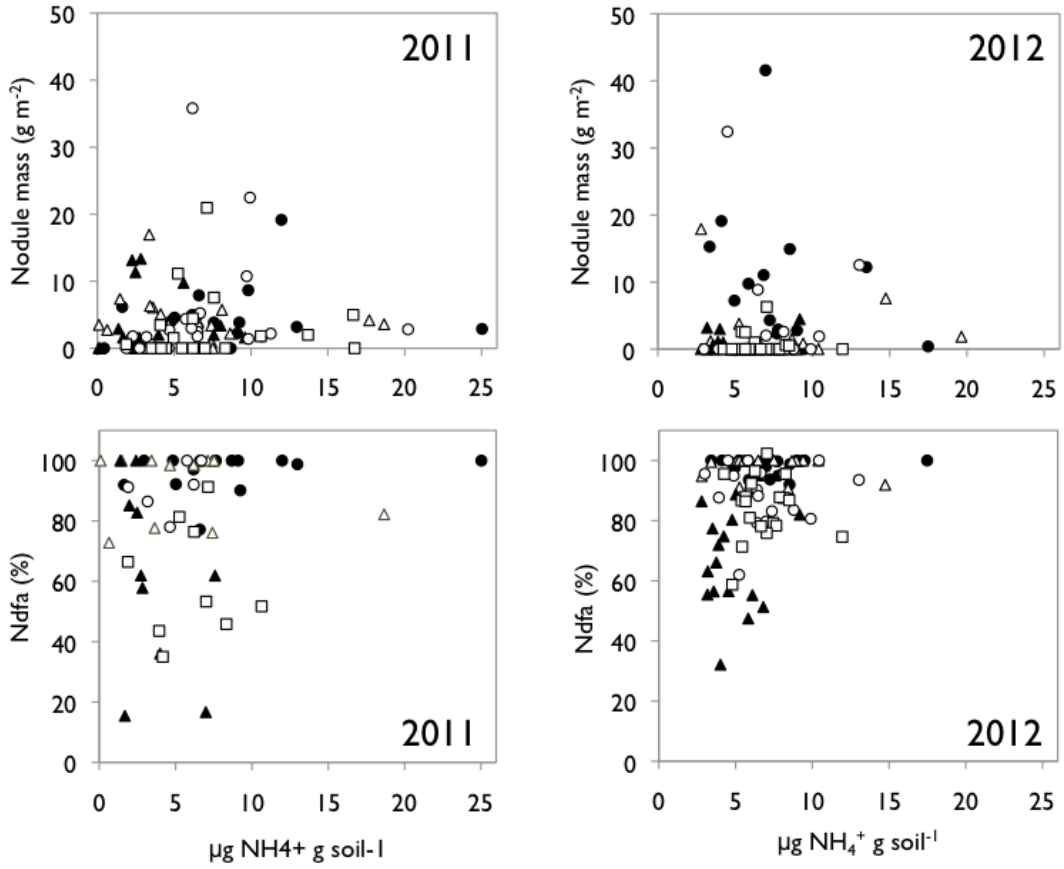
Figure 4-5



Legend:

- ● Acosmium
- △ Dalbergia
- ▲ Enterolobium
- ○ Gliricidia
- □ Lysiloma

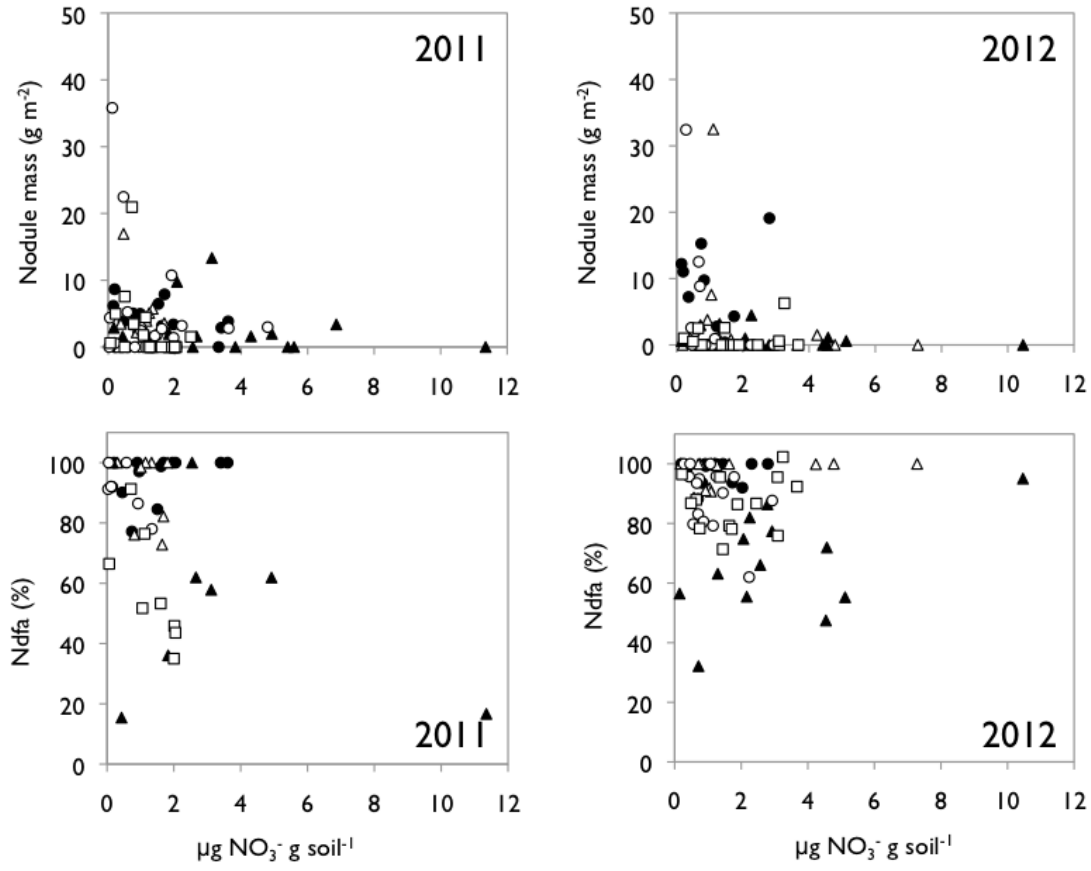
Figure 4-6



Legend:

- ● Acosmium
- △ Dalbergia
- ▲ Enterolobium
- ○ Gliricidia
- □ Lysiloma

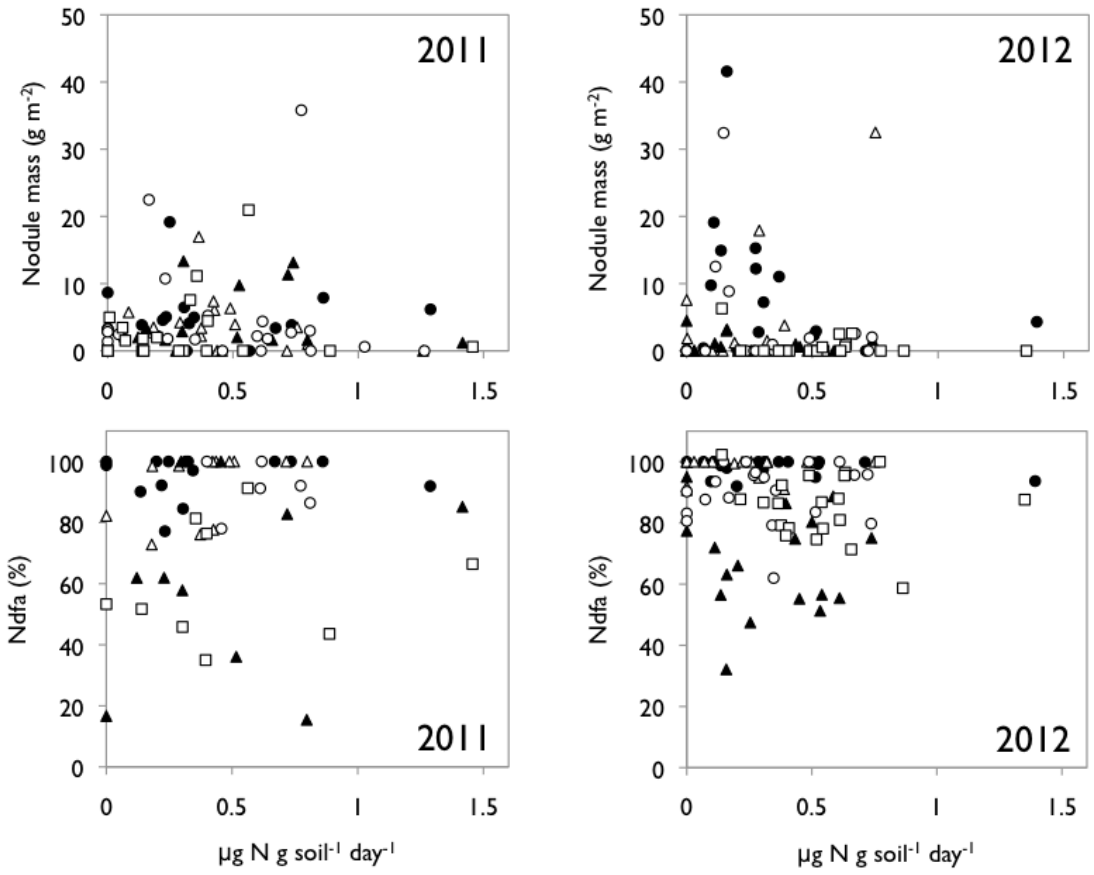
Figure 4-7



Legend:

- ● Acosmium
- △ Dalbergia
- ▲ Enterolobium
- ○ Gliricidia
- □ Lysiloma

Figure 4-8



Legend:

- ● Acosmium
- △ Dalbergia
- ▲ Enterolobium
- ○ Gliricidia
- □ Lysiloma

Figure 4-9

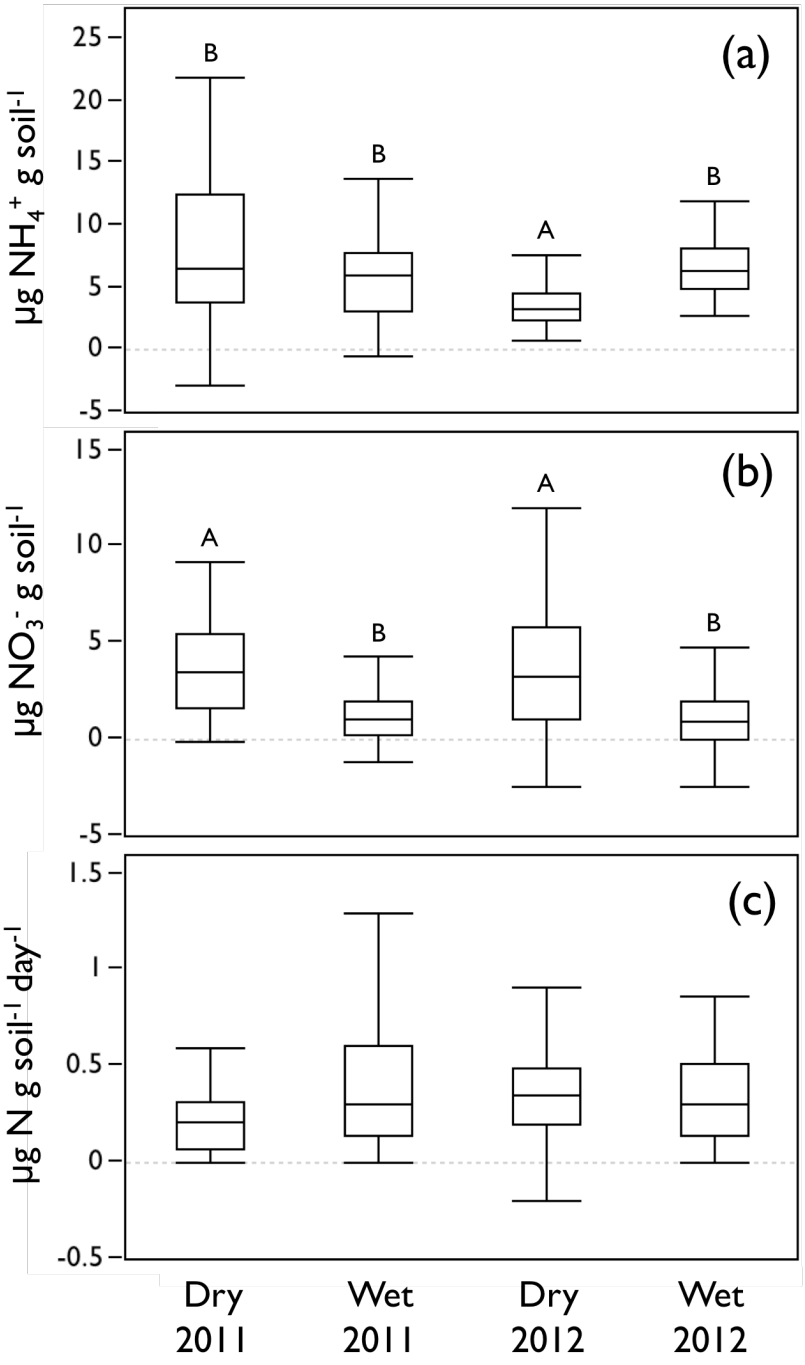

(a)
(b)
(c)
μg NH_4^+ g $soil^{-1}$
μg NO_3^- g $soil^{-1}$
μg N g $soil^{-1}$ day^{-1}
B
B
A
B
A
B
A
B
25
20
15
10
5
0
-5
15
10
5
0
-5
1.5
1
0.5
0
-0.5
Dry 2011
Wet 2011
Dry 2012
Wet 2012

Figure 4-10

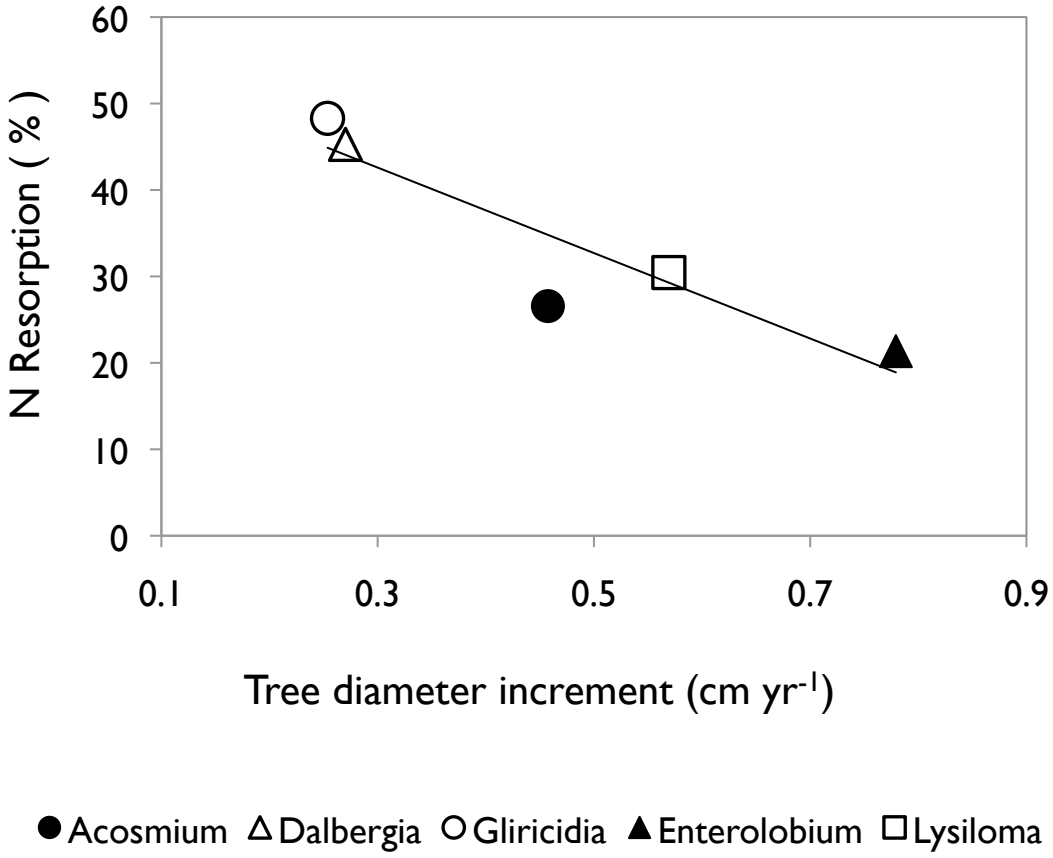

60
50
40
30
20
10
0
N Resorption (%)
0.1
0.3
0.5
0.7
0.9
Tree diameter increment (cm yr-1)
Acosmium
Dalbergia
Gliricidia
Enterolobium
Lysiloma

Figure 4-11

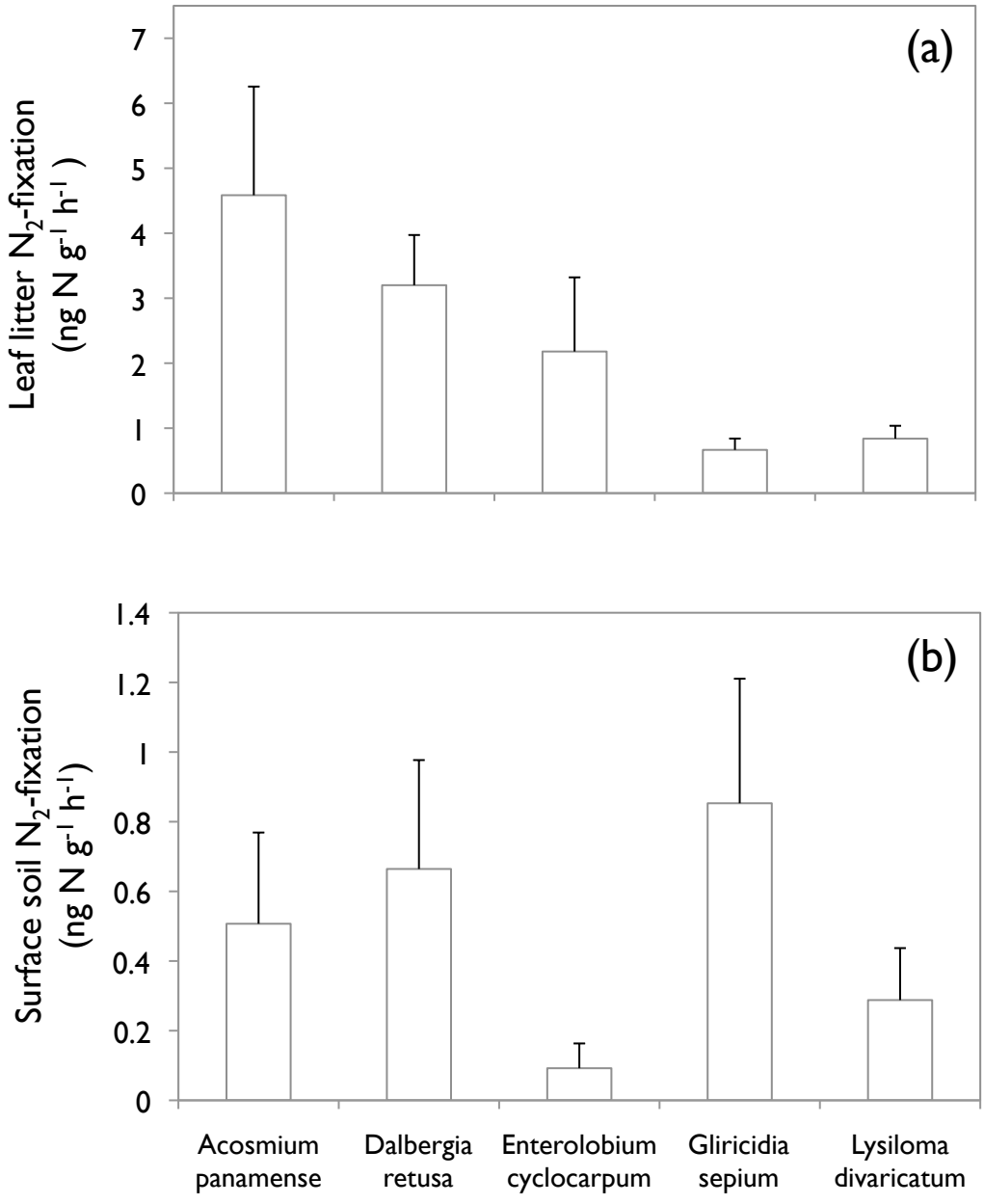

(a)
Leaf litter N_2-fixation (ng N g^{-1} h^{-1})
0
1
2
3
4
5
6
7
(b)
Surface soil N_2-fixation (ng N g^{-1} h^{-1})
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Acosmium panamense
Dalbergia retusa
Enterolobium cyclocarpum
Gliricidia sepium
Lysiloma divaricatum

Figure 4-12

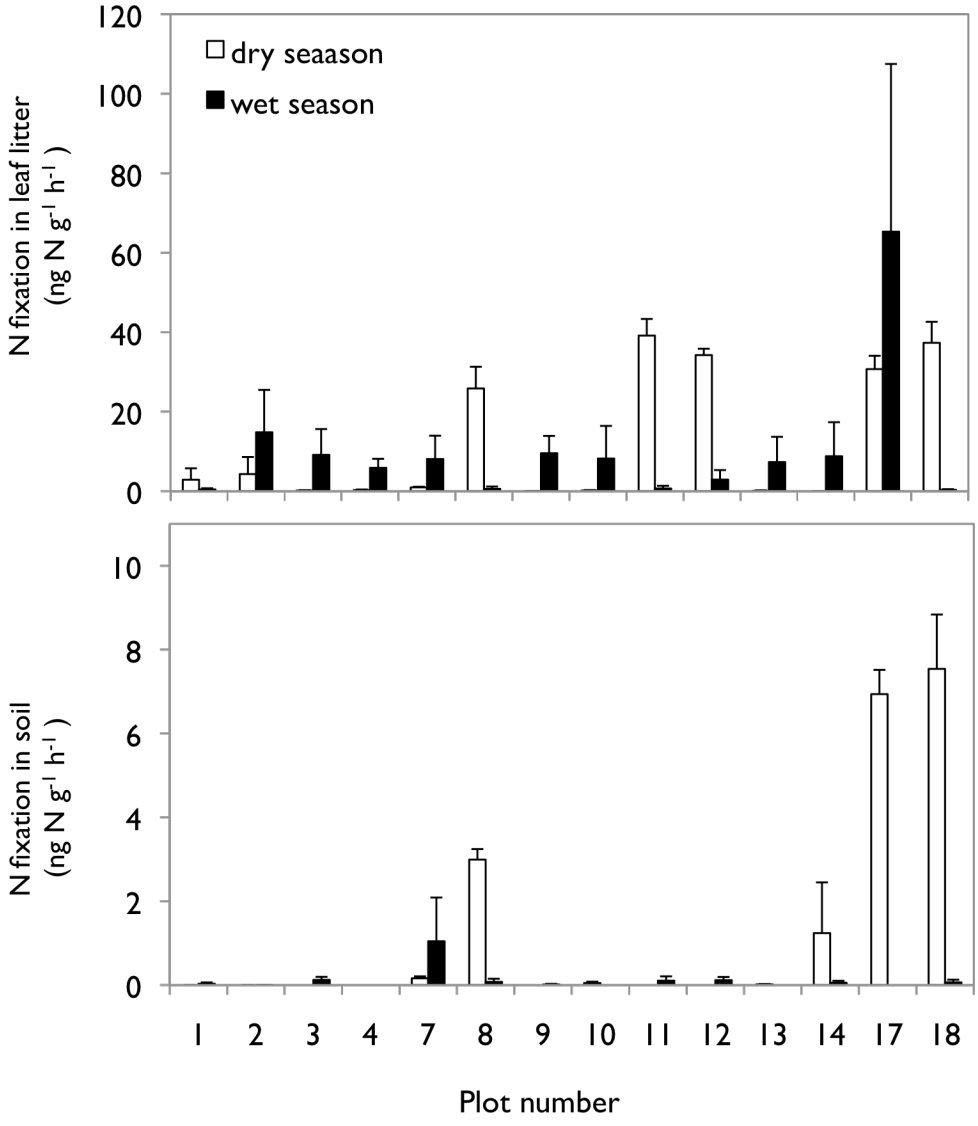

dry seaason
wet season
N fixation in leaf litter (ng N g-1 h-1)
N fixation in soil (ng N g-1 h-1)
0
20
40
60
80
100
120
0
2
4
6
8
10
1
2
3
4
7
8
9
10
11
12
13
14
17
18
Plot number

Figure 4-13

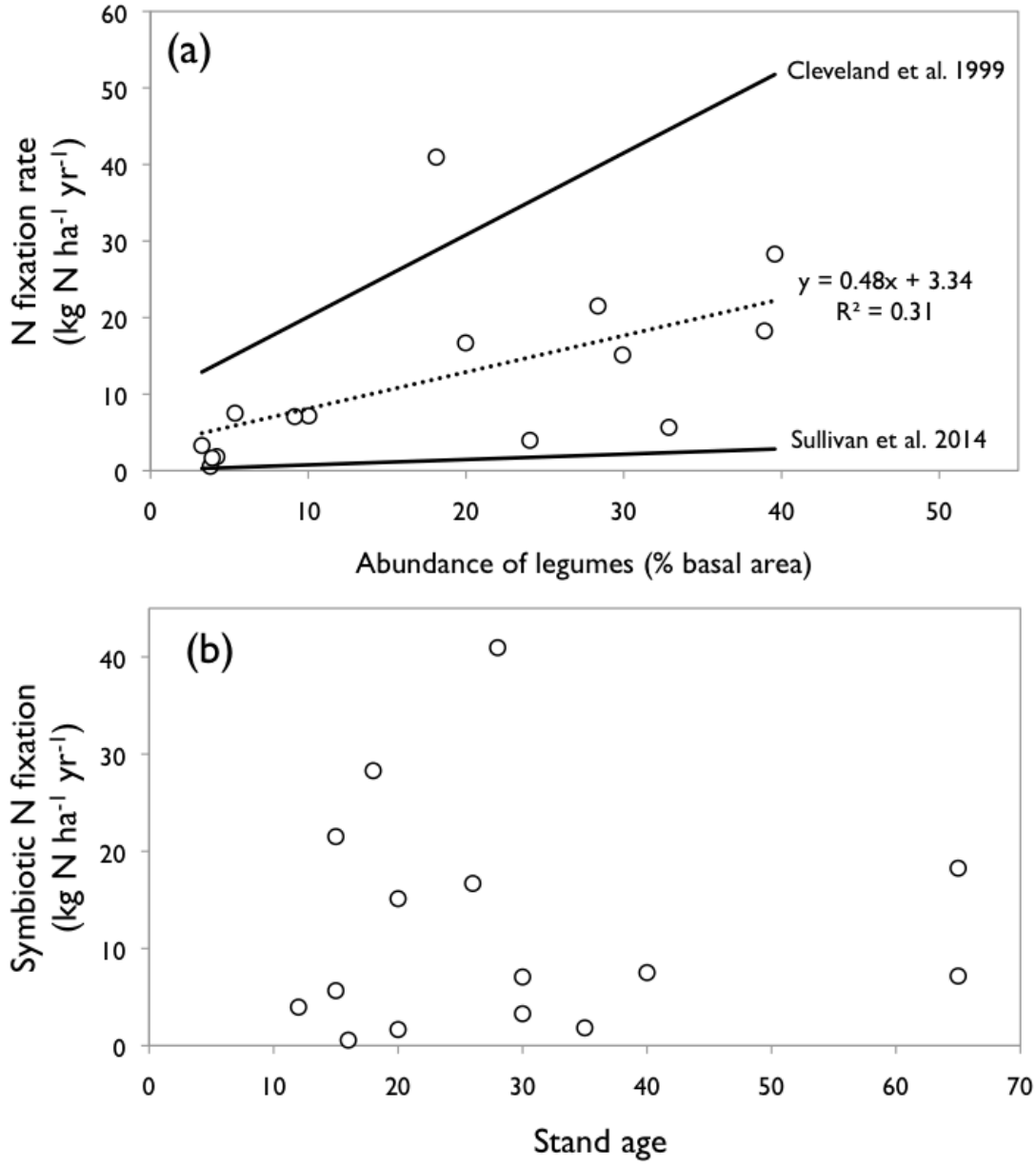

(a)
N fixation rate (kg N ha⁻¹ yr⁻¹)
Abundance of legumes (% basal area)
Cleveland et al. 1999
y = 0.48x + 3.34
R² = 0.31
Sullivan et al. 2014
0
10
20
30
40
50
60
(b)
Symbiotic N fixation (kg N ha⁻¹ yr⁻¹)
Stand age
0
10
20
30
40
50
60
70

Figure 4-14

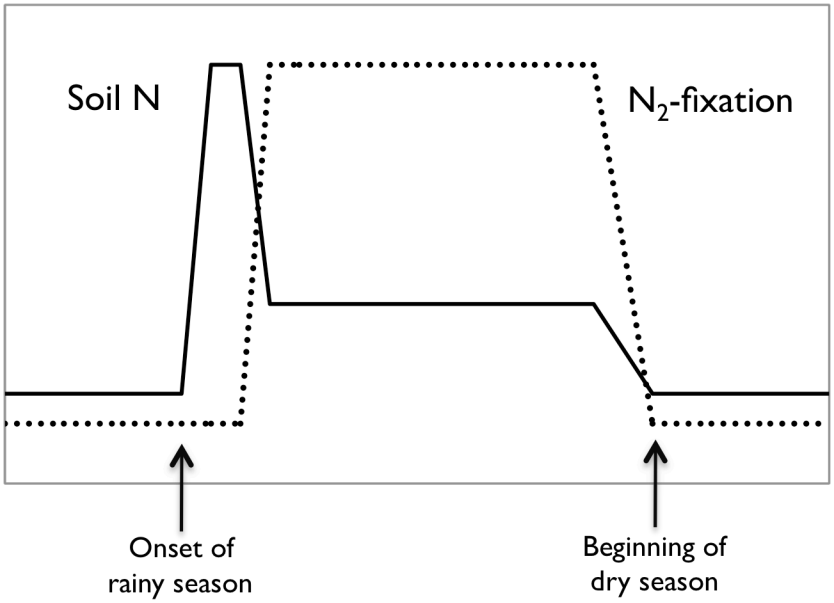

Soil N
N_2-fixation
Onset of rainy season
Beginning of dry season

Table 4-1. Species taxonomy and ecological features of four N fixing and one non-N fixing legume species common in the dry tropical forests of Área de Conservación Guanacaste, Costa Rica

Species	Subfamily	Tribe	Nodule type[1]	Height at maturity[2]	Local abundance[3]	Leaf P (%)[4]	Leaf C (%)[4]	SLA ($cm^2\ g^{-1}$)[4]	Wood density ($g\ cm^{-3}$)[4]
Acosmium panamense	Faboideae	Sophoreae	Indeterminate	20 m	2.53	0.068	48.96	92.19	0.768
Dalbergia retusa	Faboideae	Dalbergieae	Determinate	20 – 25 m	1.93	0.084	47.64	67.70	0.803
Enterolobium cyclocarpum	Mimosoideae	Ingeae	Indeterminate	25 – 35 m	1.62	0.101	47.81	145.51	0.377
Gliricidia sepium	Faboideae	Robinieae	Determinate	10 – 12 m	4.82	0.125	47.56	137.82	0.776
Lysiloma divaricatum	Mimosoideae	Ingeae	Indeterminate	15 m	3.81	0.069	48.68	113. 83	0.733

[1]Frioni et al. (1995), Parveen et al. (1997), González-Ruiz et al. (2008)

[2] From Cordero and Boshier (2003)

[3] Importance values from Powers and Becknell, unpublished.

[4]Data collected in 2008 from individuals in Área de Conservación Guanacaste. Values represent means of 1-7 trees, adapted from Powers and Tiffin (2010)

Table 4-2. Results of non-linear mixed models exploring species differences in nodule density in mature trees of five N fixing legume species (*Acosmium panamense*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, and *Lysiloma divaricatum*) across four sampling events (May 2011, August 2012, March 2012, and June 2012). Degrees of freedom: 392

	Value	Standard error	*P*-value
Intercept	10.1372	1.9346	0.0000
Dalbergia retusa	-7.2700	2.7359	0.0082
Enterolobium cyclocarpum	-8.8370	2.7359	0.0013
Gliricidia sepium	-7.6514	2.7359	0.0054
Lysiloma divaricatum	-8.7135	2.7359	0.0016

Table 4-3. Nitrogen wet deposition in an open area of Área de Conservación Guanacaste during 2011

2011	Rainfall (mm or L m^{-2})	NH_4^+ (ppm or mg L^{-1})	mg N m^{-2}	NO_3^- (ppm or mg L^{-1})	mg N m^{-2}
January	0.1				
February	10.2				
March	0				
April	9.6				
May	195.7	0.38	73.51	0.24	47.73
June	408.1	n.a.	n.a.	n.a.	n.a.
July	375.9	0.02	6.04	0.00	0.00
August	191.3	0.02	4.21	0.00	0.00
September	232.4	0.10	23.88	0.17	40.59
October	1040.7	0.01	7.18	0.00	0.00
November	87.6	0.03	2.79	0.14	12.22
December	0				
Total			117.61		100.54

Table 4-4. Stand-level estimates of nitrogen inputs to tropical dry forest in Área de Conservación Guanacaste and Parque Nacional Palo Verde, Costa Rica. All data is reported in units of kg N ha^{-1} yr^{-1}

Variable	Estimate	Range
Inputs		
Wet deposition (NO_3^- and NH_4^+)	2.18	
Symbiotic N fixation via legume basal area – Low nodulation	5.02	0.19 – 21.76
Symbiotic N fixation via legume basal area – High nodulation	20.24	1.65 – 43.22
Free-living N fixation in litter via ARA	0.37	0.02 – 1.41
Free-living N fixation in soil via ARA	0.62	0 – 2.35
Total free-living N fixation	0.99	0.02 – 3.74
Internal Transformations		
Litterfall	101.92	26 – 172
Fine roots	47.51	24.82 – 91.32

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Concluding remarks

Human activities are altering the global nitrogen (N) cycle at a rate and magnitude that exceeds even anthropogenic effects on the global carbon cycle (Galloway *et al*., 2004). Uncertainties around the N budgets from terrestrial ecosystems prevent us from accurately modeling of key fluxes in the global carbon cycle such as net primary productivity. In this rapidly changing world, robust estimates of the rates of biological N fixation in natural ecosystems are urgently needed not only to serve as a benchmark for comparison but also to predict and manage the consequences of anthropogenic global change (Vitousek *et al*., 2013). Biological N fixation by legumes is considered the largest natural source of new N to forest ecosystems. In the tropics, this N input alone could sustain a significant proportion of net primary productivity (Cleveland *et al*., 2013). Because tropical forests play a mayor role in the regulation of nutrient cycles and the global climate (Townsend *et al*., 2008), understanding the role of the group of legumes in tropical forests is crucial. To address these outstanding issues, I examined the contribution of legumes to the N cycle of one of the largest remaining protected dry forests in the Neotropics.

The group of N fixing legumes is frequently thought as a homogeneous functional group of species that share distinct characteristics like higher leaf N or the ability to fix N. However, I found several pieces of evidence that show that in tropical dry forests, this is not the case. First, results from the study of monospecific plantations of legumes in Chapter 1 suggest that individual species have measurable influences on a number of soil

properties, but that this effect is more pronounced than the influence of legumes as a functional group. In the same plantations, I observed species-specific variation in belowground foraging strategies (rooting depth) and in the timing and degree of nodulation (Chapter 2). In the shade house experiment (Chapter 3), species differed in their nodulation effort and in how they regulated N fixation with respect to available resources. In other words, species displayed a continuous range of phenotypes between obligate and facultative N fixation. These five legume species could be arrayed along a continuum defined by strategies of nutrient conservation and nutrient acquisition, which coincided with degrees of regulation of N fixation. Finally, in the field study (Chapter 4), I also observed a variety of strategies of N fixation where some legume species nodulation varied seasonally while other species maintained high nodule density throughout the year. The differences among species were consistent at different life stages and in experimental and observational studies: throughout these four studies *Acosmium panamense* showed less regulation of fixation and a more conservative nutrient strategy while *Enterolobium cyclocarpum* seems to fix N in a facultative way and invests more resources in nutrient acquisition. The group of legumes features great morphological and ecological diversity, with large ranges of character variation, life forms, life histories and geographical distributions (McKey, 1994) and my results suggest that the versatility of legumes includes their strategy of fixing N.

Besides from the fact that legumes in dry forests have a variety of strategies of N fixation, the conceptual models of how N fixation works in tropical wet forests may not necessarily be the same in seasonally dry forests. First, the nodulation patterns that I

gathered from plantations (Chapter 2) or diverse forests (Chapter 4) indicate that water is a strong control on the process of fixation. I did not find evidence of down-regulation of fixation with soil N, however it is possible that the adjustment of N fixation to soil nutrients occurs indirectly and is mediated by water availability and its effects on nutrient pulses. Furthermore, the considerable response of N fixation to phosphorus (P) additions at the seedling stage (Chapter 3) but the apparent disconnect between N fixation in mature trees and a wide range of soil P availability in the forest (Chapter 4) suggests the possibility that mycorrhizal associations have an important and unexplored role on P acquisition for legumes species.

My stand-level estimates symbiotic N fixation by legumes showed that legumes are responsible for the largest contribution of new N inputs to this ecosystem relative to other inputs such as free-living fixation or N wet deposition, but which are modest relative to N recycling through leaf litter and fine root decomposition (Chapter 4). Because in this region legume abundance does not vary consistently along gradients of forest age, I did not find directional trends in the contribution of N fixation to ecosystem N budgets with succession. Given that down-regulation of fixation is more likely driven by seasonality than by the availability of soil N, in tropical dry forests a decrease in N fixation with succession could only be driven by legume species replacement. Among the legume species that I studied, I found that species sorted along an axis of traits that typically defines whole-plant nutrient economies, between ‘slow’ nutrient conservation strategies and ‘fast’ investment in nutrient acquisition (Reich 2014). If the slow and fast legume strategies indeed represent different ways of dealing with the transient and

seasonal water availability of this ecosystem, slow legumes could be more adapted to survive long dry seasons but fast legumes able to quickly adjust N fixation and recover faster as soon as conditions become favorable. I hypothesize that in dry forests early pioneer legumes could have a fast strategy and are eventually replaced by slow, old growth legumes.

Last, conceptual frameworks of ecosystem ecology point towards environmental resources (soil N, P, molybdenum) as the main controls on N fixation. However, the possibility that the regulation of N fixation by environmental conditions depends upon the quality of the bacterial symbiont and the efficiency of that relationship, has received much less attention notably in tropical research. If we want to understand the biogeochemical consequences of a mutualistic relationship between plants and bacteria, accounting for the role of symbiont identity is a promising next step.

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