FIXING NITROGEN IN A CHANGING CLIMATE: HOW DROUGHT AND RISING TEMPERATURE AFFECT THE ECOLOGY OF *ROBINIA PSEUDOACACIA*

by

JEFFREY MICHAEL MINUCCI

(Under the Direction of Nina Wurzburger)

ABSTRACT

Climate change is increasing drought frequency, aridity, and temperature, which may affect symbiotic nitrogen fixation (SNF), a process which facilitates recovery from disturbance in terrestrial ecosystems. The impacts of these climate stressors may occur through their effects on the physiology, competitive ability, and geographic distribution of symbiotic N_2-fixing plants. In this dissertation, I examine how these stressors affect *Robinia pseudoacacia* L., the dominant N_2-fixing tree in Eastern US forests. I first tested how drought frequency affects the growth, physiology, and SNF rate of *R. pseudoacacia*, by growing seedlings in the greenhouse under soil moisture regimes which varied in drought frequency but not mean moisture. I found that, overall, *R. pseudoacacia* growth and SNF rate were resistant to both single prolonged droughts and frequent but brief droughts events, however drought frequency determined the physiological drought response strategy. I then examined how increased growing season aridity altered the competitive ability of *R. pseudoacacia* in an early successional forest, by experimentally reducing soil moisture over three years. I found that while *R. pseudoacacia* presence promoted greater soil nitrogen (N) availability and productivity of non-fixing trees, its growth and abundance were reduced in drier soils. Finally, I asked how aridity interacts with temperature and other abiotic factors to constrain the geographic distribution of *R. pseudoacacia* across the Eastern US, using forest inventory data. I found that presence of *R. pseudoacacia* was strongly associated with high mean annual temperature but only moderate summer temperature, conditions which generally promote a high value of N relative to carbon and minimize water stress. Using my model to project habitat suitability of *R. pseudoacacia* in 2050, I found that this species may gain substantial suitable habitat at the north edge of its range, while losing habitat at the southern edge as a result of higher temperatures. Collectively, the findings of this dissertation suggest that increasing aridity and temperature due to climate change will affect growth, competitive ability, and geographic distribution of the biogeochemical keystone species *R. pseudoacacia*, with implications for future N cycle dynamics and forest resilience to disturbance.

INDEX WORDS: *Robinia pseudoacacia* (black locust); symbiotic nitrogen fixation; drought; climate change; nitrogen cycle; Fabaceae; ecosystem ecology; resilience; disturbance

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DEDICATION

For Mom, Dad, Erin and Mumpo.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Global climate change is resulting in increased drought frequency, aridity and temperature (Sheffield & Wood, 2008; IPCC, 2013), which could alter the ability of terrestrial ecosystems to store carbon (C) and recover from disturbance by affecting symbiotic nitrogen fixation (SNF). SNF is carried about by plants which form symbiotic associations with nitrogen fixing bacteria (hereafter: N_2-fixers), which can access the essentially limitless pool of atmospheric nitrogen (N), thereby providing new inputs of reactive N to terrestrial ecosystems (Pearson & Vitousek, 2001; Vitousek *et al.*, 2002). Through this mechanism, N_2-fixers facilitate the build-up of N in these systems and also serve to replace N lost during disturbances (Menge, 2011; Batterman *et al.*, 2013a). If climate stressors reduce the rate of symbiotic nitrogen fixation (SNF) in terrestrial ecosystems, the ability of these systems to store C and recover from disturbance may be undermined due to increased N limitation (Norby *et al.*, 2010; Gerber *et al.*, 2013; Vitousek *et al.*, 2013).

Climate change may affect SNF by altering the abundance of N_2-fixing plants in terrestrial ecosystems, or their SNF activity. The ultimate constraint on SNF in a given ecosystem is the overall abundance of N_2-fixing plants (Vitousek & Field, 1999; Rastetter *et al.*, 2001), and thus any factor which reduces growth of N_2-fixers or their ability to compete with non-fixing plants may limit ecosystem N accumulation. However, there can also be variation in the amount of SNF activity per N_2-fixer biomass. Acquiring N through SNF is more costly than soil N uptake (Gutschick, 1981; Vitousek & Field, 1999) and while some N_2-fixers maintain a relatively stable rate of SNF (obligate N_2-fixers), many N_2-fixers regulate their carbon (C) investment in SNF in response to environmental conditions (facultative N_2-fixers) (Barron *et al.*, 2011; Menge *et al.*, 2015). When SNF is energetically favorable (*e.g.*, when soil N supply is low), facultative N_2-fixers can allocate more C to their bacterial symbionts and rely less on soil N uptake. Conversely, when SNF is less favorable (*e.g.*, when soil N supply is high) they may downregulate C investment to SNF and rely entirely on soil N uptake. As a result of this plasticity, if climate stressors such as drought or rising temperatures reduce N_2-fixer investment in SNF, ecosystem N inputs will decline, even if total abundance of N_2-fixers remains constant.

In Eastern US forests, only one N_2-fixing tree, *Robinia pseudoacacia* L., is widespread and achieves high abundance (Boring & Swank, 1984; Liao *et al.*, 2017). While temperate forests are typically dominated by obligate N_2-fixers of the *Alnus* genus and Rhamnaceae family, *R. pseudoacacia* is a facultative N_2-fixer of the legume subfamily Papilionoideae, a group whose trees occur mainly in tropical and subtropical areas (Menge *et al.*, 2014, 2017; Sheffer *et al.*, 2015). *R. pseudoacacia* is a deciduous, medium-sized, fast-growing tree, which rapidly colonizes areas disturbed by fire, logging or windfall and is often replaced by competing non-fixing trees after around 30 years (Boring & Swank, 1984; Shure *et al.*, 2006). It reproduces both sexually and asexually, with asexual reproduction occurring through clonal root-suckering from horizontal root spread (Jung *et al.*, 2009; Cierjacks *et al.*, 2013). Its native range is centered in the humid forests of the Appalachian Mountains, with a small out-population in the Ozark Mountains (Little, 1976). However, *R. pseudoacacia* has been widely planted outside its native range and occurs sporadically across the entire Eastern US. Additionally, *R. pseudoacacia* has become widely naturalized in the British Isles, continental Europe, temperate Asia, Australia and

New Zealand and has been classified as one of the worst invasive species worldwide (Richardson & Rejmánek, 2011; Cierjacks *et al.*, 2013; Vítková *et al.*, 2017).

In addition to being the only widespread N_2-fixing tree in Eastern US forests, *R. pseudoacacia* acts as biogeochemical keystone species by enhancing N cycling, and therefore it is vital to understand its ability to tolerate increased drought, aridity, and temperature. *R. pseudoacacia* is a prolific N_2-fixer, with SNF rates as high as 110 kg N ha^{-1} yr^{-1} in pure stands (Danso *et al.*, 1995), and its leaf and root litter contain high concentrations of N compared to other temperate trees (Alonso *et al.*, 2010). Soils beneath *R. pseudoacacia* stands may have greater soil N pools, as well as elevated N mineralization and nitrification rates, and streams draining *R. pseudoacacia* stands may have greater nitrate concentrations (Boring & Swank, 1984; Montagnini *et al.*, 1986; Swank *et al.*, 2001). As a result of this N enrichment, presence of *R. pseudoacacia* has been linked to greater productivity of a co-occurring non-fixing tree species, *Liriodendron tulipifera* (Apsley, 1987). While *R. pseudoacacia* can achieve very high SNF rates, investment in SNF can vary based on N supply and demand (Minucci *et al.*, 2017). The main mechanism for controlling SNF is differential investment in root nodules, the organs where SNF takes place (Pearson & Vitousek, 2001; Barron *et al.*, 2011). However, rapid fine-tuning of SNF rate is possible through adjustment of carbon supply to existing nodules (Johnsen & Bongarten, 1991). Because *R. pseudoacacia* alters forest N cycling so dramatically, the goal of this dissertation is to understand how its growth, competitive ability and SNF rate will be affected by increased drought frequency and greater growing season aridity, and how these climate factors interact with increased temperature to control its geographic distribution.

Drought frequency is increasing across the globe as a result of rising temperatures and greater temporal variability of precipitation (Sheffield & Wood, 2008; IPCC, 2013), and this stressor could affect the ability of *R. pseudoacacia* to bring new reactive N into Eastern US forests, by altering its investment in SNF or by decreasing its biomass through reduced growth rate. While individual drought events have been shown to increase SNF by *R. pseudoacacia* (Wurzburger & Miniat, 2014; Mantovani *et al.*, 2015), frequent drought events interspersed with re-wetting periods may have unexpected effects on growth and SNF rate. For example, high temporal variability in soil moisture may shift the balance between soil N supply and plant N demand, and *R. pseudoacacia* may respond by altering its overall investment in SNF. Frequent droughts may also affect *R. pseudoacacia* by causing time lags in the regulation of SNF. If soil moisture fluctuates more rapidly than *R. pseudoacacia* can regulate SNF, investment in SNF may be mismatched with soil N supply, resulting in suboptimal resource allocation and therefore reduced growth rate (Menge *et al.*, 2009a). Finally, frequent fluctuations in soil moisture could reduce growth by imposing greater costs associated with the regulation of SNF (Menge *et al.*, 2009a, 2011). For example, *R. pseudoacacia* may respond to frequent droughts by repeatedly constructing and senescing root nodules, and this carbon cost could be large enough to reduce its growth rate.

SNF by *R. pseudoacacia* may also be affected by increased aridity in the Eastern US, as long-term reductions in the mean soil moisture could shift competition between *R. pseudoacacia* and non-fixing trees, resulting in a decline in N_2-fixer biomass. Competition between *R. pseudoacacia* and non-fixers may be largely mediated by the resource trade-offs inherent to SNF. While SNF allows *R. pseudoacacia* to easily acquire N, this increased availability comes at a larger C cost than soil N uptake (Gutschick, 1981; Vitousek & Field, 1999; Vance, 2008). Therefore, the net benefit of SNF depends on the both the relative values of C and N. If low mean soil moisture results in greater stomatal limitation of photosynthesis for *R. pseudoacacia*, access to C will be limited, resulting in a greater relative value of C to N and a drop in the net benefit SNF provides over soil N uptake (Rastetter *et al.*, 2001; Vose *et al.*, 2016). Even if *R. pseudoacacia* responds by lowering investment in SNF and relying mainly on soil N uptake, non-fixing trees may be favored, as N_2-fixers tend to maintain high foliar N concentrations even when they do not fix N (Wright *et al.*, 2004), which makes them more susceptible to herbivory (Vitousek & Field, 1999; Knops *et al.*, 2000).

The physiological effects of drought on *R. pseudoacacia* and the impact of increased aridity on competition may combine with other factors like temperature, light availability, and soil nutrient content to shape the geographic distribution of this important species. Because SNF is the most obvious competitive advantage N_2-fixers hold over non-fixers, it is reasonable to predict that *R. pseudoacacia* presence is limited to conditions where SNF is favorable over soil N uptake (i.e. the value of N is high relative to the value of C) (Vitousek & Field, 1999). As previously discussed, soil moisture can mediate C supply to plants (Vose *et al.*, 2016), therefore *R. pseudoacacia* may be more likely to occur where moisture availability is high. Temperature could also play a significant role in determining success of *R. pseudoacacia.* As a member of a largely tropical and subtropical N_2-fixing subfamily, *R. pseudoacacia* may have a low cold tolerance compared to its Eastern US competitors (Menge *et al.*, 2009a). Nitrogenase, the enzyme that performs N_2-fixation, is temperature-dependent and achieves maximum activity at 26ºC or higher (Houlton *et al.*, 2008). However, high maximum temperatures during the growing season could also reduce soil moisture, which could limit C supply, as discussed above. Light availability and the ability of soils to hold N could also help shape *R. pseudoacacia* distribution, by altering the relative value of C, and N respectively (Menge *et al.*, 2010). Better understanding how these diverse factors combine to govern the geographic distribution of *R. pseudoacacia* will help us to predict the future range of this species and its impact on N cycling dynamics in Eastern US forests.

The goal of this dissertation is to examine how changes to drought frequency, mean soil moisture, and temperature will affect the role of *R. pseudoacacia* as a biogeochemical keystone species in Eastern US forests. In Chapter 2, I examine the effect of drought frequency on the growth, physiology and SNF rate of *R. pseudoacacia* by growing seedlings in the greenhouse under soil moisture regimes that differ in the frequency of droughts but not overall mean soil moisture. In Chapter 3, I examine how mean soil moisture shifts competition between *R. pseudoacacia* and non-fixing trees, through a 3-year field study where precipitation was manipulated to regenerating forest plots. Finally, in Chapter 4, I use forest inventory data and machine learning algorithms to examine what factors best predict the presence or absence of *R. pseudoacacia* in forest plots across the Eastern US. I then use the resulting model to forecast habitat suitability for this species under future climate scenarios. The results of this dissertation will contribute to our understanding of how climate shapes the realized niche of *R. pseudoacacia,* and therefore how it will respond to future climate stressors. More broadly, these findings will help to elucidate the ecological constraints on symbiotic N_2-fixation, which allow for the persistence of N limitation in many ecosystems and will shape the effects of global climate change on terrestrial N cycle dynamics.

CHAPTER 2

TOLERANCE OR AVOIDANCE: DROUGHT FREQUENCY DETERMINES THE RESPONSE OF AN N_2-FIXING TREE[1]

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[1] Minucci JM, Miniat CF, Teskey RO, Wurzburger N. 2017. Tolerance or avoidance: drought frequency determines the response of an N_2-fixing tree. *New Phytologist* 215: 434–442. (doi:10.1111/nph.14558).
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Abstract

- Climate change is increasing drought frequency, which may affect symbiotic N_2-fixation (SNF), a process that facilitates ecosystem recovery from disturbance. Here we assessed the effect of drought frequency on the ecophysiology and SNF rate of a common N_2-fixing tree in eastern US forests.
- We grew *Robinia pseudoacacia* seedlings under the same mean soil moisture but with different drought frequency due to wet-dry cycles of varying periodicity.
- We found no effect of drought frequency on final biomass or mean SNF rate. However, seedlings responded differently to wet and dry phases depending on drought frequency. Under low frequency droughts plants fixed C and N at similar rates during wet and dry phases. Conversely, under high frequency droughts plants fixed C and N at low rates during dry phases, and at high rates during wet phases.
- Our findings suggest that *R. pseudoacacia* growth is resistant to increased drought frequency because it employs two strategies– drought tolerance or drought avoidance followed by compensation. SNF may play a role in both by supplying N to leaf tissues for acclimation and by facilitating compensatory growth following drought. Our findings point to SNF as a mechanism for plants and ecosystems to cope with drought.

Keywords: acclimation, climate change, compensatory growth, ecosystem, lag times, *Robinia pseudoacacia* (black locust), symbiotic nitrogen fixation, temporal variability

Introduction

Global climate change is increasing the temporal variability of precipitation to terrestrial ecosystems (IPCC, 2013), resulting in greater frequency of drought events (Sheffield & Wood, 2008), and posing a threat to the services these systems provide (Vose *et al.*, 2016). Increased drought frequency may have direct negative effects on ecosystem primary productivity as plants are exposed to more frequent fluctuations in soil moisture and periods of low water availability (Knapp *et al.*, 2002). However, increased drought frequency may indirectly reduce productivity via symbiotic N_2 fixation (SNF), a key ecosystem process facilitated by plants. Symbiotic N_2 fixation supplies the majority of new nitrogen (N) to terrestrial ecosystems (Vitousek & Howarth, 1991), particularly those recovering from disturbances (Boring & Swank, 1984; Batterman *et al.*, 2013), but is sensitive to changes in soil N supply and plant N demand that may be driven by fluctuations in soil moisture. Thus, if increased drought frequency negatively affects SNF, it could reduce terrestrial ecosystem productivity and resilience to disturbance.

The response of SNF to increased drought frequency depends on how N_2-fixing plants (hereafter: N_2-fixers) respond to repeated drought. While N_2-fixers appear to increase SNF during individual drought events (Tobita *et al.*, 2010; Wurzburger & Miniat, 2014; Mantovani *et al.*, 2015; but see Serraj *et al.*, 1999), field studies have presented conflicting evidence on whether SNF increases or decreases across ecosystems with declining annual precipitation (Schulze *et al.*, 1991; Aranibar *et al.*, 2004; Soper *et al.* 2015). Additionally, it remains unclear how repeated drought and recovery cycles affect growth and SNF activity. A critical consideration is how drought frequency influences the soil N cycle. Since SNF is more costly than soil N uptake (Gutschick, 1981; Vitousek & Field, 1999), facultative N_2-fixers regulate SNF rate in response to soil N availability by constructing or excising root nodules, the organs where

SNF takes place. Thus, how SNF responds to increased drought frequency depends on how wet and dry phases affect: (1) the balance between soil N supply and plant N demand; (2) time lags in SNF regulation; and (3) the costs associated with regulating fixation.

Variation in soil moisture may shift the balance between soil N supply and plant N demand, altering the extent to which N_2-fixers carry out SNF. During periods of low soil moisture, reductions in N mineralization (Stanford & Epstein, 1974) and mass diffusion (Chapin, 1991) can decrease the supply of N to plant roots. Concurrently, plant N demand can decrease, remain stable, or increase depending on the physiological response to drought. Plant N demand may decrease if stomatal limitation on photosynthesis leads to decreased growth, but may recover if plants acclimate to drought and return to an ambient growth rate. Plant N demand may even increase during drought if plants acclimate by investing N in photosynthetic enzymes that increase the maximum rate of carboxylation (V_{cmax}) and thus increase water-use efficiency (WUE) (Wright *et al.*, 2001, 2005; Kitao *et al.*, 2007). However, as soils rewet, N supply may increase as moisture limitation of mineralization and diffusion are alleviated. In fact, rewetting events are often accompanied by large, transient pulses of mineral N that can exceed pre-drought levels (Tiemann & Billings, 2011). N_2-fixers may respond to these changes in N availability by adjusting investment in SNF (Barron *et al.,* 2011), because SNF is substantially more costly than soil N uptake (Vitousek & Field, 1999). As such, N_2-fixers may increase C investment to symbionts (either through construction of root nodules or greater flow of C to existing nodules), when soil N supply is limited (Tobita *et al.*, 2010; Wurzburger & Miniat, 2014), and decrease C supply to symbionts when soil N is more abundant. Thus, the effect of increased drought frequency on SNF depends on moisture-induced changes in both N supply and demand.

Frequent fluctuations in soil moisture may create time lags in the regulation of SNF. If soil moisture changes more rapidly than N_2-fixers can respond, allocation to SNF becomes suboptimal (Menge *et al.*, 2009). Such a mismatch between N acquisition strategy and the environment could result in reductions in N_2-fixer growth. Time lags are difficult to predict because we lack an understanding of the time scales of SNF regulation. Most N_2-fixers adjust SNF rate by constructing or excising root nodules (Pearson & Vitousek, 2001; Barron *et al.*, 2011). However, some species may rapidly fine-tune SNF by adjusting photosynthate supply to existing nodules, thereby altering nodule mass-specific SNF rate on a timescale of minutes to hours (Johnsen & Bongarten, 1991). Thus, the significance of time lags may depend on how N_2-fixers track changes in N availability.

Increased drought frequency could reduce the growth and biomass of N_2-fixers because of the carbon costs associated with regulating SNF (Menge *et al.*, 2009, 2011). The up- or down-regulation of SNF may result in energetic costs relating to cell growth, metabolism or protein synthesis. Nodule construction, in particular, represents a substantial “start-up” cost to SNF, which can be balanced by the benefits of increased N acquisition (Gutschick, 1981). If N_2-fixers construct and excise nodules more frequently, the “return on nodule investment” may decline due to shorter nodule lifespan. These costs may grow with increasing drought frequency (Menge *et al.*, 2011), and could result in reduced N_2-fixer growth, and thus reduced SNF.

Here we examined the effect of drought frequency on SNF with a manipulative experiment on a model N_2-fixing tree, *Robinia pseudoacacia* L., a widespread and ecologically important N_2-fixer in eastern United States forests (Boring, 1984; Boring & Swank, 1984). We hypothesized that seedling biomass and growth would be lower under high versus low drought frequency, even though both treatments had the same mean soil water content over the duration of the study, due to lag times in regulation and the costs of frequent nodule construction and excision. We also hypothesized that SNF rate and water use efficiency would be lower for seedlings under high versus low drought frequency, as a result of lag times in physiological response.

Materials and Methods

Experimental design

To assess the effect of drought frequency on SNF rate by *Robinia pseudoacacia* L., we conducted a greenhouse experiment in Athens, Georgia, USA. We germinated 140 *R. pseudoacacia* individuals from seed (Sheffield's Seed Co., Locke, NY) in 5 litre pots. Each pot contained a nutrient-poor potting mix consisting of a 1:1 ratio of local forest soil (Cecil series; Typic Kanhapludult), passed through a 4 mm sieve to remove roots and coarse organic matter, and coarse granite sand. To ensure seedlings had access to N_2-fixing symbionts, we inoculated each pot with 10 mL of a slurry consisting of field-collected *R. pseudoacacia* root nodules homogenized with deionized water. After 4 months of growth, we fertilized all pots with a ¼ strength Hoagland solution minus nitrogen.

Drought frequency treatments

After five months of growth, we randomly assigned each seedling to one of four 14-week drought frequency treatments: always wet control (AW), and three variable soil moisture treatments that cycled through "wet" and "dry" phases at low (LF), medium (MF), and high (HF) frequency (treatment *n* = 33; see Figure 2.1). The three variable soil moisture treatments had identical mean volumetric water content (VWC), averaged over the 14-week experiment, and the same total number of weeks in dry phase, but differed in the frequency of their wet/dry cycles. We maintained AW treatment pots and variable soil moisture pots during wet phases at 15% VWC, which was 70% of field capacity for our soil. Variable soil moisture pots during dry phases were held at 5% VWC, a level which caused early stomatal closure of *R. pseudoacacia* seedlings in preliminary tests but was above the wilting point for our soil (3% VWC). HF, MF, and LF treatments cycled between wet and dry phases every two, four and eight weeks, respectively. These treatments mimic conditions observed in *R. pseudoacacia* stands in Cowee, NC, where two-week droughts occurred multiple times per growing season and eight-week droughts occurred once in three years (J.M. Minucci *et al.*, unpublished data). While we exposed seedlings to similar levels of water stress during all "dry" periods, it is possible that the 8-week drought of the LF treatment posed an additional stress on seedlings due to carbon depletion, and the short but frequent droughts of the HF treatment may have stressed plants due to increased temporal variability. We randomly arranged all seedlings on a single row of tables in the greenhouse, and table was used as a blocking variable.

We randomly selected two seedlings from each treatment and outfitted their pots with 12 cm-long time domain reflectometer probes to estimate soil moisture (CS655, Campbell Scientific, Logan, UT). Since seedling roots reached the bottom of the pots by harvest one, these probes integrated moisture for the entire soil volume seedlings were accessing. We used the average soil VWC of these pairs to infer the water status of the treatment group and did not harvest or otherwise measure these seedlings at any point during the experiment. When a pair of moisture probe-equipped pots dropped 3% below the target VWC, we supplied every seedling in the treatment group with 0.25 l of water to increase VWC by approximately 6%. Drought frequency treatments began on 10 July 2014 and ran for 14 weeks under ambient light conditions.

Ecophysiological measurements and biomass determination

Every two weeks, we randomly selected five individuals from each treatment group for ecophysiological measurements and destructive harvesting. To quantify plant-available soil moisture at the time of harvest, we measured pre-dawn leaf water potential (Ψ_{pd}, MPa, PMS Instruments, Albany, OR) on one fully-extended leaf per individual. We measured leaf net carbon assimilation rate (A_{net}, µmol CO_2 m^{-2} leaf area s^{-1}) at saturating light (1800 µmol photons m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), and transpiration (E, mol H_2O m^{-2} s^{-1}) between 11 am and 1 pm, on the day of harvest (LI-6400XT, Li-Cor, Lincoln, NE). Measurements were taken on the terminal leaflet of the fully-extended leaf closest to the apical meristem. When the leaflet did not fill the chamber, we estimated leaflet area by measuring the width and length with calipers and using an allometric relationship previously established with greenhouse-grown *R. pseudoacacia* seedlings (Wurzburger & Miniat, 2014). Intrinsic water use efficiency or carbon gain per unit water lost (WUE_i, µmol CO_2 mol^{-1} H_2O) was estimated as A_{net} divided by g_s.

We then destructively harvested the five seedlings to estimate leaf, stem, root and nodule biomass pools. Biomass pools were separated and oven dried at 65 °C to constant weight and then weighed to the nearest 0.001 g. Because our results indicated strong relationships in nodule mass fraction and A_{net} in some treatment groups but not others, we estimated leaf N content (% dry weight) of HF and LF seedlings, and a subset of AW seedlings (50% randomly selected across all harvest dates) *post hoc*. For those samples, we ground the dried leaves to a fine powder in a ball-mill grinder. We then packed 15 mg of ground tissue into tin capsules and combusted samples according to the Dumas method on an elemental analyzer (Flash EA 1112 NC analyzer; Elantech, Lakewood, NJ).

Immediately upon harvest, we removed a subset of live root nodules from each seedling with forceps for estimate N_2 fixation rate via acetylene reduction assay (ARA, µmol N_2 fixed g^{-1} hr^{-1}; Barron *et al.*, 2011). We immediately placed each set of excised nodules into a tightly sealed 250 mL glass jar fitted with a rubber septum. We then replaced 10% of the jar headspace with C_2H_2, and incubated nodules for 20 minutes, taking 10 mL gas samples at 10 and 20 minutes with a syringe. We also ran control incubations without nodules to account for background C_2H_4 concentration. All samples were measured for ethylene production using a gas chromatograph equipped with a flame ionization detector (SRI Instruments, Torrance, CA). The ratio of nodule ethylene production to N_2 fixation was determined with a one-time set of ten 98% atom ^{15}N (Sigma-Aldrich, St. Louis, MO) incubations run in parallel with acetylene reduction assays. The mean (and SE) conversion ratio was 0.06 (± 0.01) µmol C_2H_4 : µmol N_2, which is at the low end of the range of values observed for woody N_2-fixers (Anderson *et al.*, 2004).

Soil moisture and nutrient analysis

To determine pot-level variability in soil moisture, we estimated soil moisture at the time of harvest by sampling ~20 g of homogenized soil from the pot of each harvested seedling. Samples were weighed and oven dried at 100 °C to a constant weight to determine percent gravimetric soil moisture. To test whether soil DIN pools changed in response to treatments, we quantified ammonium and nitrate pools with a 2M KCl extraction on the first and last harvest. For each seedling harvested, we sampled 25 g of soil, agitated it in 50 mL of 2M KCl for 4 h, and filtered it through 1 µm glass fiber filters. Ammonium and nitrate concentrations in the filtrate were quantified using continuous flow analysis (RFA300 Autoanalyzer, Alpkem Corp., Clackamas, OR).

Statistical analysis

To confirm that seedlings in wet and dry phases had different VWC and Ψ_{pd} at the time of harvest we used one-way analysis of variance (ANOVA). To test whether drought frequency treatment and water status (*i.e.*, wet/dry phase) affected our response variables, we used a two-way ANCOVA with type III sums of squares (R; car package). Explanatory variables in our models were drought frequency treatment, water status (*i.e*., wet or dry phase), and their interaction. The AW treatment was not included when assessing the interaction term, as AW seedlings experienced only the wet phase. We included total seedling biomass as a covariate to account for scaling relationships between plant size and response variables. Greenhouse table position was initially included as a blocking variable to account for spatial heterogeneity in greenhouse conditions, but it was not a significant predictor and was subsequently removed from analyses. When we detected a significant interaction between drought frequency treatment and water status, suggesting that seedlings responded differently to drought depending on their treatment, we used a set of linear contrasts to compare mean response value of wet and dry phase within each drought frequency treatment. We also compared means for each water status and treatment combination to the AW control. Family-wise type I error rate for contrasts was controlled at $\alpha = 0.05$ using a Bonferroni correction. If there was no interaction, post-hoc separation of means was carried out with Tukey's HSD test. All statistical analyses were performed with R 3.1.1 (R Development Core Team, 2016).

Results

Soil resources

Soils in wet phases had greater soil moisture than those in dry phases ($F_{1,129}$=366.5, P<0.001), with 14.96% soil moisture compared to 5.79% (Table 2.1). Mean pre-dawn water potential was also higher for the wet phase soils compared to the dry phase soils ($F_{1,130}$=66.4, P<0.001). Mean pre-dawn water potential for dry phases fell within the range of pre-dawn water potentials we observed on summer days in natural *R. pseudoacacia* stands in Cowee, NC (J.M. Minucci *et al.*, unpublished data). Dissolved inorganic nitrogen (DIN) soil pools did not differ from the first harvest to the final harvest (NH_4^+: $F_{1,31}$=0.003, P=0.42; NO_3^-: $F_{1,31}$=0.007, P=0.93), nor did they vary among drought frequency treatments (NH_4^+: $F_{3,31}$=0.3, P=0.83; NO_3^-: $F_{3,31}$=1.3, P=0.30) or between wet and dry phases (NH_4^+: $F_{1,31}$=0.7, P=0.41; NO_3^-: $F_{1,31}$=0.01, P=0.91).

Biomass partitioning and final biomass

At the end of the experiment, there were no differences in total biomass among drought frequency treatments ($F_{3,16}$=0.44, P=0.73; Figure 2.2). However, biomass partitioning among plant organs, specifically roots, differed throughout the experiment depending on treatment (drought frequency by water status interaction $F_{2,90}$=3.43, P=0.04; Table S2.1). The proportion of root biomass to total biomass (*i.e.*, root mass fraction) increased for seedlings in the low frequency drought (LF) treatment during wet phase. For this treatment, all weeks of wet phase occurred consecutively following an 8-week drought. There was no effect of drought frequency treatment or water status on the fraction of biomass partitioned to leaves (drought frequency: $F_{3,124}$=2.33, P=0.08; water status: $F_{1,124}$=0.60, P=0.44; Table S2.1), and we did not observe a significant amount of leaf shedding during dry phases.

Symbiotic N_2 fixation

Seedlings responded to wet and dry phases by up- or down-regulating nodule biomass rather than changing nodule mass-specific SNF rates, and the direction of this response depended on drought frequency (drought frequency by water status interaction: $F_{2,90}$ =12.6, P<0.001, Figure 2.3). For seedlings in the HF treatment, nodule mass fraction increased by 49% during wet phases compared with dry phases. Conversely, LF treatment seedlings increased nodule mass fraction 43% during the dry phase. MF treatment seedlings exhibited an intermediate response, with no differences in nodule mass fraction between wet and dry phases. When averaged across wet and dry phases, drought frequency did not affect nodule mass fraction ($F_{2,90}$=1.53, P=0.22). Nodule mass-specific SNF rate was consistent across drought frequency treatments and water statuses, with a mean rate of 16.8 (± 1.3) μmol C_2H_4, $g^{-1}hr^{-1}$, or 264.9 (± 20.6) μmol N_2 fixed $g^{-1}hr^{-1}$ based on our empirically-derived C_2H_4:N_2 conversion factor.

Leaf gas exchange

Drought frequency altered how net C assimilation responded to variation in soil moisture (drought frequency by water status interaction: $F_{2,90}$=3.1, P=0.048; Figure 2.4). Seedlings in the HF treatment assimilated 92% more C in wet versus dry phases, while MF and LF seedlings assimilated a similar amount of C in both phases. When averaged across wet and dry phases, drought frequency did not alter net C assimilation ($F_{2,90}$=0.28, P=0.76).

Stomatal conductance and transpiration depended on water status (g_s: $F_{1,90}$=33.7, P<0.001; E: $F_{1,90}$=36.1, P<0.001) and were not affected by drought frequency. Seedlings in wet phases had 113% and 105% higher g_s and E than those in dry phases, respectively. However, drought frequency altered how WUE_i responded to variation in soil moisture (drought frequency by water status interaction $F_{2,90}$=4.77, P=0.01; Figure 2.5). Seedlings in the LF treatment increased WUE_i by 68% during the dry phase, while MF and HF seedlings maintained consistent WUE_i across wet and dry phases.

Leaf N content

Drought frequency altered how leaf N content responded to dry versus wet phases (drought frequency by water status interaction $F_{1,80}$ =8.446, P=0.005; Figure 2.6). Seedlings in the LF treatment increased leaf N content by 21% during dry phases compared to wet phases, and by 10% compared to AW control seedlings. Leaf N content for HF seedlings was not significantly different than AW control for either wet or dry phases.

Discussion

R. pseudoacacia seedlings did not differ in their final biomass across treatments, and thus, we did not find support for our hypothesis that increased drought frequency would reduce N_2-fixer growth through costs associated with lag times or SNF regulation. Interestingly, SNF and other physiological traits responded differently to dry and wet phases depending on whether drought occurred at low or high frequency. Plant response strategies to drought include those that promote tolerance of water stress (*e.g.*, acclimation) and those that promote avoidance of water stress (McDowell *et al.*, 2008). In our study, seedlings exposed to low frequency drought cycles exhibited traits associated with drought tolerance, while those exposed to high frequency drought cycles exhibited traits associated with drought avoidance followed by compensatory growth upon soil rewetting. *R. pseudoacacia* may employ these contrasting strategies depending on how drought affects plant physiology and the availability of soil N.

Drought tolerance response

Seedlings under low drought frequency (LF) responded to a single 8-week drought by increasing WUE_i, root mass fraction, leaf N content, and SNF rate (via greater nodule mass fraction). These responses reached maximum values relative to the AW control after six or more weeks of drought (Figure S2.1), suggesting that drought tolerance was achieved through acclimation. Seedlings attained greater WUE_i by reducing g_s while maintaining a similar net assimilation to seedlings in constant well-watered conditions. Photosynthetic acclimation can result from increased N investment in Rubisco (Wright *et al.*, 2001), allowing for greater maximum rate of carboxylation (V_{cmax}) (Pankovic *et al.*, 1999; Kitao *et al.*, 2007) and WUE, and SNF may facilitate this response (Tobita *et al.*, 2010; Adams *et al.*, 2016). In support of this mechanism, we observed higher leaf N content in LF seedlings under dry versus wet phases. Additionally, we observed a significant positive relationship between leaf N content and nodule mass fraction (Pearson's $r = 0.43$, $P<0.001$), which suggests that investment in SNF may provide *R. pseudoacacia* the N required to acclimate to drought.

Both soil N supply and plant N demand can help explain plant investment in SNF under drought. Prolonged drought can reduce the N available to plants (Rennenberg *et al.*, 2009; He & Dijkstra, 2014) by suppressing microbial activity (Stanford & Epstein, 1974) and mass diffusion (Chapin, 1991), thus triggering an increase in SNF investment (Wurzburger & Miniat, 2014). In our study, although we did not detect differences in soil NH_4^+ or NO_3^- supply in response to drought frequency or water status, we observed an increase in nodule mass fraction in dry phases of the LF treatment, suggesting differences in plant N demand were driving the response of SNF. Plant N demand is largely determined by net C assimilation rate, as plants must acquire sufficient N to maintain C:N stoichiometry during growth (Elser *et al.*, 2010). Thus, N demand may decrease during drought, as a result of stomatal limitation on carbon assimilation, and hence, growth. However, if plants acclimate to drought, as observed in our LF treatment, A_{net} may recover to rates observed in well-watered plants (Flexas *et al.*, 2006)**,** and thereby maintain N demand. If, however, plants acclimate to drought specifically by increasing N investment in Rubisco, as above, N demand may become even greater than under wet conditions, and result in up-regulation of SNF (Wright *et al.*, 2001, 2005; Tobita *et al.*, 2010).

Drought avoidance and compensatory growth response

In contrast to LF seedlings, individuals grown under high drought frequency (HF) did not acclimate during repeated 2-week droughts, and they fixed C and N at low rates during these periods. This finding supports our hypothesis that seedlings would show reduced drought acclimation under high drought frequency. The lack of acclimation in HF seedlings is consistent with the idea that acclimation may require longer time periods of drought (*i.e.*, weeks to months) (Flexas *et al.*, 2006; Zhou *et al.*, 2016), and thus may be inhibited when droughts are interspersed with brief wet periods, as in our HF treatment. However, the lack of acclimation did not reduce final biomass, as HF seedlings exhibited a strong compensatory growth response, with rapid net photosynthesis and SNF rates during wet, inter-drought periods.

Seedlings grown under high frequency drought fixed N more rapidly during wet, inter-drought periods than during drought periods, a pattern which may be related to the effect of drought cycles on plant N demand. Short (2-week) drought periods in our HF treatment may have induced stomatal limitation of photosynthesis, and hence, reduced plant N demand due to a diminished growth rate. Upon rewetting however, SNF increased to a rate even greater than plants under constant moisture, and this response may be explained by greater plant N demand associated with rapid growth. Greater SNF following rewetting was not accompanied by increased leaf N content, which suggests that fixed N was not stored in leaf tissues, but instead fueled new growth. Interestingly, this increase in growth compensated for reductions during dry phases, resulting in no differences in final biomass relative to the control. Our findings of rewetting-induced compensatory growth may be vital for plant resistance to increased drought frequency. Such a response may be triggered by root production of cytokinins, which are translocated to shoots to promote rapid cell division (Wang *et al.*, 2016). Interestingly, cytokinin production is dependent on sufficient N supply to roots (Tamaki & Mercier, 2007), which suggests the possibility that N_2-fixing plants may be particularly adapted for drought-induced compensatory growth.

Ecosystem scale implications

Our findings suggest that *R. pseudoacacia* seedlings are resistant to both prolonged drought and increased drought frequency, and that SNF facilitates this resistance. Since ecosystem-level SNF is determined by the resource allocation strategies of individual plants, our findings may reveal the physiological basis of ecosystem SNF response to drought. If ecosystem-level SNF is unaffected by drought frequency, it lends natural resilience to terrestrial ecosystems recovering from disturbance (Boring & Swank, 1984; Menge *et al.*, 2012; Batterman *et al.*, 2013). However, applying our findings on tree seedlings to natural systems depends on how drought mediates the effects of competition between N_2-fixers and non-fixers (Wurzburger & Miniat, 2014), which may depend on access to deep water sources (Giordano *et al.*, 2011), susceptibility to herbivory (Gaylord *et al.*, 2013), and the successional stage of the ecosystem (Menge & Hedin, 2009; Batterman *et al.*, 2013). Additionally, since we fertilized our seedlings with all nutrients except

N, it is possible that other nutrients such as phosphorus may limit SNF rates in natural settings. Finally, drought frequency may affect the timing of SNF (*i.e.*, whether it occurs during dry or wet phases) as it did in our experiment, and it is unclear if this difference affects how fixed N enters and cycles within the plant-soil system.

Conclusions

In our study, growth and mean SNF rate of *R. pseudoacacia* seedlings were unaffected by increased drought frequency, and we found no evidence for costs associated with time lags and SNF regulation. However, we found that drought frequency determined how seedlings responded to individual drought events, where seedlings employed a tolerance strategy under low frequency drought and an avoidance strategy followed by rapid growth under high frequency drought. Interestingly, N fixation may supply the N needed in both strategies, by facilitating rapid growth following drought, and by accumulating N in leaf tissues for acclimation. While the response of natural forests to drought will depend on how N_2-fixing and non-fixing trees compete for, and acquire, water and soil N, our findings point to the potential role of SNF in ecosystem resistance to increased drought frequency.

Acknowledgements

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Author Contributions

J.M.M., N.W., C.F.M. and R.O.T designed the research. J.M.M. performed the research. J.M.M., N.W. and C.F.M analyzed the data and wrote the manuscript. R.O.T. edited final drafts of the manuscript.

Table 2.1: Effect of water status on gravimetric soil moisture (%SM), pre-dawn leaf water potential (Ψ_{pd}) and KCl-extractable ammonium and nitrate across treatments.

	Wet Phase	Dry Phase	*P*-value
% SM	14.96% (±0.36)	5.79% (±0.24)	<**0.001**
Ψ_{pd}	-0.13 MPa (±0.01)	-0.46 MPa (±0.05)	**<0.001**
NH_4^+	0.17 ppm (±0.03)	0.13 ppm (±0.03)	0.41
NO_3^-	0.08 ppm (±0.06)	0.07 ppm (±0.07)	0.91

Values represent means (±SE). *P*-values are given for the water status effect in ANOVA. Bold P-values indicate a statistically significant difference (P<0.05).

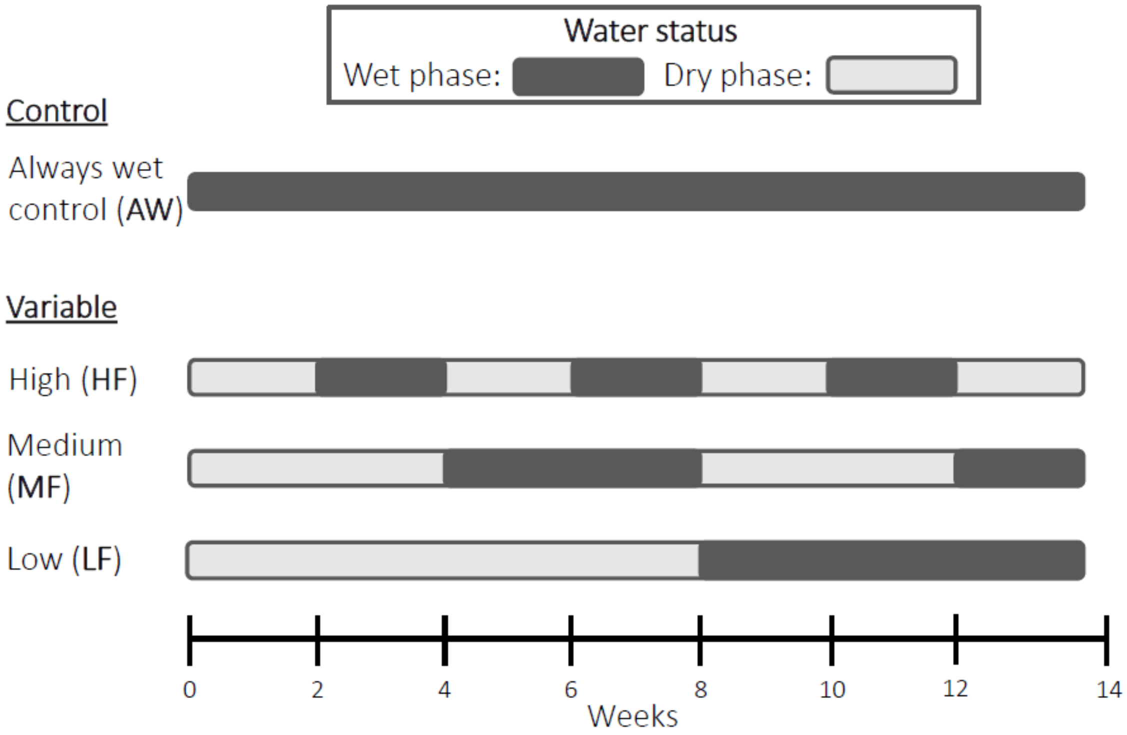

Figure 2.1: Schedule of the water status for each drought frequency treatment. Wet phases and dry phases corresponded to 15% and 5% volumetric water content, respectively. All "variable" treatments consisted of a total of 8 weeks in the dry phase and 6 weeks in the wet phase.

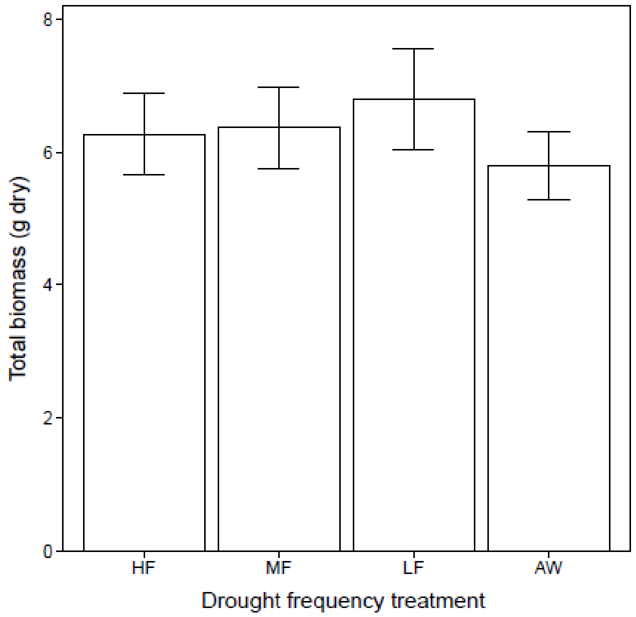

Figure 2.2: Final biomass for *R. pseudoacacia* plants in each drought frequency treatment. Bar heights represent untransformed means and error bars denote SE. Treatment abbreviations: HF = high frequency, MF = medium frequency, LF = low frequency, AW = always wet control.

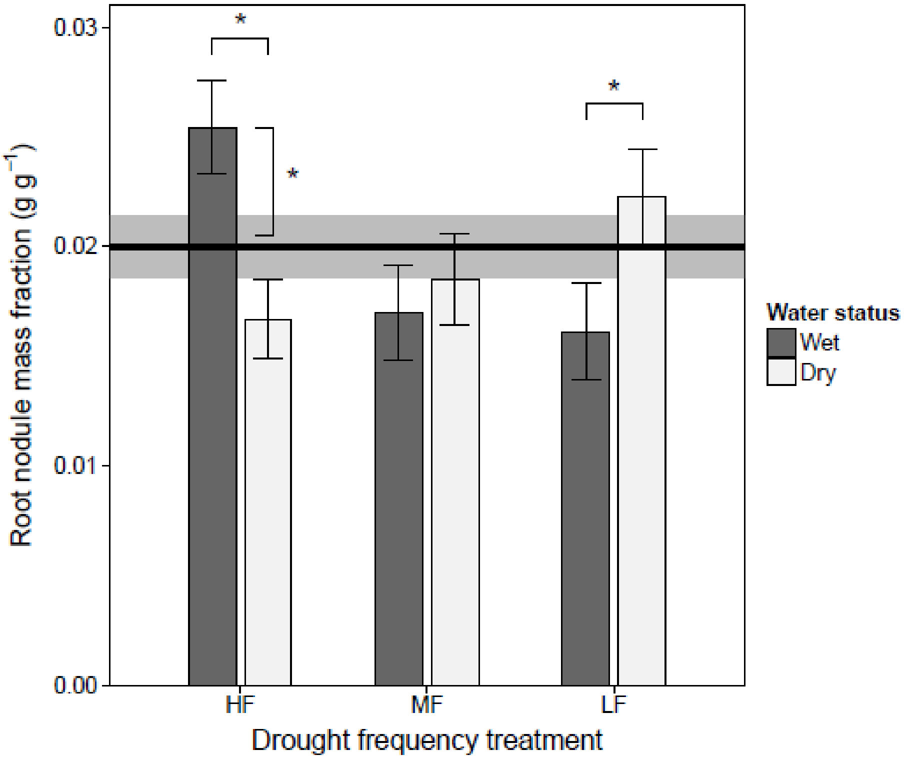

Figure 2.3: Effect of drought frequency and water status on *R. pseudoacacia* root nodule mass fraction. Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g dry weight). Error bars represent SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatment abbreviations: HF = high frequency, MF = medium frequency, LF = low frequency.

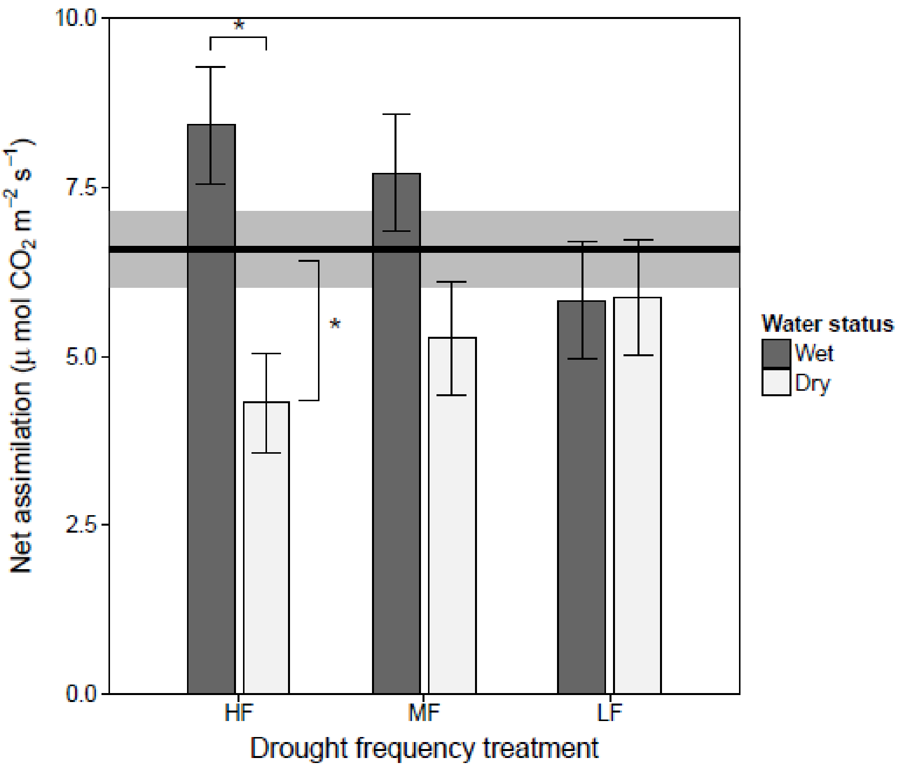

Figure 2.4: Effect of drought frequency and water status on *R. pseudoacacia* net carbon assimilation (net assimilation). Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g dry weight). Error bars represent SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatment abbreviations: HF = high frequency, MF = medium frequency, LF = low frequency.

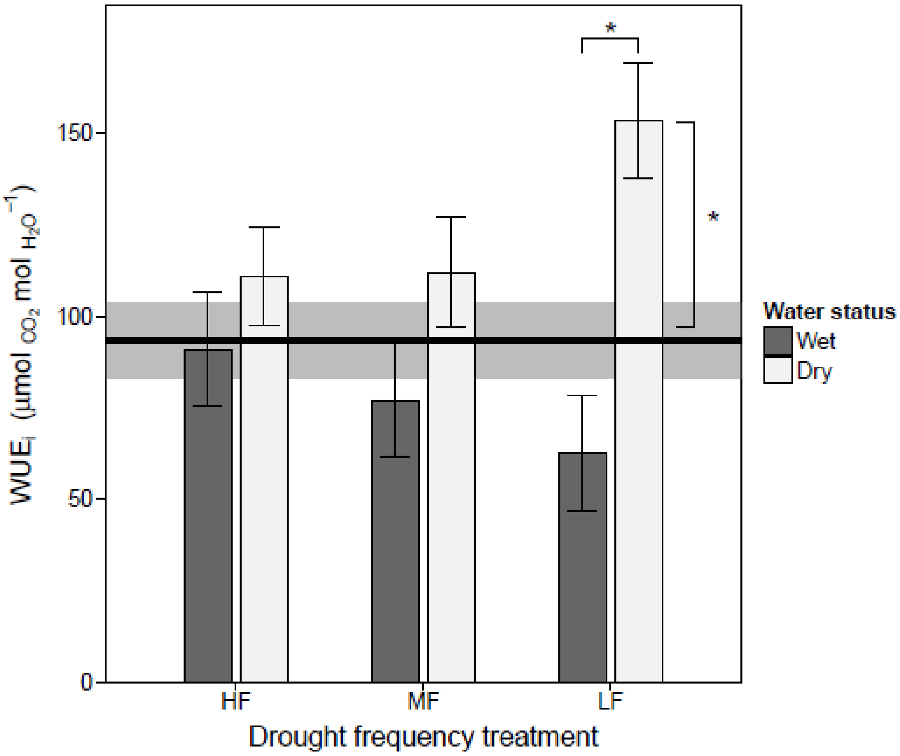

Figure 2.5: Effect of drought frequency and water status on intrinsic water use efficiency (WUE$_i$) of *R. pseudoacacia*. Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g dry weight). Error bars represent SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatment abbreviations: HF = high frequency, MF = medium frequency, LF = low frequency.

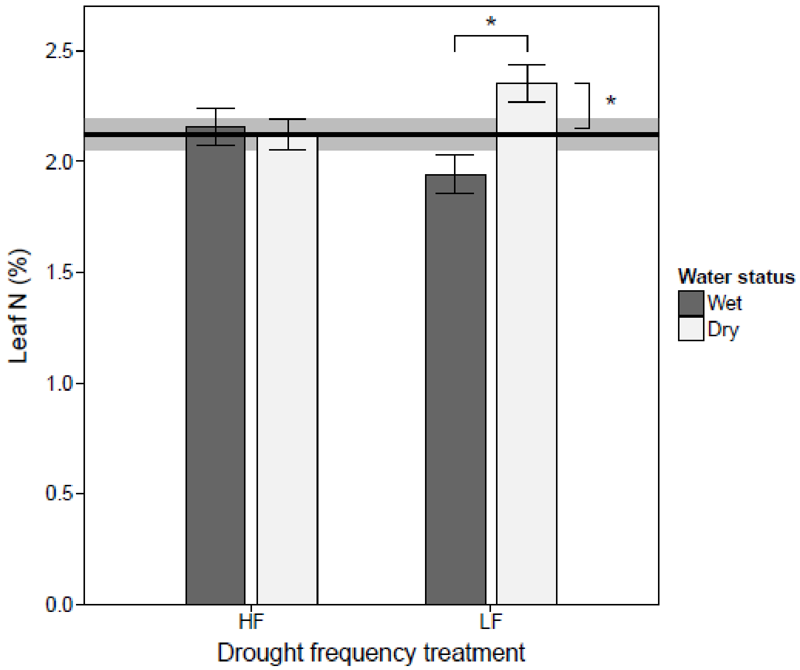

Figure 2.6: Effect of drought frequency and water status on *R. pseudoacacia* leaf N concentration (%). Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g dry weight). Error bars represent SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatment abbreviations: HF = high frequency, LF = low frequency.

CHAPTER 3

DROUGHT SENSITIVITY OF N_2-FIXING TREES MAY INHIBIT FOREST RECOVERY FROM DISTURBANCE[2]

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[2] Minucci JM, Miniat CF, Wurzburger N. To be submitted to *Ecology*.

Abstract

Increased aridity and drought frequency due to climate change has the potential to negatively affect forest productivity by reducing the abundance of nitrogen (N_2) fixing plants, and therefore the rate of new N inputs to the ecosystem. Here, we manipulated growing season precipitation across a series of plots in an early successional forest and determined the relationships between mean soil moisture, soil nitrogen availability, and light on aboveground productivity of the N_2-fixing tree *Robinia pseudoacacia* and three non-fixing tree competitors. We found that low soil moisture was associated with reduced productivity for *R. pseudoacacia* but not non-fixing trees. As a result, the relative abundance of *R. pseudoacacia* declined in drier soils over time. We also found a positive relationship between the biomass of *R. pseudoacacia* and total soil N, extractable inorganic N, N mineralization rates, and the productivity of non-fixing trees. Our results demonstrate that *R. pseudoacacia* promotes the growth of non-fixing trees during early succession through its effect on the N cycle. However, the sensitivity of *R. pseudoacacia* to dry soils suggests that its ability to fix N_2 will diminish under scenarios of increasing aridity, demonstrating a mechanism by which drought may indirectly suppress forest productivity and recovery from disturbance.

Introduction

Global climate change is expected to reduce mean soil moisture and increase drought frequency in terrestrial ecosystems (Dai, 2013; IPCC, 2013), which may impose water stress on trees and reduce forest productivity (Allen *et al.*, 2010; Vose *et al.*, 2016). Drought may also indirectly affect forest productivity through the nitrogen (N) cycle, by reducing the supply of available N to trees. Early successional temperate forests may be particularly sensitive to such indirect effects, as productivity is highly N limited (Vitousek & Howarth, 1991; Wilson & Shure, 1993). In these systems, symbiotic N_2 fixation (SNF) is often the main source of new N (Boring & Swank, 1984; Pearson & Vitousek, 2001; Vitousek *et al.*, 2002), providing a recovery mechanism from prior disturbance. However, this process may be sensitive to reductions in soil moisture, which could diminish the SNF activity of N_2-fixing plants (hereafter: N_2-fixers) or cause the abundance of N_2-fixers to decline.

N_2-fixers may be more sensitive to drought than non-fixers due to their unique N acquisition strategy. Through symbiotic N_2-fixation (SNF), N_2-fixers trade carbon (C) to root nodule-associated bacteria for access to the effectively limitless atmospheric N pool. However, SNF is energetically expensive and the benefits are potentially offset by greater C cost per unit N gained (Gutschick, 1981; Vitousek & Field, 1999; Vance, 2008). The net benefit of SNF therefore depends on the relative value of C, which varies based on plant C supply. Since light is plentiful in the early stages of forest development, water may be the primary constraint to photosynthesis and therefore a key limiting factor for plant C supply. If water stress reduces C assimilation via photosynthesis, the relative value of C will increase, thereby reducing the benefit of SNF over soil N uptake (Rastetter *et al.*, 2001). Though many N_2-fixers regulate investment in SNF versus soil N uptake based on soil N supply (Menge *et al.*, 2015) and C availability (Barron *et al.*, 2011; Minucci *et al.*, 2017), there are C costs to maintain this plasticity (Menge *et al.*, 2009a, 2011). As a result of these factors, N_2-fixers may be poor competitors with non-fixing plants when water constraints make SNF unfavorable.

At the ecosystem level, the abundance of N_2-fixers represents the ultimate control on SNF, making it necessary to understand how soil moisture affects competition between N_2-fixers and non-fixers. Non-fixers may be less sensitive to soil moisture than N_2-fixers because their growth is largely constrained by soil N supply and the C cost of soil N uptake is small relative to SNF (Vitousek & Field, 1999). If the benefit of SNF versus soil N uptake is diminished, it could reduce the ability of N_2-fixers to compete with non-fixers and result in a decline in the abundance of N_2-fixers in early successional forests. Indeed, N_2-fixer abundance and SNF activity often decline along aridity gradients (Schulze *et al.*, 1991; Aranibar *et al.*, 2004; Soper *et al.*, 2015). However, our understanding of how moisture constrains N_2-fixers and SNF activity is generally limited to arid and semi-arid ecosystems, thus it remains unclear how climate change-induced variation in soil moisture affects more mesic ecosystems where drought tolerance may not be a selected trait.

If reductions in soil moisture disproportionately affect N_2-fixer abundance and SNF rate, they could constrain forest productivity and resilience. N often limits temperate forest productivity (LeBauer & Treseder, 2008), especially after disturbance events which are accompanied by losses of N (Menge *et al.*, 2009b; McLauchlan *et al.*, 2014). SNF represents the largest natural source of N for these systems and N_2-fixing plants can stimulate local N cycling rates. For example, N_2-fixers can locally elevate N mineralization and nitrification rates (Montagnini *et al.*, 1986) and inorganic N pool sizes (Goergen & Chambers, 2009), potentially facilitating the growth of neighboring non-fixers (DeBell *et al.*, 1997). Although N_2-fixer biomass declines after early succession, N limitation is often maintained into late succession (Vitousek & Howarth, 1991; Vitousek *et al.*, 2013). Thus, the amount of N fixed in the early years of stand regeneration can determine the severity of future N limitation, and the trajectory of forest growth, decades after N_2-fixers are excluded (von Holle *et al.*, 2013). As a result, SNF rates in early succession may determine the ability of forests to recover from disturbance and store C as mature forests.

Here we examined the direct and indirect effects of drought on forest productivity via a rainfall reduction experiment in an early-successional Southern Appalachian forest. In these ecosystems, a single tree species, *Robinia pseudoacacia* L., is the dominant N_2-fixer and can significantly elevate soil N cycling rates and N availability in dense stands (Boring & Swank, 1984; Montagnini *et al.*, 1986). Over a three-year field experiment, we determined the effects of growing season soil moisture and soil N availability on the growth and competitive ability of *R. pseudoacacia* and three co-occurring non-fixers. We hypothesized that: (1) Growth and SNF rate of *R. pseudoacacia* will be greatest at high soil moisture, while growth of non-fixing trees will be greatest under high N availability; (2) As a result of hypothesis 1, we hypothesize that the relative abundance of *R. pseudoacacia* will decline over time under low soil moisture; and (3) Greater *R. pseudoacacia* biomass will be associated with elevated soil N availability and higher productivity of neighboring non-fixing trees.

Materials and Methods

Study site

Our study was conducted in a small, west-facing 30-ha watershed located in Cowee, North Carolina, within the Nantahala National Forest (35°16’43” N, 83°21’43” W). Elevation in the watershed ranges from 704 to 760 m above sea level. The watershed was a mature Southern Appalachian deciduous forest prior to harvesting in 2010 using the seed tree method. Soils in the watershed are categorized in two series: the Cowee-Evard complex, a fine-loamy, parasesquic, mesic, Typic Hapludult, and the Saunook series, a fine-loamy,mixed mesic Humic Hapludult. Mean annual temperature and precipitation are approximately 12.3 °C and 1,270 mm, respectively (PRISM Climate Group, 2016).

Experimental design

We established eighteen 9 m^2 rainfall manipulation plots in spring of 2012. We distributed three pairs of plots within each of three landscape positions: the north-facing slope, the south-facing slope, and the basin. Paired plots were located within 10 meters of each other. To hydrologically isolate plots from the surrounding soil, we buried 3 mil HDPE plastic liner around each plot to a depth of 0.6 meters. After the 2012 growing season, we tagged and measured the stem diameter and height of all individuals of four focal species: the N_2-fixing tree *Robinia pseudoacacia,* and three non-fixing species common in early succession, *Liriodendron tulipifera*, *Acer rubrum* and *Quercus velutina*. *R. pseudoacacia* and *L. tulipifera* are both fast-growing, shade intolerant trees, while *A. rubrum* and *Q. velutina* are slower growing and shade tolerant. All four species sprout readily from stumps or roots following disturbance (Shure *et al*., 2006). While the three non-fixing species often persist into late succession, *R. pseudoacacia* typically declines following canopy closure (Boring & Swank, 1984; Cierjacks *et al*., 2013).

In July of 2013 we installed rainfall manipulation panels that diverted either 0%, 20%, or 40% of incoming rainfall to augment the natural level of soil moisture variability in our study site. Panels were randomly assigned to plots within each landscape position, so that landscape positions contained two of each rainfall reduction level. Plots were fitted with four clear, corrugated plastic roofing panels which covered 40% of the plot surface area at a height of ~1.5 meters and sloped downward to a gutter system which carried water outside and downslope of the plot. For 20% and 0% rainfall reduction, two or four panels, respectively, were perforated to allow water to fall onto plots while minimizing differences in humidity and PAR from 40% reduction plots. Panels remained in place during the 2013, 2014, and 2015 growing seasons (mid-May to mid-October), and were removed each winter. Stand age was 3 years at the start of the treatment period and 5 years at the end of the experiment. To track soil moisture in our plots, we installed a soil water content reflectometer (CS-655, Campbell Scientific, Logan UT) at the center of each plot and logged hourly measurements with three solar-powered datalogger stations (CR1000 and CR10X, Campbell Scientific, Logan UT) for the duration of the study.

Tree biomass and growth rates

To quantify tree growth rates, we measured tree aboveground biomass at the end of each growing season. For each tagged tree we measured the height of the apical meristem and the stem diameter at 5-cm height, and converted these measurements to total aboveground biomass using allometric equations previously established for each species (Boring & Swank, 1984; Elliott *et al.*, 2002; Wurzburger & Miniat, 2014; Minucci *et al.*, 2017). We also tagged and measured newly recruited seedlings and noted cases of mortality for previously tagged individuals. Relative aboveground productivity (g g^{-1} yr^{-1}) was calculated at the plot level for each species according to the equation:

$$(1)\quad \text{Rel. aboveground productivity} = \frac{\ln(\frac{X_2}{X_1})}{t}$$

where X_2 = final biomass, X_1 = initial biomass, and t = time in years.

Soil N cycling and availability

To assess how plant available N varied across plots, we sampled several measures of soil N availability during the 2015 growing season. We quantified extractable ammonium and nitrate pools three times during the summer with 2M KCl and carried out 28-day *in situ* N mineralization assays using a buried bag method. For these analyses we collected and homogenized six randomly located soil cores (20 cm deep, 2.5 cm diameter) from each plot, which were divided into 0-10 cm and 10-20 cm depths. After removing roots with forceps, we sampled 25 g of wet soil from each depth for KCl extraction. This soil was immediately extracted with 50 mL of 2M KCl for 4 h, and filtered through 1 µm glass fiber filters. Ammonium and nitrate concentrations in the filtrate were later quantified using continuous flow analysis (RFA300 Autoanalyzer, Alpkem Corp., Clackamas, OR). The remaining soil was sealed in a high-density, gas permeable polyethylene membrane bag (StarPac; AgriStar Inc., Conroe, Texas) and buried 10 cm deep at the center of the plot. We retrieved N mineralization bags 28 days later and re-measured ammonium and nitrate pools with KCl extraction, as above. Net N mineralization rate was calculated as the change in DIN pool size divided by the assay period in days.

We also assessed soil N availability by quantifying the potential activity of leucine aminopeptidase (LAP) and β-N-acetylglucosaminidase (NAG), two enzymes involved in the release of N from organic material, using a modified fluorometrically-labeled substrate method (*Sayia-Cork et al. 2002)*. During the final sampling point of 2015, we collected 2 g of soil per plot from the same pooled soil samples used for KCl extractions and N mineralization assays and immediately transported samples in sealed plastic bags to the laboratory for analysis. We then mixed each soil sample with 125 mL of 50 mM sodium acetate buffer and homogenized the soil and buffer mixture in a blender for 1 minute. These soil slurries were transferred in four 200 µL aliquots to a 96-well plate and 200 uM of labeled substrate was added to each well. We then incubated plates at 25°C for 3 hours before measuring fluorescence on a spectrofluorometer (Synergy H1; BioTek, Winooski, VT) set at 365 nm excitation and 450 nm emission. Fluorescence was converted to potential enzyme activity (nmol g^{-1} dry soil h^{-1}) using standard curves of 7-amino-4-methylcoumarin for LAP and 4-methylumbelliferone for NAG.

We also determined total C and N stocks in our soils during the 2015 growing season, as well as $\delta^{15}N$ of the total N pool. In 2015, we subsampled approximately 2 grams of soil from the same pooled soil samples used for KCl extractions and N mineralization assays. We dried these soil samples at 70°C to constant weight and ground the soil to a powder in a ball-mill grinder. We then packed 15 mg of ground soil into tin capsules and combusted samples in an elemental analyzer (NA1500 CHN Analyzer; Carlo Erba, Milan, Italy) interfaced with an isotope-ratio mass spectrometer (IRMS; Delta V, Thermo-Finnigan, Bremen, Germany).

Tree foliar sampling

As another measurement of soil N availability to trees, we determined foliar N content in August 2015. We also measured $\delta^{13}C$ to assess water use efficiency and $\delta^{15}N$ and to examine whether trees acquired N from different sources. Leaf samples were obtained from 3 individuals per species per plot, with 3 fully extended, healthy and sun-exposed leaves sampled from each individual. We dried these samples at 70°C to constant weight and ground the leaves to a fine powder in a ball-mill grinder. We then packed 1.5 mg of ground leaf tissue into tin capsules and combusted samples in an elemental analyzer (NA1500 CHN Analyzer; Carlo Erba, Milan, Italy) interfaced with an isotope-ratio mass spectrometer (Delta V, Thermo-Finnigan, Bremen, Germany). SNF rate by *R. pseudoacacia* was calculated with the $\delta^{15}N$ natural abundance method and a two end-member mixing model using *A. rubrum* as a reference for the $\delta^{15}N$ signature of soil N uptake.

Light availability

To account for variability in light availability across plots, we measured canopy light transmittance on July 7th 2015. At each plot we sampled photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}; LI-250A with LI-193 sensor, Li-Cor, Lincoln, NE) above the canopy and at three random points below the canopy at 75 cm height. All readings were taken between 10:30 and 13:30. We calculated canopy transmittance as below canopy PAR divided by above canopy PAR.

Tree ecophysiological response to soil moisture

In the summer of year two (2014), we measured pre-dawn and mid-day leaf water potential, transpiration, and stomatal conductance to determine whether tree species had physiological responses to variation in soil moisture. On July 29th and September 25th of 2014, we measured pre-dawn and mid-day leaf water potential on fully-extended, sun exposed leaves for one individual per species per plot. (MPa; PMS Instruments, Albany, OR, USA). On August 12th and September 25th-26th of 2014, we measured transpiration (E) and stomatal conductance (g_s) under ambient light conditions on fully-extended, sun exposed leaves for two individuals per species per plot (E: μg H_2O cm^{-2} s^{-1}, g_s: mmol H_2O m^{-2} s^{-1}; LI-1600, LI-COR Inc., Lincoln NE).

Statistical analysis

To determine the sensitivity of the N_2-fixer *R. pseudoacacia* and its three non-fixing competitors to soil moisture, N availability, and light, we modeled 3-year relative aboveground productivity for each species and performed stepwise model selection with the Bayesian Information Criterion (BIC). We used BIC as an alternative to the Akaike Information Criterion (AIC) because it gives a greater penalty to free parameters, thereby favoring more parsimonious models (Neath & Cavanaugh, 2012). We constructed linear mixed effects models (R; nlme package) with the following potential fixed effects: 3-year mean growing season soil moisture (*%SM*), canopy transmittance, N mineralization rate (N_{min}), total inorganic N pool size (DIN) and all possible two-way interactions. Fixed effects were square-root transformed when the relationship with the predictor was clearly non-linear. Each model included landscape position (north-facing slope, south-facing slope, and basin) and plot pair number (1-9) as categorical random effects to account for spatial autocorrelation and variation in moisture and soil properties associated with topographic position within the watershed. While we originally intended to use rainfall reduction level (0%, 20%, or 40%) as a categorical treatment effect, we found that background variance in *%SM* was high compared with our treatment effect. *%SM* ranged from 18.3% to 28.9% and differences between plots were consistent across years. As a result, we used *%SM* as a continuous predictor which integrated both the pre-existing variability in moisture and the effect of our rainfall manipulation.

All other analyses were carried out with linear mixed-effects models, with landscape position and plot pair number as categorical random effects. Response variables measured at the tree-level (e.g. foliar N content) were averaged to produce one mean value per plot. Response variables and explanatory fixed effects were log or square root transformed when necessary to achieve residual error normality. To determine whether variables were significant predictors in each model, we used likelihood ratio tests comparing the full model and a model with the term of interest dropped. All statistical analyses were performed with R 3.1.1 (R Development Core Team, 2016).

Results

Variation in soil moisture among plots

We found that 3-year mean growing season soil moisture (*%SM*) varied widely across plots. The range observed was 18.3% to 28.9%, with a mean of 23.3% (Table S3.1). For plots with 40%, 20% and 0% rainfall reduction, *%SM* was 21.6%, 25.3% and 23.1%, respectively. Variance in *%SM* was explained more by landscape position (44% of variance) than by rainfall reduction treatment (22% of variance), and thus we used *%SM* as a continuous variable in our analyses to capture both natural variation and the effect of rainfall exclusion. Further, there was a high degree of correlation between yearly mean soil moisture measurements, indicating that relative differences in moisture were consistent throughout the study (Pearson's correlation: $0.72 < r < 0.94$), and this motivated our use of 3-year mean soil moisture as an integrative representation of moisture conditions.

Tree physiological response to soil moisture variation

To determine if *R. pseudoacacia* and its non-fixing competitors differed in their physiological response to low soil moisture, we examined how moisture affected pre-dawn and mid-day leaf water potential, transpiration, and stomatal conductance for each species. Low soil moisture reduced pre-dawn and mid-day leaf water potentials for all species, indicating that our soil moisture measurements reflected differences in water availability to trees (Figure S3.1; ψ_{pd}: χ^2_1=10.5, P=0.001; ψ_{md}: χ^2_1=5.7, P= 0.02). There were no differences among species in how leaf water potential responded to soil moisture (species x *%SM* interaction: ψ_{pd}: χ^2_3=0.8, P= 0.85; ψ_{md}: χ^2_3=6.01, P= 0.11).

Tree species differed in their adjustment of stomatal conductance to soil moisture conditions (species x *%SM* interaction: χ^2_3=9.4, *P*= 0.02). Only *L. tulipifera* reduced stomatal conductance in response to low soil moisture (Figure S3.2; χ^2_1=4.1, *P*= 0.04). No tree species reduced transpiration in response to low soil moisture ($\chi^2$1=2.1, *P*= 0.15), although species differed in their baseline transpiration rates (χ^2_1=49.4, *P*<0.001). Transpiration of *R. pseudoacacia* and *L. tulipifera* was approximately 73% greater than *A. rubrum* and 48% greater than *Q. velutina*. Mean soil moisture did not affect foliar δ^{13}C for any of the species examined (Figure S3.2; *%SM* effect: χ^2_1=1.57, *P*=0.21).

Factors affecting aboveground productivity of R. pseudoacacia *and non-fixers*

To address our hypothesis that productivity of *R. pseudoacacia* and non-fixing trees would be controlled by different factors (water and nitrogen, respectively), we used model selection to find the factors that best explained productivity for each group. The best model to explain relative net aboveground productivity, as determined by Bayesian information criterion (BIC), differed between the N_2-fixer *R. pseudoacacia* and non-fixing trees (Table 3.1). The best model to explain productivity of the N_2-fixing tree *R. pseudoacacia* included two main effects: a positive effect of mean SM and a negative, non-linear effect of net N mineralization rate (N_{min}) (Table 3.1, Figure 3.1), indicating that productivity was highest for this species at high moisture but low N_{min}. In contrast, non-fixer productivity was best explained by a model that included only a positive main effect of N_{min} (Table 3.1, Figure 3.1), signifying that productivity increased with N_{min} and that this relationship was consistent across all moisture and light conditions.

When non-fixing tree species were modeled individually, there were more nuanced relationships between productivity and the predictors (Table 3.1). For *L. tulipifera*, the best model included a positive a positive main effect of N_{min} and a positive interaction between mean growing season soil moisture (*%SM*) and canopy transmittance. This indicates that the greatest growth rates occurred when both soil moisture and transmittance were high, and that greater N_{min} was correlated with higher growth across all conditions. The best model for *A. rubrum* included only a positive N_{min} term, indicating that greater N availability was correlated with greater growth across all moisture and light conditions. The best model for *Q. velutina* included only a negative main effect of canopy transmittance.

Change in relative abundance of R. pseudoacacia

To address our hypothesis that relative abundance of *R. pseudoacacia* would decline over time under low soil moisture, we calculated the change in relative abundance of each species over the three years of the experiment. Mean relative abundance of *R. pseudoacacia*, as a percent of biomass, increased slightly in year 1 (from 17.1% to 19.0%), but then declined in years 2 and 3 to a final relative abundance of 13.7%. The decline in years 2 and 3 was greater in plots with low *%SM* (Figure 3.2; $\chi^2_1=5.7$, $P= 0.02$). There was a similar decline in relative stem abundance during this period (from 13.4% to 7.2%), with the greatest decrease also occurring in plots with low *%SM* (Figure S3.3; $\chi^2_1=4.9$, $P= 0.03$). In contrast, the relative abundance of *L. tulipifera* increased in plots where *%SM* was low between years 2 and 3 ($\chi^2_1=4.0$, $P= 0.04$), while there were no significant changes in the abundance of *Q. velutina* or *A. rubrum*, and no changes in relative stem abundance for any single non-fixing species.

Effect of R. pseudoacacia biomass on forest N cycle and non-fixer productivity

To determine whether *R. pseudoacacia* increased soil N availability and productivity of non-fixers, we first examined the relationship between *R. pseudoacacia* biomass and several metrics of N availability. Biomass of *R. pseudoacacia* was a significant predictor of two measures of soil N availability: N_{min} (Figure 3.3; χ^2_1=13.9, $P<0.001$) and extractable DIN (Figure 3.3; χ^2_1=20.2, $P<0.001$). However, it was not a predictor of potential activity of the N-acquiring enzymes NAG (χ^2_1=1.4, P= 0.23) and LAP (χ^2_1=0.2, P= 0.64). We then assessed how non-fixer relative net aboveground productivity related to *R. pseudoacacia* biomass, and found that non-fixing trees responded positively to *R. pseudoacacia* biomass (Figure 3.4; χ^2_1=7.9, P= 0.005). When modeled by individual species, this relationship was significant for *A. rubrum* and *L. tulipifera*, but not for *Q. velutina*. Foliar N content (Figure 3.3c; χ^2_1=8.82, P=0.02) and δ^{15}N of non-fixers (Figure 3.3d; χ^2_1=17.3, $P<0.001$) also increased with greater *R. pseudoacacia* biomass, suggesting that non-fixers assimilated fixed N. Foliar N content was significantly higher for *R. pseudoacacia* (2.9%±0.33) than for non-fixers (1.73% ±0.27; Welch's t-test: t=10.4, $P<0.001$).

We also examined whether the abundance of *R. pseudoacacia* across plots was associated with differences in total soil C and N stocks and the δ^{15}N signature of the total N. We found that *R. pseudoacacia* biomass was positively correlated with total soil N (χ^2_1=3.97, P=0.046), but not C (χ^2_1=3.37, P=0.07) or C:N ratio (χ^2_1=0.08, P=0.78). However, we found no relationship between *R. pseudoacacia* biomass and the δ^{15}N of the total N pool (χ^2_1=0.02, P=0.88), which was 3.0‰, on average (Table S3.1).

Effect of soil moisture on SNF rate of R. pseudoacacia

In order to assess our hypothesis that SNF rate by *R. pseudoacacia* would decline at low soil moisture due to the high cost of SNF, we calculated the percent of N that *R. pseudoacacia* derived from the atmosphere using the $\delta^{15}N$ natural abundance method. However, non-fixing trees foliar $\delta^{15}N$ increased with increasing abundance of *R. pseudoacacia* (i.e., it approached 0) (Figure 3.3d; $\chi^2_1=17.3$, $P<0.001$), suggesting that SNF affected the $\delta^{15}N$ of the soil available N pool, which prevented our ability to distinguish between N derived from soil versus atmosphere in high *R. pseudoacacia* biomass plots. Although we could not reliably calculate N derived from the atmosphere, foliar N content decreased with declining soil moisture for *R. pseudoacacia* ($\chi^2_1=9.833$ $P=0.02$), but not for non-fixers ($\chi^2_1=0.36$ $P=0.54$).

Discussion

Our manipulative study demonstrates that low soil moisture can reduce the relative abundance of an N_2-fixing tree in an early successional temperate forest. We also found that the N_2-fixer was positively associated with available soil N, and the growth of non-fixing trees, confirming its role as a facilitator of forest recovery. However, increasing aridity due to climate change appears to shift competition between this N_2-fixing species and non-fixers, and could undermine this role. Our findings point to the high C cost of SNF compared to soil N uptake as a potential mechanism driving the greater sensitivity of N_2-fixers to low soil moisture.

Divergent constraints on productivity of the N_2-fixer R. pseudoacacia vs. non-fixers

Relative productivity of the N_2-fixing tree *Robinia pseudoacacia* was controlled by different resources than were non-fixing trees. *R. pseudoacacia* was most productive under high soil moisture but low soil N mineralization rate, while non-fixers were unresponsive to soil moisture and most productive under high soil N mineralization rate, supporting our first hypothesis. Under high soil moisture but low N_{min}, *R. pseudoacacia* was more productive than its non-fixing competitors (Figure 3.1), which supports the idea that SNF is most beneficial when the value of N is larger than the value of C. However, this competitive advantage appeared to decline at low soil moisture, supporting the idea that the SNF is too costly when C acquisition is limited by water. Non-fixers appeared to outcompete *R. pseudoacacia* under high soil N availability, where SNF may no longer confer a competitive advantage (Barron *et al.*, 2011; Batterman *et al.*, 2013b). Even if *R. pseudoacacia* downregulated SNF when soil N availability was high, it still may be a poor competitor due C costs of maintaining N uptake plasticity (Menge *et al.*, 2009a, 2011). Another possible explanation for the poor performance of *R. pseudoacacia* at high N is that N_2-fixers tend to have higher foliar N concentrations than non-fixers (Knops *et al.*, 2000; Wright *et al.*, 2004), leading to lower N use efficiency (Menge *et al.*, 2008) and greater susceptibility to herbivory (Vitousek & Field, 1999; Knops *et al.*, 2000). In our study, foliar N content was approximately 65% greater for *R. pseudoacacia* than for non-fixers.

While productivity of the non-fixer community as a whole was correlated only with N_{min}, there was variation in the best predictors of productivity for each species. Productivity of *L. tulipifera* was strongly correlated with water and light, with growth increasing only when both were in high supply. However, high N_{min} was positively associated with productivity across all water and light conditions, suggesting that soil N availability may be the primary limiting factor for this species. In contrast, productivity of *Q. velutina* was not explained by our measures of soil N availability, and light was the only significant predictor. The negative relationship between light and *Q. velutina* may reflect the poor ability of many oaks to compete with faster growing shade intolerant species (such as *L. tulipifera*) under high light (Kaelke *et al.*, 2001; Rebbeck *et al.*, 2012). In addition, *Q. velutina* was the only non-fixer species that did not increase productivity, foliar N content, or $\delta^{15}N$ under conditions of greater *R. pseudoacacia* biomass. One potential explanation for this lack of N sensitivity is that *Q. velutina* associates with ectomycorrhizal fungi, while all other species in this study associate with arbuscular mycorrhizal fungi. While arbuscular mycorrhizal fungi scavenge mineral N, ectomycorrhizal fungi can additionally mine N from stable organic matter via the excretion of extracellular enzymes (Read & Perez-Moreno, 2003), and therefore may be less sensitive to changes in mineral N pools.

Soil moisture shapes competition between R. pseudoacacia and non-fixers

We found that relative abundance of the N_2-fixer *R. pseudoacacia*, in terms of both biomass and stem number, declined over time under low soil moisture, supporting our hypothesis that *R. pseudoacacia* would be outcompeted by non-fixers at low moisture due to the additional C cost of SNF. Concurrent with this decline, we found an increase in relative abundance of *L. tulipifera*, suggesting that this particular non-fixer replaces *R. pseudoacacia* in drier conditions. The high relative aboveground productivity of *L. tulipifera* (approximately 60% greater than the other non-fixers) makes it the most likely candidate to outcompete *R. pseudoacacia* for light or other resources over our 3-year study period (Apsley, 1987). The relative cost of SNF versus soil N uptake may be responsible for this shift in competition under low soil moisture. If consistent dry conditions reduce C assimilation via photosynthesis, the scarcity of C would make SNF even more expensive relative to soil N uptake (Gutschick, 1981; Vitousek & Field, 1999; Rastetter *et al.*, 2001), potentially making the costs of SNF outweigh the benefits. While we did not observe significant changes in stomatal conductance or $\delta^{13}C$ (a measure of water-use-efficiency) under low soil moisture, *R. pseudoacacia* is known to reduce stomatal conductance and increase its WUE under dry conditions (Mantovani *et al.*, 2014a; Wurzburger & Miniat, 2014; Minucci *et al.*, 2017). Additionally, we found that low soil moisture was associated with a decline in foliar N content of *R. pseudoacacia*, supporting that idea that reductions in SNF may explain the reduced competitive ability of *R. pseudoacacia*.

R. pseudoacacia drives soil N availability and non-fixer productivity

Our findings demonstrate that *R. pseudoacacia* enhances soil N availability and the productivity of non-fixing trees, supporting our third hypothesis. We also found that non-fixing trees had elevated δ^{15}N and foliar N content when *R. pseudoacacia* was most abundant, suggesting that SNF was the cause of enhanced soil N availability and was facilitating the growth of non-fixing trees. While it has previously been recognized that woody N_2-fixers can increase productivity of non-fixing trees in mixed-species plantations (DeBell *et al.*, 1997) and natural forest settings (Apsley, 1987), we provide direct evidence of N pool enrichment by *R. pseudoacacia* and subsequent uptake of this N by non-fixers as the mechanism for this facilitation.

Interestingly, we observed that plots containing *R. pseudoacacia* had not only greater DIN pool sizes, but also greater total soil N stocks, which suggests that turnover of *R. pseudoacacia* litter in our five-year old stand was rapid enough to enrich the total soil N pool. In contrast, Boring and Swank (1984) found that while nitrate pool size increased in young stands, total soil N was not elevated until 38 years after disturbance, suggesting that turnover of *R. pseudoacacia* biomass into stable soil N pools occurs very slowly. Since *R. pseudoacacia* often sprouts from root stock that remains viable for many years (Boring & Swank, 1984; Shure *et al.*, 2006), it is also possible that the enhanced N availability and soil N stocks associated with *R. pseudoacacia* in our study was, at least in part, a legacy of SNF from prior disturbance events. In support of this mechanism, soil N availability has been found to be elevated over a decade after extirpation of *R. pseudoacacia* (von Holle *et al.*, 2013). Additionally, although total N stocks were greater, we did not find that any change in total soil $\delta^{15}N$ with *R. pseudoacacia* presence, which suggests that the contribution of fixed N to the total N pool was sufficiently cycled by soil microbes to enrich its $\delta^{15}N$ signature and remove evidence of atmospheric origin (Robinson, 2001). However, the increase in foliar $\delta^{15}N$ of non-fixers, suggests that these trees are taking up a significant amount N that was recently fixed.

Implications for forest productivity and recovery from disturbance

Our study provides evidence that drought may reduce forest productivity and recovery from disturbance indirectly, by reducing the abundance of N_2-fixing plants. In temperate forests, N_2-fixers are typically abundant in early succession but are excluded shortly after canopy closure, when light availability declines and the relative cost of C increases (Vitousek & Howarth, 1991). As a result, SNF cannot supply sufficient N over the course of ecosystem development, leading to widespread and sustained N limitation in mature temperate forests (LeBauer & Treseder, 2008). Nevertheless, the large amount of N fixed by SNF early in succession helps to drive forest growth decades later, due to the high rate of N recycling in non-polluted forests (Johnson & Turner, 2014). More arid or drought-prone growing seasons, and thus reduced SNF in early succession, could further exacerbate N limitation throughout forest development, resulting in decreased forest productivity and C sink potential.

N limitation could be further magnified by repeated disturbance events that release N from forest ecosystems. Disturbances such as forest harvest or fire trigger the loss of plant biomass, volatilization of N, and leaching losses of DIN at times where plant N uptake is low (Vitousek & Matson, 1984; Wan *et al.*, 2001; Rastetter *et al.*, 2013). N fixed through SNF during the early stages of forest regrowth allows for the replacement of N lost during these events. Our findings suggest that reductions in soil moisture during early succession may therefore degrade the ability of forests to recover from disturbance if it leads to declines in N_2-fixer abundance.

Conclusions

Our study demonstrates the potential for drought to indirectly affect forest productivity through its impacts on symbiotic nitrogen fixation. We found that low soil moisture was associated with reduced aboveground productivity of the N_2-fixing tree, *Robinia pseudoacacia*, and a decline in the relative abundance of this species over time. Reductions in the abundance of N_2-fixing trees could have long-term impacts on forest productivity, as the presence of *R. pseudoacacia* enhanced soil N cycling rates, soil N stocks and non-fixer productivity. Our findings suggest that if drought becomes more frequent under future climate, the ability of forests to recovery from disturbances may be constrained by diminished N inputs through SNF, and therefore reduced soil N availability to non-fixing trees.

Table 3.1: Best linear mixed effects models of relative net aboveground productivity, as selected by stepwise BIC. Random effect of landscape aspect included in all models.

Species	**Predictor**	**Estimate**	**SE**	**r^2**
R. pseudoacacia	Intercept	-1.99	1.16	
	%SM	0.12	0.05	
	$\sqrt{(N_{min})}$	-1.44	0.99	
	Total Model Fit			**0.48**
Non-fixing trees	Intercept	0.67	0.07	
	N_{min}	1.04	0.55	
	Total Model Fit			**0.18**
L. tulipifera	Intercept	1.65	0.78	
	%SM	-0.04	0.03	
	N_{min}	2.19	0.77	
	Transmittance	-6.56	2.40	
	%SM x Transmittance	0.32	0.12	
	Total Model Fit			**0.44**
A. rubrum	Intercept	0.43	0.11	
	N_{min}	2.23	0.55	
	Total Model Fit			**0.47**
Q. velutina	Intercept	0.72	0.07	
	Transmittance	-0.75	0.20	
	Total Model Fit			**0.51**

r^2 = marginal r^2 or the variance explained by fixed effects only.

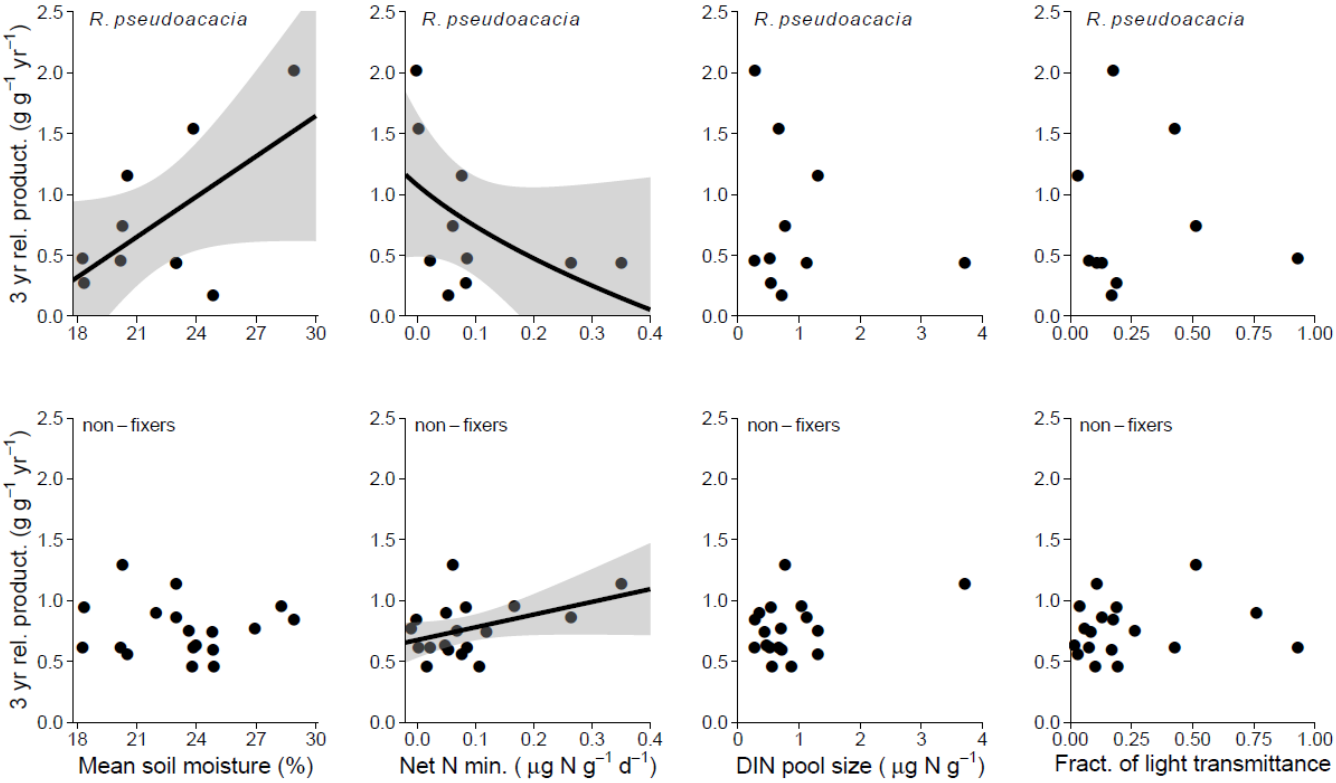

Figure 3.1: Effect of 3-year mean growing season soil moisture, soil N availability, and light availability on the relative aboveground productivity of the N_2-fixing tree *R. pseudoacacia* (top) and non-fixing tree species (bottom). Regression lines with 95% confidence intervals are included when the effect was present in the best model (as selected by BIC).

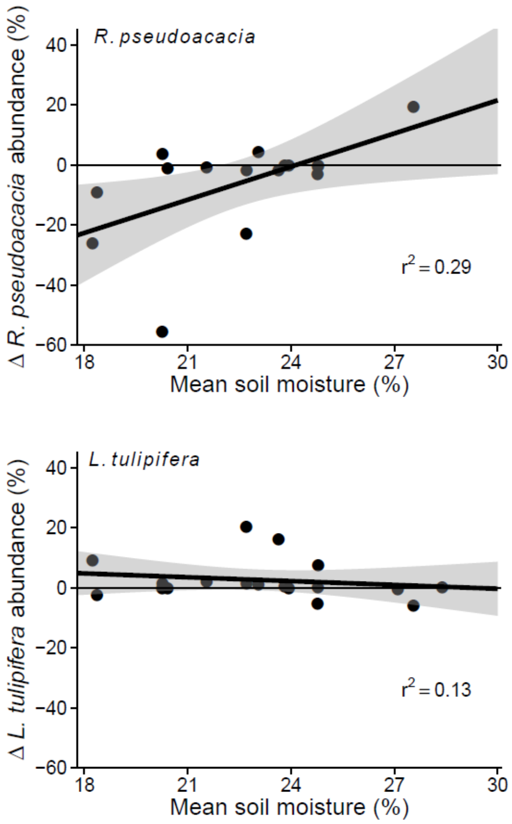

Figure 3.2: Change in relative abundance (percent of total plot biomass) of *R. pseudoacacia* (top) and *L. tulipifera* (bottom) during years 2 and 3 was related to mean soil moisture during that period. Lines represent regression fits with 95% confidence intervals and marginal r^2 values.

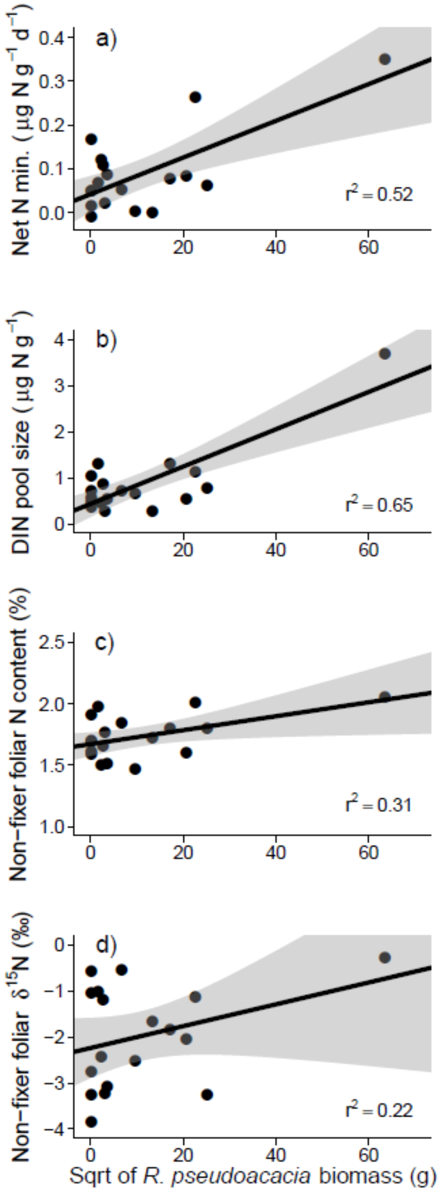

Figure 3.3: Greater *R. pseudoacacia* biomass is related to increases in net N mineralization rate (a), soil DIN pool size (b), foliar N content of non-fixing trees (c), and foliar δ^{15}N of non-fixing trees (d). Biomass of *R. pseudoacacia* refers to biomass in year 2, the midpoint of the study. Regression fits with 95% confidence intervals and marginal r^2 values are included for significant relationships.

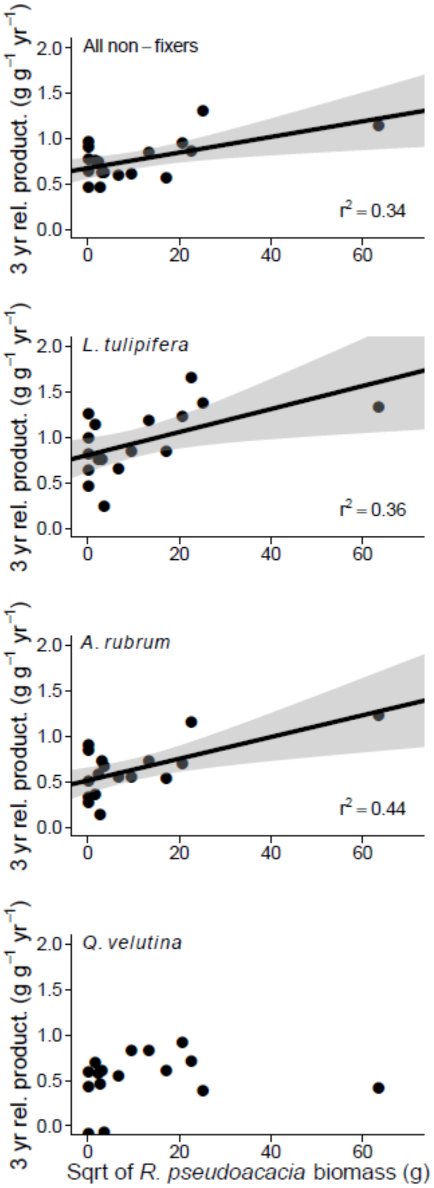

Figure 3.4: Greater *R. pseudoacacia* biomass is related to increased relative net aboveground productivity of non-fixing trees (top). When non-fixers were modeled individually, there was a significant relationship for *L. tulipifera* and *A. rubrum*, but not *Q. velutina.* Regression fits with 95% confidence intervals and marginal r^2 values are included for significant relationships.

CHAPTER 4

TEMPERATURE SENSITIVITY MAY DRIVE NORTHWARD MIGRATION OF A KEY N_2-FIXING TREE[3]

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[3] Minucci JM, Schmidt JP, Wurzburger N. To be submitted to *Ecological Applications*.

Abstract

Symbiotic N_2-fixing plants provide key nitrogen (N) inputs to ecosystems and significantly alter N cycling rates, yet the factors driving their distribution across the landscape remain unclear. One dynamic that could shape the distribution of N_2-fixing plants is the resource economic trade-off inherent to symbiotic N_2-fixation, a process which provides greater access to N but at a greater carbon cost. Here, we used data on forest inventory and structure, climate, and soil factors to model the distribution of an ecologically important N_2-fixing tree, *Robinia pseudoacacia,* in the Eastern US. We found that presence of *R. pseudoacacia* was associated with factors that are likely to promote a greater value of N relative to carbon, including high mean annual temperature, high light availability and low soil N availability. Our findings indicate that the basic resource economic trade-off of utilizing symbiotic N_2-fixation over soil N uptake poses constraints that help to shape N_2-fixer distribution across the landscape. We also used our model and projections of climate for the year 2050 to predict future habitat suitability for *R. pseudoacacia*, revealing a likely northward migration of high quality habitat due to increased temperatures. The gain or loss of this important N_2-fixing tree could have major impacts on future N cycling rates, forest productivity, and the rate of recovery from disturbance.

Introduction

Symbiotic N_2-fixing plants (hereafter N_2-fixers) act as biogeochemical keystone species in forest ecosystems, providing large inputs of nitrogen (N) during early succession (Pearson & Vitousek, 2001; Vitousek *et al.*, 2002). N_2-fixers can even allow forest ecosystems to overcome N limitation and recovery more rapidly from disturbance if they occur in sufficient density (Menge, 2011; Batterman *et al.*, 2013a). In the Eastern US, one leguminous N_2-fixing tree species, *Robinia pseudoacacia* L. (or black locust), increases soil N availability and productivity of non-fixing species (Montagnini *et al.*, 1986; von Holle *et al.*, 2013; Chapter 3). Despite the importance of this species in altering forest biogeochemistry, its geographic distribution pattern is largely unexplained and it is unclear what ecological factors control the range and abundance of this species. The distribution of *R. pseudoacacia* is generally limited to the Appalachian Mountains, despite the fact that available soil N is generally low throughout Eastern US forests (LeBauer & Treseder, 2008; Rennenberg *et al.*, 2009) and other N_2-fixing tree species are largely absent (Menge *et al.*, 2014). Those woody N_2-fixers that do occur in the Eastern US are generally either small shrubs (*e.g*., *Ceanothus americanus* L.), are rare (*e.g.*, *Robinia viscosa* L.), or occur only in certain habitats (*e.g*., *Alnus serrulata* (Aiton) Willd. in riparian zones). It is crucial to understand the ecological factors controlling the distribution of *R. pseudoacacia,* so that we can predict how global change factors will alter the presence of this important N_2-fixer in Eastern US forests and lead to declines in some regions and invasion into others. By examining how the presence or absence of *R. pseudoacacia* across the landscape is related to various abiotic factors, we can gain insight into the realized niche of this key species and project future geographic distribution patterns under global change (Elith & Leathwick, 2009).

The lack of N_2-fixing tree species throughout much of the Eastern US, despite widespread N limitation to forest productivity, raises questions about what ecological factors control *R. pseudoacacia* distribution across the region (Menge *et al.*, 2010, 2017). One potential explanation is that abiotic conditions in these areas cause *R. pseudoacacia* to be outcompeted by non-fixers, and this competition may be driven, in part, by resource economic trade-offs inherent in using symbiotic N_2-fixation (SNF) over soil N uptake. By trading carbon (C) to root nodule-associated bacterial symbionts, N_2-fixers can bypass the soil N cycling pathway and access atmospheric N_2. However, SNF is more energetically expensive than soil N uptake and its benefit to the plant depends on the relative values of C and N (Gutschick, 1981; Vance, 2008). Due to this trade-off, SNF should be favored over soil N uptake when soil N supply is low but C supply is high, because the additional C cost of SNF is dwarfed by the value of the N gained (Vitousek & Field, 1999).

The distribution of *R. pseudoacacia* could also be driven, in part, by traits characteristic of leguminous trees, which are largely tropical and subtropical in range (Liao *et al.*, 2017; Menge *et al.*, 2017). *R pseudoacacia* is an extremely fast-growing, shade-intolerant tree of medium size, which makes it a rapid colonizer of disturbed areas but a poor competitor once canopy closure occurs (Boring & Swank, 1984; Shure *et al.*, 2006). Additionally, it maintains high leaf N concentrations, which causes increased susceptibility to herbivory (Knops *et al.*, 2000; Alonso *et al.*, 2010). While these traits cannot be disentangled from the ability to fix nitrogen, and are likely consequences of high N availability, they could help to determine the competitive ability of *R. pseudoacacia* under a given set of abiotic conditions. The economic trade-offs inherent to SNF and the traits associated with this N uptake strategy may regulate the distribution of *R. pseudoacacia*, as the relative values of C and N, and therefore the net benefit of SNF, may be altered by: (1) temperature, (2) soil moisture (3) light availability, and (4) soil characteristics.

Temperature may drive *R. pseudoacacia* distribution through its effects on soil N availability and N_2-fixer N demand. The mineralization of N from organic matter is a temperature-dependent microbial process, and as temperature increases, plant-available N is released more rapidly (Brookshire *et al.*, 2011). However, warmer temperatures are also associated with longer growing seasons and therefore greater potential annual growth rate of plants, which may result in increased N demand for N_2-fixers (Goulden *et al.*, 1996). Therefore, SNF should be favored over soil N uptake under warmer climates if N mineralization does not increase in proportion to N demand, or if soil N pools are depleted. Additionally, the activity of nitrogenase, the enzyme responsible for SNF, is also temperature-dependent and maximum SNF rates may be achieved at 26ºC or higher (Houlton *et al.*, 2008; Liao *et al.*, 2017). The importance of temperature is supported by tree inventory data from the US and Mexico suggesting that N_2-fixing tree abundance generally increases with mean annual temperature (Liao *et al.*, 2017). These data also demonstrate that a transition occurs between rhizobial N_2-fixers such as *R. pseudoacacia* (legumes, which associate with *Rhizobia*-type bacteria) and actinorhizal N_2-fixers (which associate with *Frankia*-type bacteria) at 35ºN latitude and this pattern has been linked to temperature (Menge *et al.*, 2014; Sheffer *et al.*, 2015). Since the range of *R. pseudoacacia* is largely above 35ºN, where rhizobial N_2-fixer abundance declines dramatically, low temperature could be expected to be a strong limiting factor at the northward range edge (Little, 1976).

Soil moisture may also affect *R. pseudoacacia* distribution by altering the value of C, and therefore the relative cost of SNF. Low soil moisture can trigger stomatal limitation of photosynthesis in this species (Mantovani *et al.*, 2014b; Minucci *et al.*, 2017), which could limit growth and C accumulation, thereby raising the value of C relative to N (Vose *et al.*, 2016). In areas where growing season soil moisture is low, *R. pseudoacacia* has been observed to be outcompeted by non-fixers, leading to declines in its abundance (Chapter 3). However, other leguminous N_2-fixing tree species are highly abundant in some hot arid regions such as southwestern United States deserts and tropical savannas (Liao *et al.*, 2017), and there is evidence that SNF can facilitate acclimation to prolonged droughts (Minucci *et al.*, 2017). In these very arid regions, this physiological drought tolerance may be a more important factor for plant success than competition for resources (Grime, 1977). Within the Eastern US, where the range of soil moistures is generally higher, we expect *R. pseudoacacia* to occur more frequently in high moisture areas than low moisture areas due to greater C availability. The variation in soil moisture that occurs within the region is likely driven on a large scale by precipitation patterns and temperature (especially during the growing season), and on a smaller scale across stands by slope, aspect, and soil texture.

High light availability may promote the presence of *R. pseudoacacia* by supporting high rates of photosynthesis, leading to rapid growth and the accumulation of carbohydrates. High growth rates and non-structural carbohydrate accumulation should decrease the value of C relative to N, making SNF energetically favored over soil N uptake (Norby *et al.*, 2010). The importance of light availability in supported by observation that N_2-fixers are most abundant in early succession across a variety of ecosystems (Menge *et al.*, 2010). Additionally, high rates of disturbance, such as fire or blowdowns, may promote N_2-fixers by creating canopy gaps where light is abundant (Barron *et al.*, 2011; Yelenik *et al.*, 2013). Since light gaps occur throughout the eastern US, light availability may not control the overall range of *R. pseudoacacia,* but it could be a key control at a finer scale, helping to determine whether *R. pseudoacacia* occurs in a given forest plot within its range. Given the importance of light in promoting N_2 fixation, we expect that *R. pseudoacacia* abundance will be greater in young forest stands, southward facing slopes, and areas with a high disturbance rate (*e.g.* steep slopes, fire-prone forests).

Soil characteristics are also likely to drive *R. pseudoacacia* distribution, by altering the supply of nutrients to plants and therefore the relative value of N. Since SNF will be favored over soil N uptake when soil N availability is low, soils with a low ability to retain N should generally promote N_2-fixers over non-fixers (Vitousek *et al.*, 2002). For example, soils with a coarse texture and low cation exchange capacity (CEC) are particularly poor are retaining ammonium, the main source of N for most plants, and therefore should promote N_2-fixer success (Castellano *et al.*, 2012). *R. pseudoacacia* may also be promoted by low pH, as N mineralization rate can be suppressed under acidic conditions (Fu *et al.*, 1987). Finally, high organic carbon content may promote N limitation for decomposers, resulting in greater N immobilization. The parent materials that soils arise from may also affect *R. pseudoacacia* success, through variation in the concentration of rock-derived nutrients. SNF rate by N_2-fixers can be limited by low phosphorus (P) availability (Batterman *et al.*, 2013b), and less frequently by low molybdenum and iron (Brear *et al.*, 2013; Wurzburger & Hedin, 2016). In summary, *R. pseudoacacia* presence may be promoted by soils with a low capacity to cycle and retain N, but high concentrations of rock-derived nutrients.

Here, we investigate how temperature, soil moisture, light availability, and soil characteristics correlate with the distribution of the ecologically important N_2-fixing tree, *Robinia pseudoacacia* (or black locust). Because *R. pseudoacacia* is distributed throughout the entire Appalachian Mountain region, across many climates, soil types, and parent materials, it should be possible to separate the effects of these individual factors on habitat suitability. We utilized forest inventory data from the USDA Forest Service Forest Inventory and Analysis program and boosted classification trees to model likelihood of *R. pseudoacacia* occurrence across eastern US forests. While Iverson *et al.* (2008) also used FIA data to model *R. pseudoacacia* distribution (along with other 134 Eastern US tree species) with the goal of assessing general range shifts with climate change, we sought to tailor a model to better fit the distribution of this single species and to interpret the most important predictors in order to build our understanding of the underlying mechanisms driving its distribution. In addition, while previous work has attempted to explain *R. pseudoacaica* distribution at a coarser scale, we modeled presence or absence in individual FIA plots, which allows us to assess predictors which vary over both large (*e.g.*, mean annual temperature) and small (*e.g.* slope, stand age) spatial scales. We hypothesized that, due to the resource economy of SNF (favoring conditions where N is scarce but other resources are abundant), presence of *R. pseudoacacia* would be associated with: (a) high mean annual temperature, (b) consistently high soil moisture (*i.e.*, high precipitation and low summer temperatures), (c) high light availability (*i.e.*, young stand age, frequent disturbance), and (d) N limited soils (*i.e.*, low cation-exchange capacity, low pH, high organic carbon content, coarse texture), derived from parent materials rich in P.

We also use the resulting models to project changes to habitat suitability in the year 2050 and detect potential areas of invasion and loss for this important species. We hypothesized that reductions in soil moisture due to high summer temperatures would cause habitat suitability for *R. pseudoacacia* to: (a) decrease in the southern Appalachian and Ozark regions, and (b) increase in the northeastern US and the upper Midwest (areas where *R. pseudoacacia* is largely absent today).

Methods

Study area and tree inventory data

Presence and absence data for *R. pseudoacacia* was taken from version 1.7 of the USDA Forest Service Forest Inventory and Analysis (FIA) database (http://www.fia.fs.fed.us). FIA plots are distributed at a density of one plot per 2,400 ha of forest land, and within each plot trees are inventoried inside four 7.3 m radius subplots. We focused our analysis on FIA plots in the continental US, east of 100º W longitude, which encompasses the entire natural range of *R. pseudoacacia.* We included only naturally regenerated forest plots surveyed after the year 2000, when plot design and sampling protocols were standardized nationally. We also restricted our data to only the most recent survey for a given plot. The resulting dataset consisted of 82,023 plots, of which 2,586 (c. 3.2%) contained *R. pseudoacacia* (Figure 4.1a).

Forest structure data

We also used data on the physical characteristics of each plot, as these factors could influence light availability and microclimate. From the FIA database, we used data on elevation, slope, aspect, stand age, and presence of fire damage. We transformed circular aspect data into the topographic radiation aspect index, a continuous variable between 0 and 1, with 0 representing cooler and wetter north-northeastern slopes and 1 representing hotter and drier south-southwestern slopes (Roberts & Cooper, 1989). To characterize the density of forest cover at each plot, we also included the 2001-2012 average maximum green vegetation fraction (MGVF), a MODIS-based metric available at 1km$_2$ resolution from the USGS Land Cover Institute (https://landcover.usgs.gov/green_veg.php/).

Climate data

To account for the impact of climate on the distribution of *R. pseudoacacia,* we matched each of our FIA plots to climate data from the WorldClim global climate database version 2 (http://worldclim.org/). Specifically, we used a set of 19 bioclimatic variables derived from monthly temperature and rainfall values from 1970 to 2000 interpolated at 1 km^2 resolution (Table 4.1; Fick & Hijmans, 2017). These bioclimatic variables are useful for modeling species distributions, as they characterize not only annual means, but also seasonal trends and potentially limiting environmental factors (e.g. precipitation of the driest season).

Soil and geological data

Since the success of N_2-fixers is likely affected by soil nutrient availability, we included several soil and geological factors in our model. For a general characterization of the land surface (e.g., colluvial sediment, extrusive volcanic rock), we extracted lithology classes from the USGS surficial lithology of the coterminous United States map (https://pubs.usgs.gov/sim/3126/). For a more specific characterization of underlying parent materials, we extracted primary and secondary rock types from the USGS geologic map of the United States (https://mrdata.usgs.gov/geology/state/). Since many of the 149 parent rock types occurred very rarely, we included the 40 most common rock types and pooled all others in a "rare rock types" level. We also included several soil characteristics known to have strong impacts on nutrient availability: pH, soil texture class, cation exchange capacity (*CEC*) and organic carbon content (*%OC*). These variables were extracted for each FIA plot location from the Harmonized World Soil Database version 1.2 (http://www.fao.org/soils-portal/soil-survey/soil-maps-and-databases/harmonized-world-soil-database-v12/), which maps soil characteristics at 1 km^2 resolution.

Boosted regression tree analysis

To link our potential predictor variables to the probability of *R. pseudoacacia* occurrence in a forest stand, we used boosted regression tree (BRT) models with a Bernoulli error distribution (Elith *et al.*, 2008). BRTs utilize gradient boosting to iteratively combine many decision tree models, thereby decreasing model bias and improving accuracy. We selected BRTs because of their excellent accuracy and their ability to flexibly learn non-linear relationships between the response and predictors and handle collinearity between predictors. Additionally, BRTs have the advantage of automatically performing model selection by including informative predictors in decision trees and ignoring non-informative predictors. To implement BRTs, we used the GBM package in R (Ridgeway, 2017).

We randomly split our data into two sets before analysis: the training set (80% or 65,620 plots) and the test set (20% or 16,404 plots). We then used the training set to optimize and fit a GBM model, while reserving the test set for an assessment of the final model's accuracy. Because *R. pseudoacacia* was present in under 4% of plots, we used the synthetic minority over-sampling technique (SMOTE) when fitting our model to the training set, using the DMwR package in R. This technique combines undersampling of the majority class (*R. pseudoacacia* absent) with oversampling of the minority class (*R. pseudoacacia* present) using synthetic cases constructed between sets of nearest-neighbors in feature space (Chawla *et al.*, 2002). Using SMOTE allowed us to improve fit by minimizing rare events bias, which can result in an overemphasis of majority class accuracy at the expense of reduced minority class accuracy. Whenever we assessed training set fit using cross validation, we applied SMOTE within each fold to avoid overfitting as a result of oversampling.

Optimization of boosted regression tree model

We selected the area under the receiver operating character curve (AUROC) as our metric of model accuracy because this metric takes into account both sensitivity (the true positive rate) and specificity (the true negative rate) and is preferable to classification accuracy when class frequencies are imbalanced (Chawla, 2010). The AUROC metric falls between 0 and 1, with an AUROC of <0.5 indicating a fit no better than random, and values > 0.9 indicating excellent fit (Baldwin, 2009; Beane *et al.*, 2013).

Finding the best BRT model involves optimizing several hyperparameters that control the boosting algorithm: the depth of each tree, the minimum observations in a node, the number of trees and the learning rate (Elith *et al.*, 2008). Since the learning rate has little effect on model accuracy as long as it is sufficiently low, we kept this hyperparameter constant at 0.01. The three other hyperparameters were simultaneously optimized using a two-step approach: (1) a random grid search of hyperparameter values, followed by (2) Bayesian optimization. First, we fit GBM models to the training set for 400 random combinations of the tree depth and minimum observations in a node hyperparameters, each evaluated between 100 and 4000 total trees, resulting in 16,000 combinations of hyperparameters.

Goodness of fit to the training set was evaluated for each model using five repeats of five-fold cross validation and the AUROC metric. The best model, as determined by the maximum AUROC value, used an interaction depth of 32, a minimum number of observations in a node of 12 and 800 trees. To test whether an untested combination of hyperparameters was better than our best randomly selected combination, we then performed Bayesian optimization with a Gaussian process (Snoek *et al.*, 2012), using the rBayesianOptimization package in R. For Bayesian optimization, we used the Matérn 5/2 kernel, the upper confidence bound acquisition function and 30 iterations, with our random grid search results as the initial samples of the target function. The best hyperparameters identified through Bayesian optimization were the same hyperparameters found with our random grid search.

Finally, we optimized the class threshold (percent occurrence probability required to predict “present”) by fitting an ROC curve to the entire training set with our final model and finding the class threshold that gave equal true positive rate and true negative rate. This allowed us to better balance the predictive value of “present” and “absent” predictions. We then used this optimized threshold to assess model accuracy on the test set and to make final predictions of *R. pseudoacacia* presence or absence over the entire dataset.

Final model interpretation

To determine which predictors were most important in driving *R. pseudoacacia* distribution, we calculated the relative importance of each predictor in our best model. Importance in determining the presence or absence of *R. pseudoacacia* was calculated using the number of times a predictor was selected for a tree split, weighted by the model improvement of each split and averaged over all trees (Friedman & Meulman, 2003; Elith *et al.*, 2008). We then scaled importance to fall between 0 and 100, with the most important predictor assigned to 100.

We also characterized the shape of the relationship between relevant predictors and the probability of *R. pseudoacacia* occurrence. The relationship of each predictor to the probability of occurrence was visualized using partial dependence plots, which illustrate the effect of a single predictor on the response after accounting for the mean effects of all other predictors (Friedman, 2001). Because no high spatial resolution measurements of soil or rock P content were available for the study area, we fit a linear regression between the predicted probability of occurrence and median P content of each parent rock type (Porder & Ramachandran, 2013), in order to explore whether the relationship between *R. pseudoacacia* occurrence probability and primary parent rock type was related to rock P content.

Modeling R. pseudoacacia distribution under future climate

To predict how the distribution of *R. pseudoacacia* may change under future climate scenarios, we utilized our final BRT model to predict probability of occurrence with current forest structure, soil and parent material data, but year 2050 climate projections. We used projections of our 19 bioclimatic variables for the year 2050, averaged across an ensemble of 16 GCMs for the Representative Concentration Pathways scenario RCP8.5 (Table S4.1; http://www.worldclim.org/cmip5v1/). Our projections represent the potential habitat suitability for *R. pseudoacacia* in 2050, since dispersal speed may constrain the actual migration rate of this species.

Results

Final model performance

Our final BRT model was able to predict presence of *R. pseudoacacia* in forest plots with excellent accuracy (AUROC > 0.90) on both training and test sets (Table 4.2). Cross-validation on the training set found an AUROC of 0.92. Using a default class threshold of 50% probability (assigning plots with >50% probability of occurrence a "present" prediction), overall cross-validated model accuracy was 91%, with a true positive rate (sensitivity) of 67%, and a true negative rate (specificity) of 91%, indicating that our model predicted presence or absence of *R. pseudoacacia* correctly 91% of the time, but predictions of absence were more likely to be correct than predictions of presence. Using the full training set ROC curve, we optimized the class threshold (probability required to predict "present") to give equal true positive and true negative rates, and we found this threshold at 40% probability of occurrence.

On the test set, which was not used for any model tuning or fitting, the final model achieved an AUROC of 0.91, indicating that our model was not over-fit to the training data. Using a class threshold of 40% (which was determined to be optimal on the training set), the overall model accuracy was 88%, with a true positive rate of 73% and a true negative rate of 88%.

Qualitatively, our model was able to accurately reproduce the distribution pattern of *R. pseudoacacia* in the Eastern US (Figure 4.1). We predicted the highest probability of *R. pseudoacacia* occurrence in the Appalachian Mountains and the Bluegrass Region of Kentucky. We also accurately identified several small populations which occur outside the main range, including two Ozark populations in Missouri and Arkansas, and a small population in the Hudson River Valley of New York. When assigning *R. pseudoacacia* as either present or absent at each plot (Figure 4.1c), our model somewhat overpredicted *R. pseudoacacia* presence at the center of its range because any plot with over 40% probability of occurrence is predicted to contain the species, while logically many of these plots could be good habitat but not contain the species by chance.

Most important factors for predicting R. pseudoacacia distribution

To determine what factors drive the distribution of *R. pseudoacacia*, we ranked the predictors in our model by their relative importance values. The most important predictor in our model was mean annual temperature (Table 4.1). The rest of the top six predictors, ranging from 81.2% to 33.2% as important as mean annual temperature, were: maximum temperature of the warmest month, primary parent rock type, plot slope and aspect, and soil organic carbon content.

We addressed our hypothesis that *R. pseudocacacia* occurrence would be greater where mean annual temperature (*MAT*) is highest by examining the partial dependence of *MAT* on probability of *R. pseudoacacia* presence. As *MAT* increased above 5ºC, probability of *R. pseudoacacia* presence increased dramatically, reaching a maximum probability above 8ºC (Figure 4.2f). *MAT* was the most important predictor in our final model, and with the exception maximum temperature of the warmest month (discussed above), no other temperature predictor was over 33% as important at *MAT*.

We assessed the relationship between soil moisture and *R. pseudoacacia* distribution by plotting the partial dependence of occurrence probability on precipitation and summer temperatures. Contrary to our expectations, mean annual precipitation was of little importance on our model (3.8%; Figure 4.2d**)**. However, the second most important predictor in our model was the maximum temperature of the warmest month, which likely correlates with drought stress. As maximum temperature of the warmest month increased, *R. pseudoacacia* occurrence became less probable (Figure 4.2e).

To examine the relationship between light availability and *R. pseudoacacia* distribution, we examined the partial dependence of occurrence probability on slope, aspect, stand age, MGVG, and fire. We found that probability of occurrence increased with slope (Figure 4.2a), which likely correlates to increased frequency of disturbance and therefore greater light availability. The direction of slopes also correlated with probability of occurrence. *R. pseudoacacia* occurs less often at both extremes of aspect: cool and wet north-northeastern slopes and hot and dry south-southwestern slopes **(**Figure 4.2b). Stand age was also a fairly important predictor (22.0% as important as the best predictor) of *R. pseudoacacia* occurrence, with increasing sharply until around 30 years after disturbance and then declining **(**Figure 4.2c**).** There was little or no correlation between *R. pseudoacacia* distribution and MGVF or presence of fire (relative importance: 3.7% and 0%, respectively).

To examine the impact of soil factors and parent material on *R. pseudoacacia* distribution, we plotted the partial dependence of *CEC*, pH, soil texture class, *%OC* and parent rock type on probability of *R. pseudoacacia* occurrence. Probability of occurrence was highest at low CEC and low pH (Figs 4.2g and 4.2h**)**, though neither effect was large, and both predictors had low relative importance (12.8% and 11.0%, respectively). Soil texture class was a poor predictor of *R. pseudoacacia* occurrence (2.9% relative importance), however probably of occurrence was approximately 20% greater on loamy sand than other texture classes. In contrast, *%OC* was one of the most important predictors (33.2% relative importance) in our model, and probability of *R. pseudoacacia* occurrence increased with *%OC* (Figure 4.2i). Primary parent rock type was also an important predictor in our final model (75.1% relative importance), and probability of occurrence varied widely, from 10% to 74%, across rock types. The five primary parent rock types associated with the highest probability of occurrence included chert, silt, and three types of schist (Table 4.3). Overall, there was no significant relationship between primary parent rocks' median P content (as reported by Porder and Ramachandran (2013)) and probability of *R. pseudoacacia* occurrence (Figure 4.3; $t = -0.56$, $P = 0.58$).

Predicted habitat suitability for R. pseudoacacia under future climate

We used our final BRT model to predict the probability of *R. pseudoacacia* occurrence under projected climate in 2050, as determined by 16 GCMs (Figure 4.4a). Our GCM-averaged predictions indicate a northward shift in *R. pseudoacacia* habitat suitability, with the largest declines in the Southern Appalachian Mountains, the Ozark Mountain Region, and the Bluegrass Region of Kentucky (Figure 4.4b). We predict the largest increase in habitat suitability in the Upper Midwest, New York, and Northern Pennsylvania.

To test whether the projected northward migration of *R. pseudoacacia* habitat was driven by temperature changes, we also predicted probability of occurrence under 2050 projected precipitation patterns but current temperature regimes. We found that projections made without increased temperatures resulted in no major northward expansion of *R. pseudoacacia* habitat (Figure 4.4c).

Discussion

Using boosted regression tree models, we were able to predict the distribution of the N_2-fixing tree *R. pseudoacacia* with high plot-level accuracy (88%) and realistic regional patterns (Figure 4.1). The relationships between *R. pseudoacacia* occurrence probability and the most important predictors (Figure 4.2) generally support the idea that this species is distributed where SNF is most energetically favorable over soil N uptake. Overall, distribution appears to be most strongly controlled by temperature, either directly or indirectly, with high but not extreme temperatures promoting *R. pseudoacacia* presence. Our models also suggest that ideal *R. pseudoacacia* habitat will migrate northward by 2050, as a result of increased temperatures. The loss or gain of this biogeochemical keystone species could have dramatic impacts on forest N cycling.

Resource economic trade-offs of SNF may drive R. pseudoacacia distribution

We found that presence of *R. pseudoacacia* was correlated with conditions where N was scarce but other resources were abundant, supporting our first hypothesis. Such conditions promote an increased value of N relative to C, which reinforces the idea that the distribution of N_2-fixers may be driven by the resource economics of SNF and the ability of N_2-fixers to compete with non-fixers.

We predicted that high mean annual temperature (*MAT*) would be correlated with greater probability of *R. pseudoacacia* occurrence, since longer growing seasons could promote higher annual growth rates and accumulation of C, thereby raising the value of N relative to C.

Moreover, potential SNF rate may be greater under high temperature as a result of the temperature dependence of the nitrogenase enzyme (Houlton *et al.*, 2008). In our BRT model, *MAT* was the most important predictor, and the probability of *R. pseudoacacia* occurrence increased dramatically between 5ºC and 10ºC before leveling off. Liao *et al.* (2017) found a similar relationship between MAT and the abundance of North American leguminous trees, but with the increase occurring between 12ºC and 25ºC. This discrepancy could be due to fact that tropical and subtropical N_2-fixers dominated their model because of their high local abundance. While warm climate appears to promote *R. pseudoacacia*, very high summer temperatures had a strong negative association with occurrence probability, and this constraint could be due to drought stress, as discussed below. However, thermal stress due to high temperature could also limit C supply, and therefore the net benefit of SNF, by increasing C loss through respiration and decreasing C gain through photooxidative damage (Teskey *et al.*, 2015).

We also predicted that high soil moisture availability would be associated with *R. pseudoacacia* presence, since water stress could limit C acquisition through stomatal limitation of photosynthesis (McDowell *et al.*, 2011). Low mean soil moisture has been shown to favor non-fixing trees over *R. pseudoacacia* in an early successional forest (Minucci *et al.* unpublished). In our BRT model, there were no variables related to precipitation among the top predictors (Table 4.1), and contrary to our prediction, there was only a very weak positive correlation between *R. pseudoacacia* presence and mean annual precipitation (Figure 4.2d). However, *R. pseudoacacia* was much more likely to occur in areas where the maximum temperature of the warmest month was lower than 32ºC, and this variable could relate to growing season as drought stress through increased evapotranspiration. Additionally, the decrease in *R. pseudoacacia* occurrence probability at very high topographic radiation aspect index, as discussed above, may also point to the role of drought stress in limiting *R. pseudoacacia* distribution.

Because of our hypothesis that *R. pseudoacacia* distribution is associated with high value of N relative to C, we also predicted that *R. pseudoacacia* presence should be associated with areas of high light availability (e.g. high slope, young stand age), as increased C supply from photosynthesis should promote SNF. High light availability should promote greater potential growth rates, and therefore N demand, as well as accumulation of carbohydrates (Wurzburger & Hedin, 2016). Our BRT model supported this assertion, as two of the most important predictors of *R. pseudoacacia* presence were slope and topographic radiation aspect index, two factors closely related to light availability. *R. pseudoacacia* occurrence probability had a strong positive correlation with slope (Figure 4.2a), which is in turn related to greater frequency of canopy gap generating disturbances (Norman *et al.*, 1995). High frequency of canopy gaps has been previously implicated in allowing N_2-fixers to persist in late-successional tropical forests (Barron *et al.*, 2011). Presence of *R. pseudoacacia* was also associated with moderate values of topographic radiation aspect index (Figure 4.2c), suggesting that high light availability could come with the trade-off of low soil moisture. Stand age was another important predictor in our model (22.0% relative importance), with *R. pseudoacacia* occurrence probability peaking at around 30 years, and then strongly declining. The rapid elimination of *R. pseudoacacia* from mid- and late-successional forest stands is likely the result of reduced light availability due to canopy closure, and this mechanism is often implicated for the broad absence of N_2-fixers in mature temperate forests (Vitousek & Field, 1999; Menge *et al.*, 2010).

Finally, we predicted that soil characteristics associated with low N availability but high availability of rock-derived nutrients like P would be correlated with greater probability of *R. pseudoacacia* occurrence. Primary parent rock type was the third most important predictor in our model (Table 4.1), which suggests that variation in soil characteristics and nutrient content plays a large role in driving *R. pseudoacacia* distribution. While we did not find a relationship between *R. pseudoacacia* occurrence probability and the median P content of primary parent rock types, wide variation in P content exists within each rock type (Porder & Ramachandran, 2013), making it difficult to accurately assess this relationship. While low P has been shown to limit SNF in tropical N_2-fixers (Batterman *et al.*, 2013b), P availability is generally much lower in highly-weathered tropical soils and P may not be a limiting factor in many temperature forest soils (Vitousek *et al.*, 2010). Another important factor in our model, slope, could correlate with differences in soil characteristics, and *R. pseudoacacia* may be promoted in high slope areas as soil N is lost downhill due to erosion (Berhe & Torn, 2017).

Implications for understanding N_2-fixer distribution patterns

Our model suggests that *R. pseudoacacia* distribution in the Eastern US may be related to the resource economic trade-off inherent to SNF (high C cost per unit N gained), and this mechanism could apply more broadly to other N_2-fixers, which should be constrained by the same basic economic principles. This may be particularly true for obligate N_2-fixers such as actinorhizal plants, which cannot regulate their SNF rate (Menge *et al.*, 2009a). Some N_2-fixers (including *R. pseudoacacia*) can down regulate their SNF rate (facultative N_2-fixation) when conditions favor soil N uptake over SNF, potentially allowing them to outcompete non-fixers in variable environments (Menge *et al.*, 2009a; Minucci *et al.*, 2017). However, under conditions which are consistently unfavorable for SNF, even facultative N_2-fixers should be outcompeted by non-fixers, as there are costs to maintaining this plasticity. Additionally, N_2-fixers typically have high leaf N content, making them more susceptible to herbivory than non-fixers (Vitousek & Field, 1999; Knops *et al.*, 2000). As a result, in ecosystems where competition between N_2-fixers and non-fixers is strong, obligate or facultative N_2-fixer success may hinge on SNF being energetically favored over soil N uptake for a considerable portion of the growing season.

While our model suggests that the energetic favorability of SNF over soil N uptake plays a role in driving the distribution of *R. pseudoacacia*, competition between N_2-fixers and non-fixers may not be the most important factor for N_2-fixer success in highly stressful environments. In these environments, ability to tolerate an abiotic stress may be the primary factor shaping plant communities (Grime, 1977; Craine, 2009). For example, N_2-fixers are the most abundant group of plants in many arid ecosystems (Liao *et al.*, 2017), where their ability to sustain high leaf N and therefore water-use-efficiency facilitates drought tolerance (Adams *et al.*, 2016; Minucci *et al.*, 2017). The energetic favorability of SNF may also be less important in systems where another resource besides N is highly limiting, for example P in tropical forests. The presence of N_2-fixers in N rich but P poor tropical forests has been hypothesized to be the result of N_2-fixers ability to acquire N to invest in P-acquiring enzymes (Houlton *et al.*, 2008). As we predicted, *R. pseudoacacia* occurrence probability was greater under high *%OC*, low CEC, and low pH (Figure 4.2g-i), conditions which can promote reduced soil N availability. Percent organic carbon (*%OC*) was one of the most important predictors in our model, and high *%OC* may indicate conditions where soil microbes are N limited, thereby favoring N immobilization over N mineralization (Frankenberger & Abdelmagid, 1985).

Projected shifts in R. pseudoacacia habitat suitability

Averaging across 17 different projections of 2050 climate in the Eastern US, our model predicts a northward migration of habitat suitable for *R. pseudoacacia*, with losses of habitat in the southern portion of the range, and gains in the Upper Midwest and New York (Figure 4.4a,b), supporting our second hypothesis. These changes disappear if we project with 2050 precipitation patterns but current temperature regimes (Figure 4.4c), demonstrating that increased temperature, not altered precipitation, is driving the projected shifts. Increases in the two most important variables in our model, *MAT* and maximum temperature of the warmest month, are the likely drivers of northward expansion and loss of habitat in southern areas, respectively (see Figure 4.2d,e). Our projections of *R. pseudoacacia* habitat in 2050 generally agree with those made by Iverson *et al.* (2008) for 2080, despite modeling at different scales and using different model types.

Our predictions represent the change in habitat suitability due to shifts in climate alone, and other factors such as dispersal limitations, migration of competitors, or significant alterations to forest age structure could also play roles in shaping the future geographic distribution of *R. pseudoacacia* (Svenning & Sandel, 2013). Northward expansion might be expected to be limited by dispersal rate, given the large size of *R. pseudoacacia* seeds and its habit reproduction through root suckers (Vítková *et al.*, 2017). However, seed dispersal and life history characteristics have been found to be surprisingly poor predictors of range shifts (Clark *et al.*, 2003; Zhu *et al.*, 2012). Additionally, *R. pseudoacacia* has been widely planted outside its native range, which has helped this species become one of the most prolific plant invaders worldwide (Richardson & Rejmánek, 2011). An analysis of the geographic range of seedlings versus large trees found evidence of both northward range expansion and southern range contraction for *R.*

pseudoacacia (Zhu *et al.*, 2012), which aligns with our predicted changes to habitat suitability. Finally, the future realized niche of *R. pseudoacacia* may also be affected by shifts in herbivore interactions, since N_2-fixers have a high susceptibility to herbivory and this species is targeted by several specialized insect pests (Athey & Connor, 1989; Knops *et al.*, 2000; Cierjacks *et al.*, 2013).

The gain or loss of *R. pseudoacacia*, a biogeochemical keystone species, will have major implications for N cycling in Eastern US forests. An invasion of *R. pseudoacacia* into Upper Midwest and Northeastern forests could trigger a significant N enrichment in forests that have previously harbored very few N_2-fixing trees (Liao *et al.*, 2017). This increase in N availability could lead to the decline of low-N adapted trees such as those forming ectomycorrhizal symbioses (Read & Perez-Moreno, 2003), and trigger an increase in invasive species (Seabloom *et al.*, 2015). In the Southern Appalachian Mountains and Ozarks, where *R. pseudoacacia* habitat is predicted to decline, the loss of the main N_2-fixing tree could diminish rates of forest productivity and recovery from disturbance (Menge, 2011; Minucci *et al*. unpublished). This is of particular significance because many Southeastern US forests will be in early succession in the near future as a result of demand for timber and biofuels (U.S. Department of Agriculture, 2012) . However, if subtropical N_2-fixing trees also respond to increased temperature by migrating northward (Liao *et al.*, 2017), loss N inputs from *R. pseudoacacia* could be compensated for by novel N_2-fixing tree species.

Conclusions

In this study we used BRT modeling to examine what factors shape the distribution of the N_2-fixing tree *R. pseudoacacia* and limit its abundance outside the Appalachian Mountain region. We found that the presence of this species was associated with conditions that may promote a higher value of N relative to C for plants, suggesting that the resource economic trade-off of acquiring N through SNF rather than soil N uptake is an important component of N_2-fixer success. The factors most strongly promoting *R. pseudoacacia* presence included high mean annual temperature but low maximum temperatures, high light availability, high soil organic carbon content and low soil cation exchange capacity. We also used our model to project habitat suitability for *R. pseudoacacia* in 2050, and we found a trend of northward habitat migration driven by temperature increases. The gain or loss of this N_2-fixing tree in Eastern US forests will have major implications for future forest N cycling and productivity.

Table 4.1: List of potential predictors included in BRT analysis and their relative importance in the final BRT model.

Predictor	**Units**	**5th percentile**	**95th percentile**	**Rel. importance (%)**
Continuous				
Mean annual temperature (*MAT*)	ºC	3.3	19.4	100.0
Maximum temp. of warmest month	ºC	24.4	34.5	81.2
Slope	Rise / 100 m	0	45	41.1
Topographic radiation aspect index	Unitless	0	1	34.6
Soil organic carbon content (*%OC*)	Percent	0.7	2.1	33.2
Mean temp. of coldest quarter	ºC	-12.1	11.0	30.7
Stand age	Years	7	100	22.0
Mean temp. of warmest quarter	ºC	16.7	27.3	21.6
Isothermality	Percent	26	43	18.9
Precipitation of warmest quarter	mm	225	455	15.5
Elevation	MASL	15.2	640.4	12.8
Soil cation exchange capacity (*CEC*)	cmol/kg	5	26	12.8
Latitude	Degrees	30.4	47.1	11.6
Soil pH	Unitless	4.5	6.6	11.0
Temperature seasonality	ºC	6.3	11.7	9.4
Mean temp. of wettest quarter	ºC	5.93	26.5	8.6
Longitude	Degrees	-96.5	-71.8	8.2
Precipitation seasonality	Percent	10	53	7.2
Mean diurnal range	ºC	10.6	13.9	7.0
Mean temp. of driest quarter	ºC	-12.1	22.9	6.1
Precipitation of driest month	mm	17	89	4.8
Temperature annual range	ºC	29.6	47.0	4.6
Minimum temp. of coldest month	ºC	-20.4	3.9	4.0
Mean annual precipitation (*MAP*)	mm	683	1506	3.8
Precipitation of wettest month	mm	93	173	3.7
Max green vegetative fraction (*MGVF*)	Percent	88	100	3.7
Precipitation of wettest quarter	mm	253	467	3.0
Precipitation of driest quarter	mm	64	302	2.5
Precipitation of coldest quarter	mm	64	418	1.9

Categorical	*# of classes*	
Primary parent rock type	41	75.1
Secondary parent rock type	41	20.6
Lithography class	17	19.5
Soil USDA texture class	7	2.9
Fire disturbance since last census	2	0.0

Table 4.2: Performance of the final BRT model on the training set (cross-validated) and test set.

	Training (cross validated)	**Test**
Number of plots	65,620	16,404
AUROC	0.92	0.91
Accuracy (%)	91%	88%
True positive rate (%)	67%	73%*
True negative rate (%)	91%	88%*

* Using optimized class cutoff determined on training set.

Table 4.3: Primary parent rock types associated with the highest probability of *R. pseudoacacia* occurrence.

Rock type	**Frequency (% of plots)**	**Probability of *R. pseudoacacia****
Biotite schist	0.9%	74.0%
Schist	0.9%	65.1%
Silt	0.7%	64.1%
Chert	0.6%	47.5%
Mica schist	0.8%	45.8%

* Probability of occurrence accounting for mean effect of all other predictors.

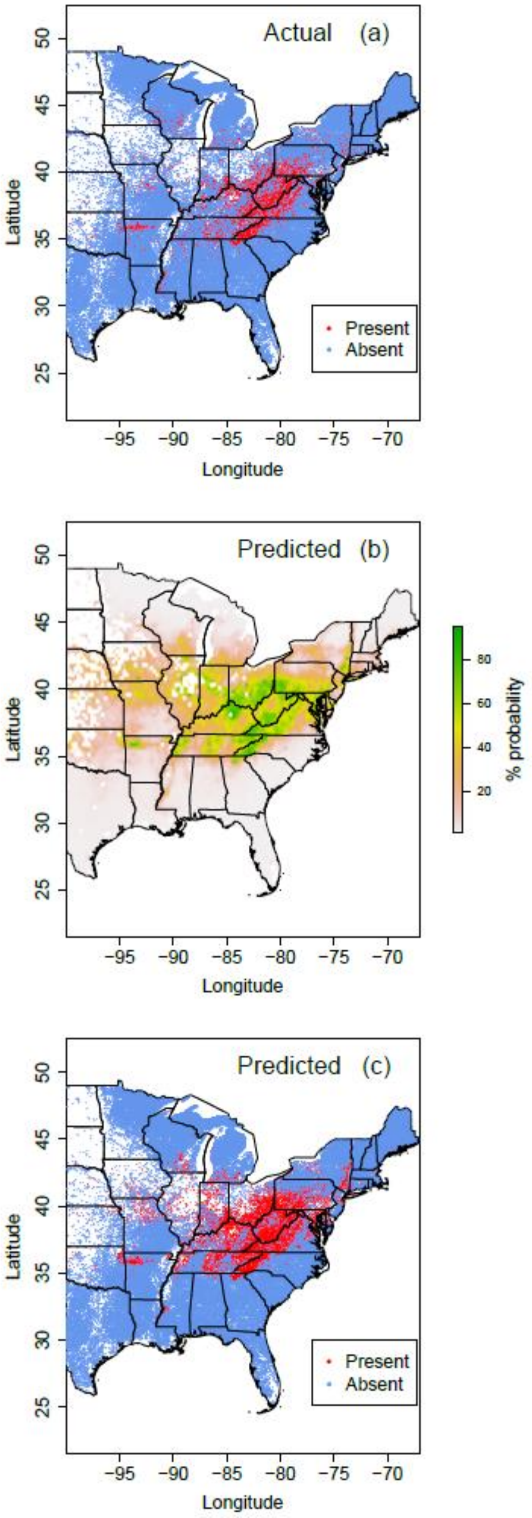

Figure 4.1: Map of all FIA plots (N=82,024) included in our analysis (a) and predicted current distribution using our BRT model (b and c). We map actual (a) and predicted (c) presence or

absence of *Robinia pseudoacacia* at each plot, with red dots indicating presence and blue dots indicating absence. We also map the predicted percent probability of occurrence, averaged over 0.25º cells and interpolated with thin plate splines (b). To determine predicted presence or absence at each plot, we used a 40% probability threshold (as optimized on the training set), meaning that any plot with greater than 40% chance of *R. pseudoacacia* occurrence was predicted to have *R. pseudoacacia* present.

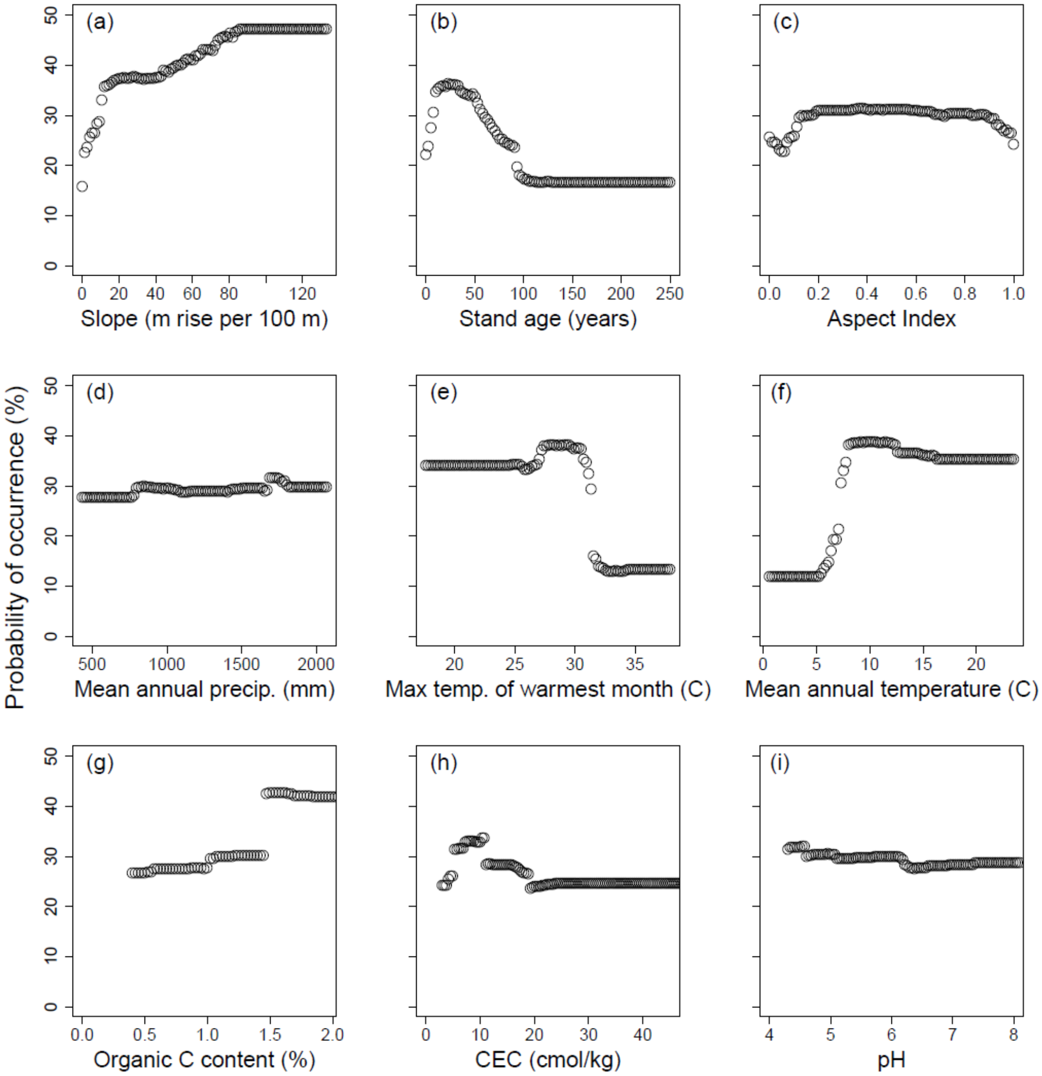

Figure 4.2: Partial dependence of *Robinia pseudoacacia* occurrence probability on: a) slope, b) stand age, c) aspect index (0 = cool, wet slopes and 1 = hot, dry slopes), d) mean annual precipitation (*MAP*), e) maximum temperature of the warmest month, f) mean annual temperature (*MAT*), g) soil organic carbon content (*%OC*), h) soil cation exchange capacity (*CEC*), and i) soil pH. Each plot illustrates the relationship between the predictor and probability of occurrence while accounting for the mean effect of all other predictors.

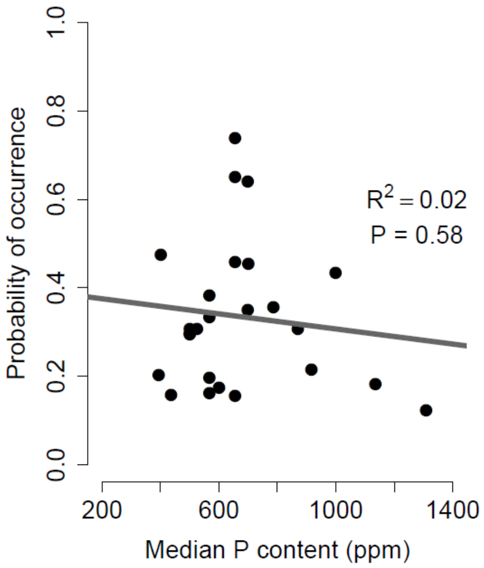

Figure 4.3: No relationship between the probability of *R. pseudoacacia* occurrence and median phosphorus (P) content of primary parent rock types. Median P content of primary parent rock types from Porder and Ramachandran (2013).

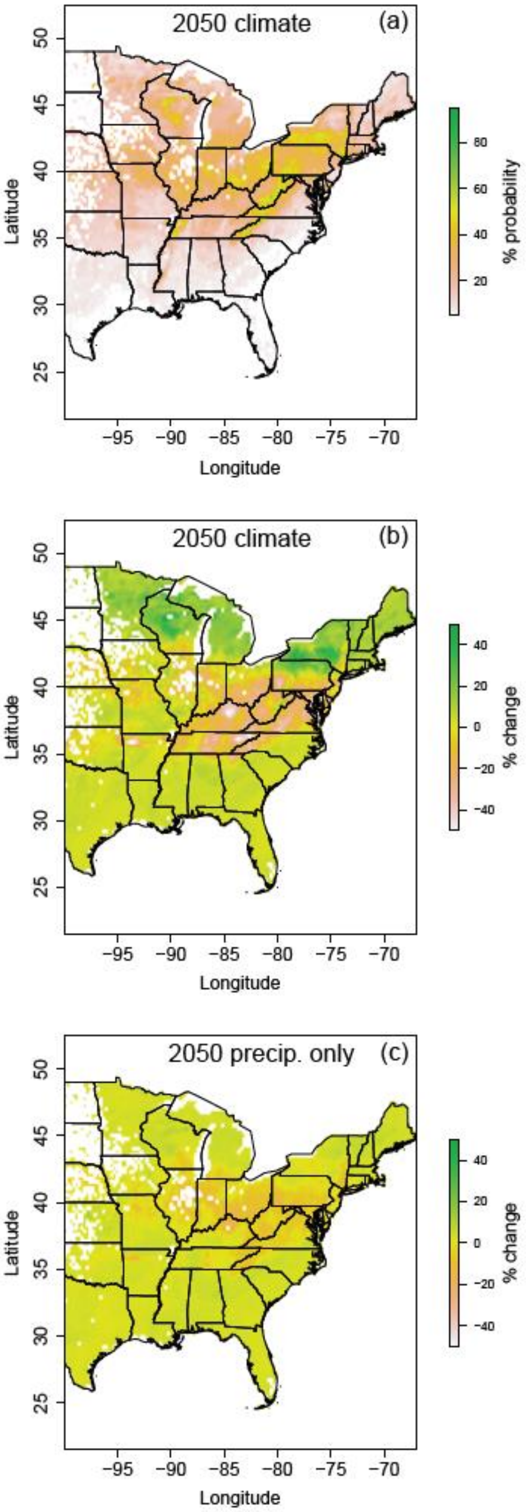

Figure 4.4: Projected habitat suitability under year 2050 climate for *Robinia pseudoacacia.*

Maps show the predicted percent probability of occurrence for 2050 (a), and change in percent probability from current (b), as well as change in percent probability from current when considering 2050 precipitation patterns but current climate (c). All maps show the average across 16 GCM climate projections (RCP 8.5; see **Table S4.1**).

CHAPTER 5

CONCLUSIONS

The overarching goal of this dissertation was to examine how changing precipitation and temperature regimes will affect growth, symbiotic nitrogen fixation rate (SNF), and competitive ability of *Robinia pseudoacacia*, a biogeochemical keystone species in Eastern US forests. Understanding the future SNF potential of this species is critical given its ability to increase forest N cycling rates (Montagnini *et al.*, 1986) and facilitate ecosystem recovery from disturbance (Boring & Swank, 1984). While SNF contributes to the productivity, nutrient limitation status and resilience of ecosystems (Menge, 2011; Batterman *et al.*, 2013a), it is a process which is regulated by stand-level competition and the resource allocation of individual plants (Vitousek & Field, 1999; Menge *et al.*, 2009a). To address this multi-level dependency, I examined how *R. pseudoacacia* responds to climate stressors at the level of individual seedlings (Chapter 2), and the early successional community (Chapter 3), and how these responses scaled up and interacted with other abiotic factors to drive the distribution of this N_2-fixer across the Eastern US (Chapter 4).

In Chapter 2, I tested the effect of drought frequency on the growth, SNF rate, and physiology of *R. pseudoacacia*, by growing seedlings in a greenhouse under soil moisture regimes which differed in drought frequency but not mean moisture. I found that overall growth and SNF rate were unaffected by drought frequency. However, drought frequency did determine the physiological response of seedlings to individual drought events, where seedlings exposed to a single, prolonged drought exhibited traits associated with drought tolerance, and seedlings exposed to brief but frequent droughts exhibited traits associated with drought avoidance. I also found evidence that SNF may supply the N required for both strategies. During a single, prolonged drought, *R. pseudoacacia* increased investment in SNF after several weeks, and this was associated with a rise in foliar N content and water-use efficiency. When exposed to brief but frequent drought events, *R. pseudoacacia* instead increased investment in SNF immediately following rewetting, during periods of rapid compensatory growth. My findings in this chapter suggest that SNF by *R. pseudoacacia* may be unaffected by increased drought frequency, but that SNF may play an important role in allowing ecosystems to resist the impacts of drought events.

In Chapter 3, I examined how a reduction in mean growing season soil moisture shifts competition between *R. pseudoacacia* and three non-fixing tree species, potentially leading to indirect effects on forest productivity via the nitrogen cycle. I approached this question through a three-year rainfall reduction study in an early successional Southern Appalachian forest. I found that low soil moisture was associated with reduced productivity of *R. pseudoacacia*, while productivity of non-fixers was largely insensitive to soil moisture but highly responsive to soil nitrogen. Because of these divergent responses, the relative abundance of *R. pseudoacacia* (in terms of biomass and stem number) decreased in areas of low soil moisture through time. We also found evidence that *R. pseudoacacia* locally enhances soil N availability, likely through inputs of N acquired through SNF, and such changes had positive effects on the growth of non-fixing trees. My findings in this chapter raise the possibility that increased aridity in the Southeastern US could indirectly reduce forest productivity, by decreasing *R. pseudoacacia* abundance and therefore reducing N inputs via SNF.

In Chapter 4, I sought to test whether the reduced competitive ability of *R. pseudoacacia* under low soil moisture at the stand level manifested in its geographic distribution pattern across the Eastern US. In addition, I considered the effects of temperature, light availability, and soil factors on *R. pseudoacacia* distribution. Using Eastern US forest inventory data from the USDA Forest Service, I created a machine learning model to predict the probability that *R. pseudoacacia* would occur in a forest stand. According to my model, the factors which were most strongly associated with *R. pseudoacacia* presence included high mean annual temperature but low maximum temperatures, high light availability, high soil organic carbon content and low soil cation exchange capacity. These conditions are generally associated with a high relative value of nitrogen and a low relative value of carbon, which promotes nitrogen acquisition through SNF instead of soil nitrogen uptake. I also used my model to project future habitat suitability in 2050, and found that the best habitat for *R. pseudoacacia* will shift northward, with habitat expansion in the Upper Midwest and New York State and habitat loss in the Southern Appalachian Mountains and the Ozarks. My findings in this chapter support the idea that the resource trade-offs inherent to SNF help to drive the geographic distribution of *R. pseudoacacia* across the Eastern US. Furthermore, my model shows that higher temperature could reduce N inputs to forest ecosystems by shifting the distribution of this key N_2-fixing tree northward.

Collectively, this dissertation provides insight into how altered in precipitation and temperature regimes associated with global climate change will affect SNF potential by *Robinia pseudoacacia*. At the individual plant level, SNF appears to confer to *R. pseudoacacia* considerable resistance to both single, prolonged drought events, and frequent but brief droughts. However, in the context of competition with other non-fixing trees, *R. pseudoacacia* growth and competitive ability is reduced by low mean growing season moisture. The decline of *R. pseudoacacia* under consistently dry conditions appears to reflect, in part, a reduced energetic benefit from SNF due to stomatal limitation of photosynthesis. In addition, the ability of non-fixing trees to benefit from the higher soil N availability that *R. pseudoacacia* promotes may contribute to this competitive shift. By modeling *R. pseudoacacia* distribution across the Eastern US, I observed that soil moisture may indeed play role in *R. pseudoacacia* success at a larger scale. However, the most important factor associated with *R. pseudoacacia* distribution was high mean annual temperature, and this relationship will likely drive a northward migration of suitable *R. pseudoacacia* habitat.

The findings of this dissertation point to a potential reduction in SNF by *R. pseudoacacia* in the Southern Appalachian Mountains, due to increased temperature and aridity. The loss of this crucial N input could negatively affect forest productivity and recovery from disturbance in this region, if another prolific N_2-fixing tree does not migrate into the area. Conversely, the potential expansion of *R. pseudoacacia* into previously unsuitable habitat in the Upper Midwest and New York State could trigger N enrichment in these forests. Future research within this system should focus on elucidating the time scale over which reductions in *R. pseudoacacia* abundance lead to diminished soil N availability, as considerable time lags may exist between incorporation of fixed N in N_2-fixer biomass and mineralization of that N into soil pools (Boring & Swank, 1984). Simulation models could also be utilized to assess whether *R. pseudoacacia* abundance will reach a new equilibrium under a more arid climate, given that N_2-fixers should be promoted by an eventual decrease in soil N availability. Additionally, because of the high susceptibility of *R. pseudoacacia* to herbivory (Hargrowe *et al.*, 1984; Knops *et al.*, 2000; Zheng *et al.*, 2003) and the fact that drought stress can reduce herbivore defenses (Gaylord *et al.*, 2013), future work should seek to understand how herbivore pressure on this species will be altered by climate change.

More broadly, the findings of this dissertation add to our growing understanding of how environmental conditions affect the physiological function and competitive ability of symbiotic N_2-fixing plants. While light and temperature have long implicated as important factors affecting SNF (*e.g.* Vitousek *et al.*, 2002; Houlton *et al.*, 2008), more recent work has begun to recognize the relationship between SNF and soil moisture and/or drought stress. Adams *et al.* (2016) found a correlation between water-use-efficiency and leaf N concentrations for legumes, but not non-fixers, suggesting that SNF may serve as a drought tolerance mechanism. In support of this idea, N_2-fixers, including *R. pseudoacacia,* tend to invest more in SNF during extended drought periods (Tobita *et al.*, 2010; Wurzburger & Miniat, 2014; Mantovani *et al.*, 2015). My findings in Chapter 2, that *R. pseudoacacia* increased nodule mass fraction, leaf N content, and water-use-efficiency during an 8-week drought provides evidence that SNF can promote drought tolerance. My Chapter 2 findings also indicate that SNF can promote a drought avoidance strategy when *R. pseudoacacia* is exposed to short but frequent droughts, by facilitating compensatory growth following rewetting.

Interestingly, despite the role of SNF in drought tolerance or avoidance, a field survey along an aridity gradient found fewer N_2-fixers in the driest areas (Aranibar *et al.*, 2004). In addition, when N_2-fixers are abundant in dry areas, they appear to fix less nitrogen than in wet areas (Schulze *et al.*, 1991; Soper *et al.*, 2015). These trends are supported by my rainfall exclusion experiment (Chapter 3), where I observed reduced growth, relative abundance, and leaf N concentrations of N_2-fixers under low moisture. High N_2-fixer abundance in some very arid regions could be driven by severe drought tolerance instead of direct competition for resources, which is likely the dominant factor in mesic conditions like my field experiment (Craine, 2009; Soper *et al.*, 2015). In this context, SNF may allow for high N inputs during the most severe droughts or following rewetting, but may be energetically unfavorable, and therefore downregulated, at most other times. This mechanism could promote N_2-fixer success even when SNF rates are low when averaged over time. Further research is needed to examine how competition between N_2-fixers and non-fixers is altered by environmental context, such as more mesic or arid conditions.

In conclusion, this dissertation provides evidence that alterations in soil moisture and temperature due to global climate change may affect growth rate, competitive ability and geographic distribution of the N_2-fixing tree *R. pseudoacacia*, a biogeochemical keystone species. As a result, these climate stressors could constrain N inputs through SNF, thereby reducing forest productivity and inhibiting recovery from disturbance. By contributing to our understanding of future N cycle dynamics in Eastern US forests, this research can help to inform management of these ecosystems and the services that they provide.

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APPENDIX A

CHAPTER 2 SUPPLEMENTAL INFORMATION

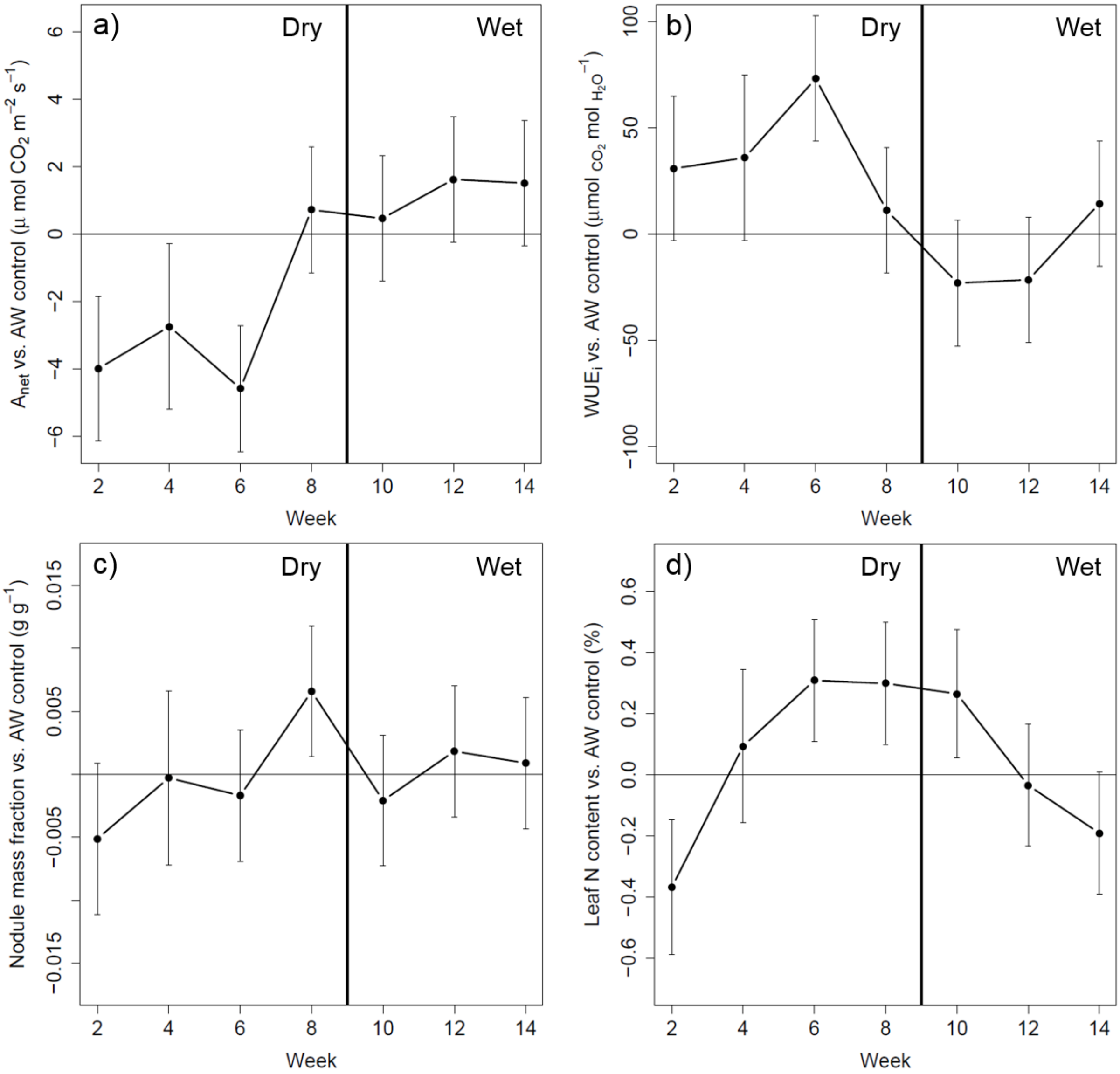

Figure S2.1: Physiological differences between low frequency drought (LF) treatment *R. pseudoacacia* seedlings and always wet control (AW) through time, including: a) net C assimilation, b) intrinsic water use efficiency, c) nodule mass fraction, and d) leaf N content. Vertical lines denote the transition between an 8-week dry phase and a 6-week wet phase. Error bars represent SE.

Table S2.1: Effect of drought frequency treatment and water status on root mass fraction, leaf mass fraction, and nodule mass-specific SNF rate of *R. pseudoacacia*. Values represent means (±SE).

Treatment	Water status	Root mass fraction (g g^{-1})	Leaf mass fraction (g g^{-1})	Nodule mass-specific SNF rate (μmol N_2 $g^{-1}hr^{-1)}$
HF	Wet	0.46 (±0.02)	0.31 (±0.02)	230.6 (±56.9)
	Dry	0.44 (±0.02)	0.32 (±0.02)	252.1 (±46.9)
MF	Wet	0.45 (±0.02)	0.30 (±0.02)	212.4 (±57.3)
	Dry	0.47 (±0.02)	0.28 (±0.02)	188.2 (±53.4)
LF	Wet	0.49 (±0.02)	0.30 (±0.02)	296.4 (±55.9)
	Dry	0.42 (±0.02)	0.36 (±0.02)	234.2 (±57.3)
AW	Wet	0.43 (±0.01)	0.34 (±0.01)	343.4 (±36.5)

APPENDIX B

CHAPTER 3 SUPPLEMENTAL INFORMATION

Table S3.1: Mean soil conditions across plots.

	Units	**Mean**	**SD**
Growing season soil moisture	%	23.3	3.0
Bulk density	g cm^{-3}	1.13	0.09
Total carbon	%	4.18	1.32
Total nitrogen	%	0.22	0.07
C:N	unitless	19.3	1.9
$\delta^{15}N$	‰	3.0	1.2

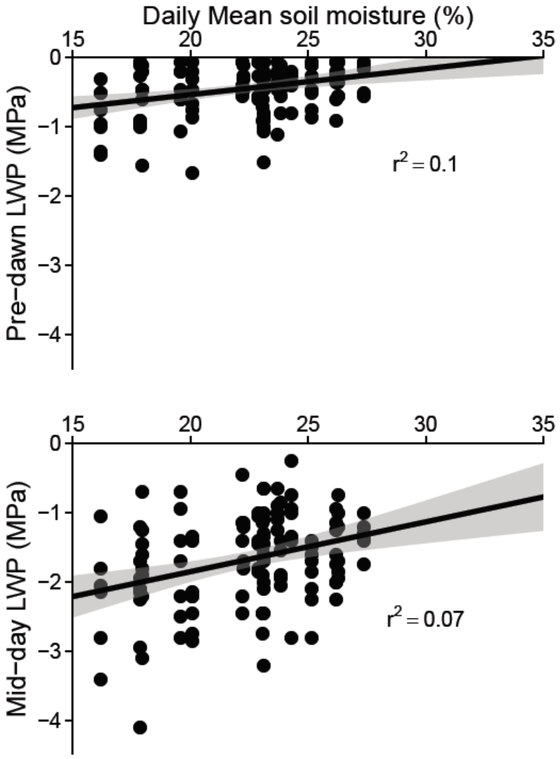

Figure S3.1: Pre-dawn and mid-day leaf water potential (LWP) was related to the mean soil moisture recorded on the day of measurement for all species. Lines represent regression fits with 95% confidence intervals and marginal r^2 values.

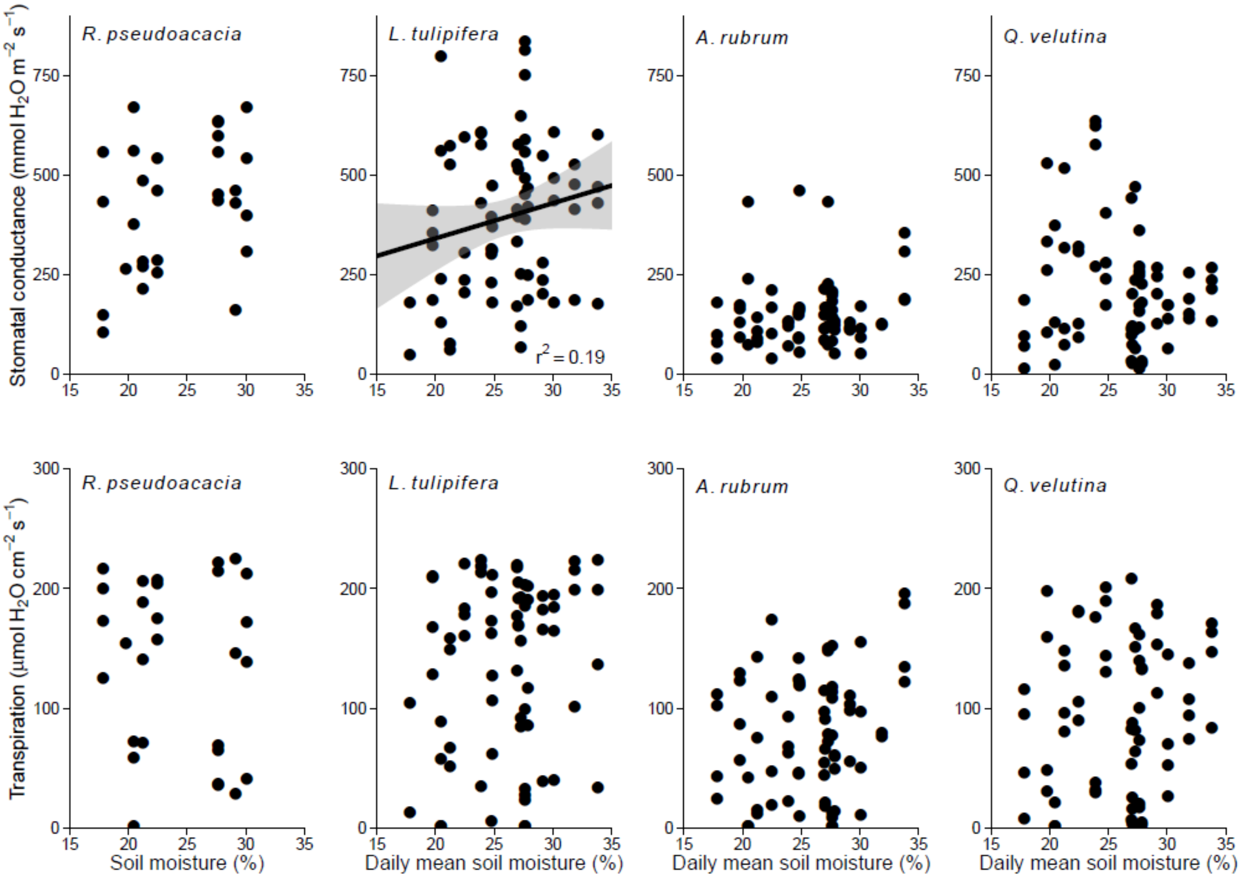

Figure S3.2: Stomatal conductance was related to the mean soil moisture recorded on the day of measurement for *L. tulipifera*, only. There was no relationship between transpiration and mean daily soil moisture for any species. Regression fits with 95% confidence intervals and marginal r^2 values are included for significant relationships.

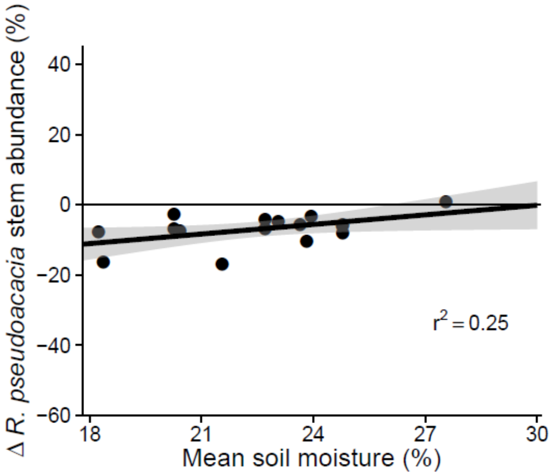

Figure S3.3: Change in relative stem abundance (percent of total plot stems) of *R. pseudoacacia* during years 2 and 3 was related to mean soil moisture during that period. Lines represent regression fits with 95% confidence intervals and marginal r^2 values.

APPENDIX C

CHAPTER 4 SUPPLEMENTAL INFORMATION

Table S4.1: List of 16 GCMs used for projection of 2050 habitat suitability. For all GCMs, the RCP 8.5 scenario was used and downscaled and calibrated bioclim variables were obtained from the WorldClim database (http://www.worldclim.org/cmip5v1/).

Model Name
Access1-0
BCC-CSM1-1
CCSM4
CNRM-CM5
GISS-E2-R
HadGEM2-AO
HadGEM2-CC
HadGEM2-ES
INMCM4
IPSL-CM5A-LR
MIROC-ESM-CHEM
MIROC-ESM
MIROC5
MPI-ESM-LR
MRI-CGCM3
NorESM1-M