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ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

Nitrogen and light regulate symbiotic nitrogen fixation by a temperate forest tree

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Abstract

Symbiotic nitrogen fixation (SNF) is a critical mechanism of ecosystem recovery, and in forests of the eastern United States, the most common tree species that supports SNF is black locust (*Robinia pseudoacacia* L.). Despite its prevalence, black locust's fixation strategy—whether it maintains fixation at a constant rate (obligate fixation) or reduces its fixation rate (facultative fixation)—is unknown. Here, we examined how nitrogen and light control SNF by black locust, by growing seedlings under two nitrogen levels and across four levels of light. Seedlings were harvested after 12 weeks to determine biomass changes, nodule activity, and photosynthetic rates. Black locust seedlings increased biomass growth with increasing light, but only in the absence of nitrogen addition, while seedling root:shoot (biomass) modestly declined with increasing light regardless of nitrogen level. We found that black locust behaved like a facultative fixer, and regulated fixation by excising or maintaining nodules, and by controlling nodule biomass and activity. Specifically, nitrogen addition reduced seedling investment in nodule biomass (g g^{-1}) by 63%, and reduced seedling allocation to nitrogen fixation (μmol C_2H_4 g^{-1} h^{-1}) by 66%. In contrast, light affected nitrogen fixation through two indirect pathways. First, light increased plant growth, and hence nitrogen demands, which caused an increase in nitrogen fixation proportional to biomass. Second, light increasd photosynthetic activity, which was positively associated with nodule activity, but only in the absence of nitrogen addition. Our findings for how black locust regulates SNF can improve predictions of ecosystem SNF under the changing environmental conditions.

Keywords *Robinia pseudoacacia* (black locust) · Succession · Disturbance · Nodule · Rhizobia

Introduction

The symbiotic relationship between plants and nitrogen-fixing bacteria is geographically extensive and is responsible for converting atmospheric dinitrogen gas to plant-available forms of nitrogen (Sheffer et al. 2015). These specialized bacteria utilize the enzyme nitrogenase to catalyze the conversion of dinitrogen to ammonia, which plants can assimilate. This critical ecosystem pathway of symbiotic nitrogen fixation (SNF) exceeds other nitrogen fluxes such as weathering and atmospheric deposition (Vitousek et al. 2013). Inputs of SNF can be substantial following forest disturbance because the combination of high light availability and nitrogen limitation supports the growth and competitive ability of nitrogen fixers (LeBauer and Treseder 2008; Levy-Varon et al. 2019). Because SNF is energetically costly, nitrogen-fixing plants have evolved distinct strategies to regulate the symbiosis in response to competition with non-fixing plants for limiting resources (Menge et al. 2015; Sheffer et al. 2015). These strategies affect the magnitude and timing of SNF following disturbance, and therefore are critical for predicting SNF across ecosystems. However, our knowledge of SNF strategies is limited to broad-scale patterns with latitude and among biomes, and experimental evidence for SNF strategies of individual nitrogen-fixing species is often unknown.

Current ecological theory suggests that SNF strategies evolved due to the costs and benefits of fixation. A "facultative" strategy allows fixers to up- and downregulate fixation in response to available nitrogen and is evolutionarily stable in tropical forests where nitrogen cycling is highly dynamic,

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and nitrogen limitation is ephemeral or spatially heterogeneous (Menge et al. 2008; Barron et al. 2011; Sheffer et al. 2015). However, this facultative strategy comes with an opportunity cost—there are carbon costs associated with the ability to turn fixation on and off—making a facultative nitrogen fixer less competitive against non-fixing plants in environments with chronic nitrogen limitation (Menge et al. 2009). As a result, an "obligate" strategy, where fixers maintain their rate regardless of environmental conditions, is evolutionarily stable in temperate forests, where nitrogen limitation tends to persist over succession (Sheffer et al. 2015).

These two strategies explain differences in how nitrogen fixers competitively interact with non-fixing species (Sheffer et al. 2015), and ultimately influence the timing and magnitude of SNF rates over succession (Menge et al. 2009). Following forest disturbance, nitrogen fixers, regardless of their strategy, tend to dominate because nitrogen and light limit productivity during this time. However, whether nitrogen fixers remain dominant over succession differs between tropical and temperate forests due to fixation strategies. In tropical forests, facultative nitrogen fixers can allocate more carbon to their bacterial symbionts when the soil supply of nitrogen is low and light is abundant (Sheffer et al. 2015; Lu and Hedin 2019). Facultative fixers can downregulate fixation rates of nodules in a matter of minutes (Johnsen and Bongarten 1991), and nodules can be excised in a time frame of weeks (Batterman et al. 2013), to reduce fixation rates in response to available nitrogen. As a result, facultative nitrogen fixers can persist in mature tropical forests (Gei et al. 2018) due to their ability to downregulate nitrogen fixation and remain competitive with non-fixing trees (Sheffer et al. 2015). However, in temperate forests, obligate nitrogen fixers, which maintain the costly process of SNF regardless of environmental conditions, tend to decline as the forest matures because they are outcompeted for light by non-fixing species (Menge et al. 2008; Liao and Menge 2016). Although theory supports facultative and obligate strategies as evolutionarily stable in tropical and temperate forests, respectively, it is unclear whether all nitrogen-fixing species conform to this pattern.

In the eastern United States, there is one widespread and ecologically important native nitrogen-fixing tree species. *Robinia pseudoacacia* L., or black locust, is one of the most common nitrogen-fixing tree species in North America (Staccone et al. 2020), has been used in reforestation efforts globally, and as a result, now has naturalized populations across Europe and Asia (Vítková et al. 2017). Black locust has the potential for high rates of fixation, especially in younger forests (Boring and Swank 1984a; Wurzburger et al. 2022). Because nitrogen fixers may have diverse methods for conserving resources and regulating SNF (Menge et al. 2009), it is important to understand the SNF strategy of black locust to predict its contribution to ecosystem nitrogen cycling. Based on ecological theory, we would expect that black locust, a temperate nitrogen fixer, has an obligate strategy. Limited available evidence appears to contradict this expectation, however. Greenhouse studies with black locust seedlings have found that nitrogen additions reduce nodule biomass (Wang et al. 2020) and reduce nitrogenase activity (Johnsen and Bongarten 1991). In the field, both the probability that black locust trees possessed nodules and nodule biomass decreased over 75 years following disturbance (Wurzburger et al. 2022). These findings suggest that black locust downregulates SNF in response to increasing soil nitrogen availability.

In addition to nitrogen controlling SNF rates in a facultative species, light may also dictate SNF allocation. In fact, recent work suggests that light can have stronger control than nitrogen on regulating nitrogen fixation in a tropical, nitrogen-fixing tree species (Taylor and Menge 2018). However, it remains unclear how light and soil nitrogen might interact to control fixation across facultative and obligate nitrogen fixers more broadly, and for black locust in particular. Black locust recruitment and growth respond positively to high light in disturbance gaps or following a stand-replacing disturbance (Elliott et al. 2017). In these disturbed conditions, light is an abundant resource that may allow black locust to allocate more carbon to nodules, thereby increasing rates of SNF. Conversely, as black locust is shaded by larger trees, SNF rates may decrease as carbon resources decline.

The abundance of black locust and its rate of nitrogen fixation in the years immediately after disturbance may influence tree growth rates and community composition when forests are mature (Minucci et al. 2019). However, the SNF inputs by black locust will depend on its fixation strategy (Wurzburger et al. 2022). For example, if black locust's ability to fix nitrogen is regulated by soil nitrogen availability, then atmospheric nitrogen deposition (Knoepp et al. 2008) and fire exclusion (Carpenter et al. 2021), which can directly or indirectly increase nitrogen availability, may cap the fixation potential of black locust as eastern forests become nitrogen-rich. In contrast, if light is a strong regulator of black locust's nitrogen fixation rate, then SNF might be limited to early succession or disturbance gaps where black locust is a canopy-dominant or has ample access to light. These two regulators of SNF are impossible to disentangle in a natural ecosystem. Even in mature forests, light availability may be lower today than it was in the past due to long-term fire exclusion, which has resulted in dense forest mid- and under-stories (Nowacki and Abrams 2008). Understanding the role of nitrogen and light in regulating fixation by black locust will, therefore, improve our ability to predict SNF at the ecosystem level given the current conditions of eastern deciduous forests.

Here, we examined how nitrogen and light control symbiotic nitrogen fixation by black locust. We designed a

greenhouse experiment to test black locust's fixation strategy by modulating light conditions at four levels and nitrogen at two levels. While there is theoretical reasoning for black locust to be an obligate nitrogen fixer, experimental and observational evidence suggests that black locust is sensitive to nitrogen availability (Johnsen and Bongarten 1991; Wurzburger et al. 2022) but the role of light independent of nitrogen remains unclear. Based on prior findings, we expected that black locust would respond like a facultative fixer to both nitrogen and light availability. We hypothesized that (1) black locust would respond to nitrogen addition by decreasing both nodule activity and nodule biomass relative to plant biomass, and (2) black locust would respond to declining light availability by decreasing both nodule activity and nodule biomass relative to plant biomass.

Materials and methods

Greenhouse conditions and plant material

We conducted a greenhouse experiment using black locust seedlings at the USDA Forest Service, Coweeta Hydrologic Laboratory in western North Carolina, USA. In May 2020, we germinated *Robinia pseudoacacia* seeds from an eastern US seed source (Sheffield Seed Company, Locke, NY) and grew them for 12 weeks before the start of the experiment. We grew seedlings in seed trays in a 1:1 ratio by volume of vermiculite and sand inoculated with a native soil slurry from local black locust trees. When seedlings were *ca.* 3 cm tall, we transplanted them into individual 4-L pots containing a 1:1 ratio by volume of vermiculite and peat and trace amounts of sand. Soil pH at the end of the experiment was similar between treatments and ranged from 4.8 to 5.5, which is similar to local field soils.

Treatments

We established eight treatments (two levels of nitrogen crossed by four levels of light, described below), with $n = 13$, except the −N, 40% light transparency treatment was $n = 12$. We randomly stratified seedlings into treatments based on initial seedling height and diameter. At the start of treatment, we measured seedling height to the apical meristem and the stem diameter at 5 cm height to estimate total aboveground biomass using data from our previously established allometric equations (Wurzburger and Miniat 2014) (Online Resource 6).

For 12 weeks, we grew seedlings individually in 4-L pots, randomly rearranging their bench position within each treatment every 2 weeks. During the experiment, average greenhouse air temperature was 22.2 ºC (range 21.1–26.7 ºC). Average soil moisture was 35% v/v (CS655, Campbell Scientific Inc., Logan UT; $n = 1$ per treatment). Average air relative humidity was 42% (HMP50, Campbell Scientific Inc., Logan, UT; $n = 1$ per light treatment). Air temperature, soil moisture, and relative humidity did not differ among the treatments.

In addition to a control 100% light transmittance group, we created three additional light levels (40, 20, and 10%) using shade cloth. Light levels were above the light compensation point for black locust on an average day in the greenhouse. Because the response of photosynthesis to light is often curvilinear due to the saturating effect of light (Evans et al. 1993), we selected shade cloth that would confer relatively low light levels—levels above the light compensation point for this species and increasing such that we would likely see a linear increase in photosynthesis with our treatment levels. We monitored photosynthetically active photon flux density (PPFD, μmol m^2 s^{-1}; LI-250A with LI-193 sensor, Li-Cor, Lincoln, NE) in each treatment to validate treatment levels (see Harvest sampling: A_{net}).

Nutrient treatments included two levels of nitrogen (+N:10 g m^{-2} y^{-1} and −N: 0) as a volumetric application rate, applied as ammonium nitrate in 100 mls every 2 weeks. To ensure that other nutrients were not limiting seedling growth, we added 100 mls of a 50% strength Hoagland's solution without nitrogen (Hoagland and Arnon 1950) to all pots midway through the experiment.

Harvest sampling: biomass

After 12 weeks of treatment, we destructively harvested the seedlings to determine nodule biomass, plant growth, and rates of SNF and photosynthesis. Over three consecutive days, we iteratively harvested three replicates from each of the eight treatments until all seedlings were harvested. We separated the aboveground system from the belowground system using shears and washed the plant biomass with tap water. Leaf and woody biomass were separated manually with shears, as were root and nodule biomass with shears and forceps, respectively. Nodules were excised with *ca.* 0.5 cm of root tissue remaining on either side of the attachment point. Nodules were stored in humidified jars briefly (15 min maximum) until activity could be assessed (described below). We dried all biomass at 65 °C for at least 14 days and weighed it to the nearest 0.001 g. We calculated biomass growth over the duration of the experiment by totaling end biomass and subtracting estimated initial biomass.

Harvest sampling: nitrogenase activity

To determine the nitrogen fixation rate of nodules, we used an acetylene reduction assay (ARA) method paired with $^{15}N_2$ incubations (Wurzburger et al. 2022). Immediately after harvest, a subset of nodules (mean of 38 mg of dried

nodule biomass) was placed into a sealed 250-mL glass jar with a rubber septum, and 10% of the headspace was replaced with acetylene (C_2H_2). We generated acetylene each day of the harvest by reacting calcium carbide with water and stored the gas in evacuated sampling bags. We sampled 10 mL of mixed headspace at the beginning of the incubation and again after 30 min, and stored samples in evacuated 20-mL glass vials. Using a gas chromatograph (Model 8610C, SRI Instruments, Torrance, California USA) with a flame-ionization detector, we determined ethylene (C_2H_4) production of each set of nodules by analyzing peaks against known standards. To correct for any potential ethylene contamination, we ran blank samples with acetylene and no nodules as well as nodules with no acetylene; we did not detect ethylene. On seven seedlings, we also performed paired $^{15}N_2$ incubations on a subset of nodules (Wurzburger et al. 2022). We replaced 10% of the jars' headspace with 99 atom % $^{15}N_2$ (Sigma Aldrich, Missouri, USA) and incubated nodules for 30 min. All nodules were dried at 60 ºC as above, and those associated with the paired $^{15}N_2$ incubations were ground to a fine powder and analyzed for $\delta^{15}N$ with an isotope ratio mass spectrometer at the University of Georgia Center for Applied Isotope Studies. We used unenriched nodules from each seedling to calculate the C_2H_4:N_2 conversion factor. The biomass of nodules used in acetylene reduction assays and $^{15}N_2$ incubations were mathematically added to the total nodule biomass. Because not all nodules had detectable nitrogenase activity, we categorized nodule biomass at the plant level into two groups: nodules, which included all seedlings with nodules, regardless of their activity, and active nodules, which only included seedlings that had nodules with detectable activity.

Harvest sampling: A_{net}

We conducted leaf gas exchange measurements (LI-6400-40, Li-Cor Biosciences, Lincoln, NE) in tandem with ARA incubations during harvest so that measurements could be paired. We measured net photosynthesis (A_{net}, µmol CO_2 m^{-2} leaf area s^{-1}) on the terminal leaflet of the fully extended leaf closest to the apical meristem. Leaf temperature was set to 23 °C, CO_2 in the sample cell was 435 µmol/mol, flow rate was 400 µmol s^{-1}, and relative humidity was between 68 and 75%. Light in the sample chamber was set to ambient PPFD prior to measurement and stayed constant during measurement. Average (SE) PPFD levels in the sample chamber for the light treatments were 47.9 (5.9), 70.4 (5.8), 75.8 (8.9), and 284.3 (19.8) mmol m^{-2} s^{-1} for the 10, 20, 40, and 100% transmittance treatments. It is notable that the weather during harvest days was overcast, and we supplemented light in the greenhouse with the high-pressure sodium lamps.

Statistical model structure

We analyzed data using generalized linear models with light as a continuous variable and nitrogen as a factor; we analyzed the main effects first with an interaction term, and if neither the main effects nor the interaction was significant, we removed the interaction. If an interaction was significant, we analyzed the separate slopes of the interactions using the R package *emmeans* (Lenth 2022). To deal with the prevalence of zeros for nodule biomass, active nodule biomass, and fixation, we created a presence/absence binomial variable which we analyzed using a logistic regression model. Then we selected all non-zero data and ran a generalized linear model as above. When appropriate, we transformed response variables using the TransformTukey function from package *rcompanion*, which conducts Tukey's ladders of power to select a power coefficient to meet normality assumptions (Mangiafico 2021), and we verified the normality of residuals. Statistically significant data were indicated by $\alpha \leq 0.05$. We analyzed all data in R statistical software version 4.2.1 (R Core Team 2022).

Results

Plant biomass growth

Overall, plant biomass growth components of black locust reflected a similar physiological response to both light and nitrogen after 12 weeks of treatment. We found that total plant biomass growth increased with increasing light ($p < 0.001$, $t = 3.58$), but only in the absence of nitrogen addition (light by nitrogen interaction $p < 0.05$, $t = -2.99$) (Online Resource 1). This trend was likely driven by the aboveground biomass growth, which had a similar positive response to light ($p < 0.01$, $t = 2.75$), and an interaction between light and nitrogen ($p < 0.05$, $t = -2.74$) (Fig. 1a, Online Resource 1). Plant belowground biomass growth increased with increasing light ($p < 0.001$, $t = 3.74$), and also displayed an interaction of light and nitrogen (light by nitrogen interaction $p < 0.001$, $t = -4.37$), where belowground biomass growth increased with increasing light in the −N treatment and slightly declined with light in the + N treatment (Fig. 1b, Online Resource 1). Seedling aboveground and belowground biomass at the end of the experiment had similar responses to those of growth (Online Resource 1). Thus, black locust growth was only positively affected by nitrogen addition at low levels of light, and the benefit of added nitrogen on biomass diminished with increasing light. Root to shoot biomass ratio declined with light ($p < 0.05$, $t = -2.74$), but was not affected by nitrogen ($p > 0.05$) (Fig. 1c, Online Resource 1).

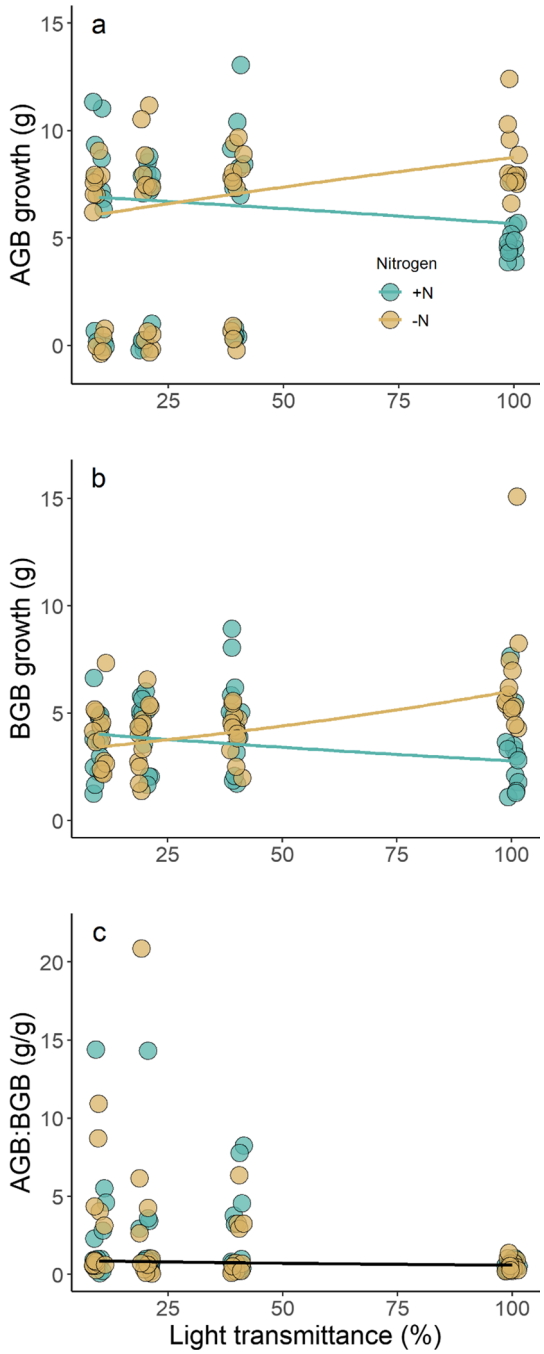

Fig. 1 Biomass growth of black locust seedlings over the 12-week experiment, for **a** aboveground biomass (AGB) growth (g), **b** belowground biomass (BGB) growth (g), and **c** root-to-shoot mass ratio (root:shoot). Points are individual seedlings, jittered for better visualization, and lines show model predictions of significant relationships ($\alpha \leq 0.05$). All predictions are back-transformed to be visualized at the original scale. In panel **c**, black line shows significant relationship with light only, and one value (light = 20%, root:shoot = 188) was excluded from the plot for better visualization

Nodule presence and biomass

The probability that a seedling had nodules was greater when nitrogen was not added ($p < 0.05$, $z = -2.02$) and with increasing light ($p < 0.05$, $z = 2.01$) (Fig. 2a, Online Resource 2), and nodule biomass increased with increasing light ($p < 0.05$, $t = 2.64$), but declined with nitrogen addition ($p < 0.05$, $t = -2.95$) (Fig. 2b, Online Resource 2). The probability that a seedling had active nodules increased with increasing light ($p = 0.013$, $z = 2.48$), but declined with nitrogen addition ($p < 0.001$, $z = -3.47$) (Fig. 2c, Online Resource 2), and active nodule biomass followed the same pattern for light ($p < 0.05$, $t = 2.39$) and nitrogen ($p < 0.05$, $t = -2.56$) (Fig. 2d, Online Resource 2). Therefore, nodule presence, nodule biomass and activity were sensitive to both light and nitrogen.

Symbiotic nitrogen fixation

In support of our hypothesis, black locust downregulated fixation in response to the addition of nitrogen. Despite the patterns of nodulation and active nodules under light and nitrogen treatments, nodule activity (ARA) was only affected by nitrogen and was greater in the −N treatment ($p < 0.05$, $t = -2.04$) (Fig. 2e, Online Resource 3). Because nitrogen fixation was only measured at the conclusion of the experiment, our study did not capture temporal differences in fixation among seedlings. We bolstered our instantaneous measures of nitrogen fixation with instantaneous measurements of A_{net} to understand how differences in seedling physiology contributed to SNF patterns across our treatments. Leaf net carbon assimilation (A_{net}), and ARA are both instantaneous rates measured on the same day. A_{net} was positively associated with our light treatments and increased with light, ranging from − 1.3 to 11.6 µmol CO_2 m^{-2} leaf area s^{-1} ($p < 0.0001$, $t = 10.67$), but was not affected by the addition of nitrogen (Online Resource 3). We found that nodule activity had a marginally significant interaction with A_{net} ($p = 0.06$, $t = -1.89$) (Fig. 2f, Online Resource 3), where nodule activity in the −N treatment increased more with A_{net} compared to the + N treatment.

Plant normalized variables of fixation and total fixation

To understand how plant investment in nitrogen fixation was regulated by light and nitrogen, we normalized nodule biomass and fixation to seedling biomass. Nodule investment (g nodules g^{-1} seedling biomass) declined with the addition of nitrogen ($p < 0.05$, $t = -2.66$), but not light (Fig. 3a, Online Resource 4). This contrasts with our finding that total nodule biomass was driven by both light and nitrogen. Further, fixation allocation (µmol C_2H_4 g^{-1} $seedling^{-1}$ h^{-1})

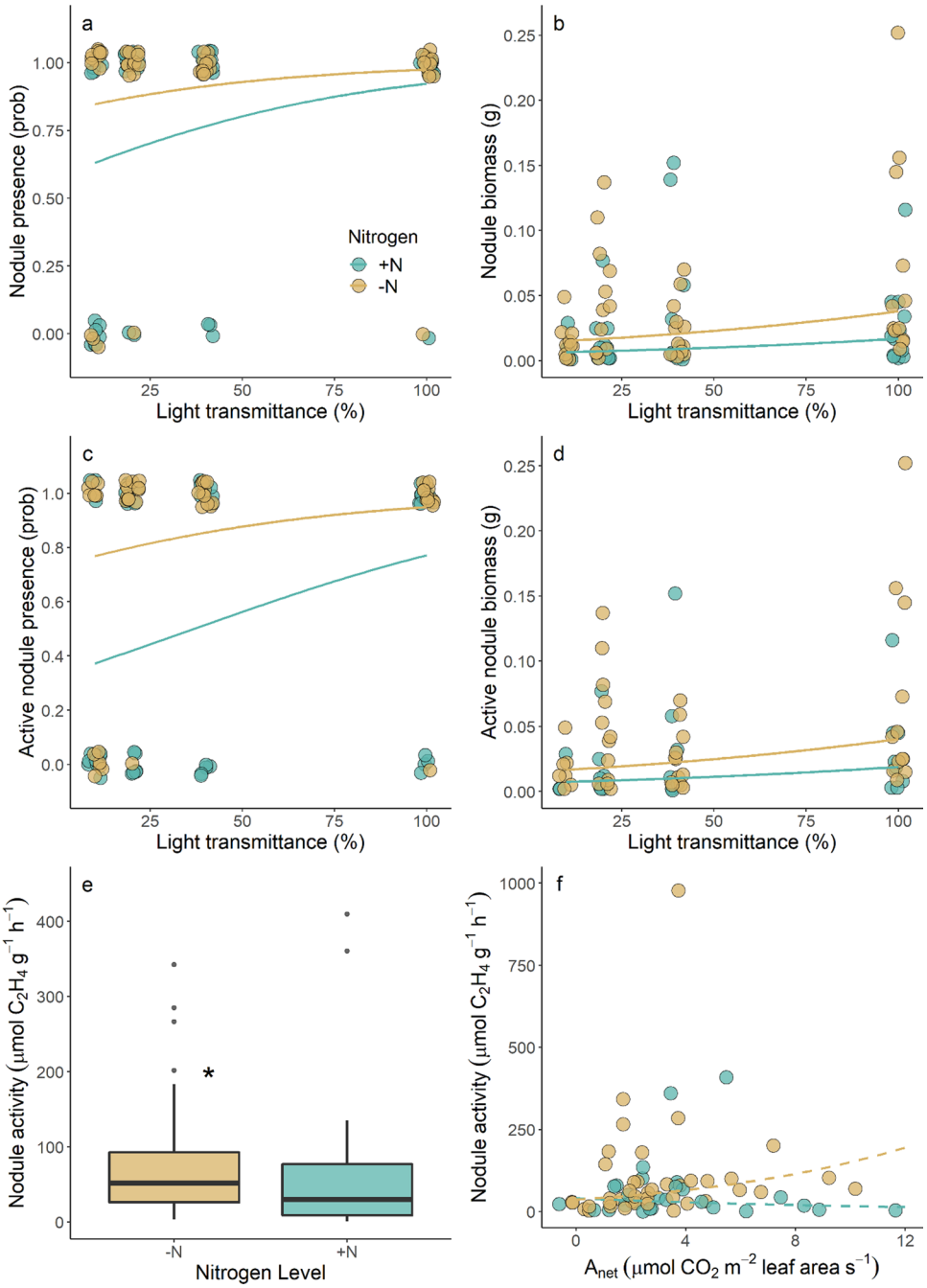

Fig. 2 Nodule presence, biomass, and activity for black locust seedlings, for **a** the probability of nodule presence, **b** nodule biomass (g), **c** probability of active nodule presence, **d** active nodule biomass (g), **e** nodule activity (µmol C_2H_4 fixed g^{-1} nodule h^{-1}), and **f** nodule activity (µmol C_2H_4 fixed g^{-1} nodule h^{-1}) related to A_{net} (µmol CO_2 m^{-2} leaf area s^{-1}). Points are individual seedlings, jittered for better visualization, solid lines show model predictions of significant relationships ($\alpha \leq 0.05$), and dashed lines show marginally significant relationships ($\alpha \leq 0.06$). All predictions are back-transformed, and data are untransformed to be visualized at the original scale. Boxplots show the median and upper and lower quartile range, points represent outliers, and asterisks denote significant differences between nitrogen treatments ($\alpha \leq 0.05$). One value of nodule activity (978) was excluded from panel e to better visualize the difference between treatments

declined under nitrogen addition ($p < 0.05$, $t = -3.83$) (Fig. 3b, Online Resource 4), which also contrasts with our finding that active nodule biomass responded to both light and nitrogen. We observed that total seedling fixation rate (µmol C_2H_4 h^{-1}) increased with increasing light ($p < 0.05$, $t = 2.44$), but declined with nitrogen addition ($p < 0.001$, $t = -4.17$) (Fig. 3c, Online Resource 4). Thus, we infer that light primarily affected fixation by increasing plant biomass, but only nitrogen regulated the relative investment that black locust made in nodulation and fixation.

Our C_2H_4:N_2 conversion factor from seven seedlings ranged from 1.66 to 139.68, with a mean value of 33 (Online Resource 5). The high end of the range was likely driven by high nodule activity values on a few seedlings. We infer that these extremely high rates may be attributed to the young age of our seedlings, and the mass-to-activity ratio of the small nodules when calculating rates. Indeed, black locust nodules are perennial and tend to have decreasing activity with age and size (Boring and Swank 1984b; Wurzburger et al. 2022). We then calculated conversion factors using

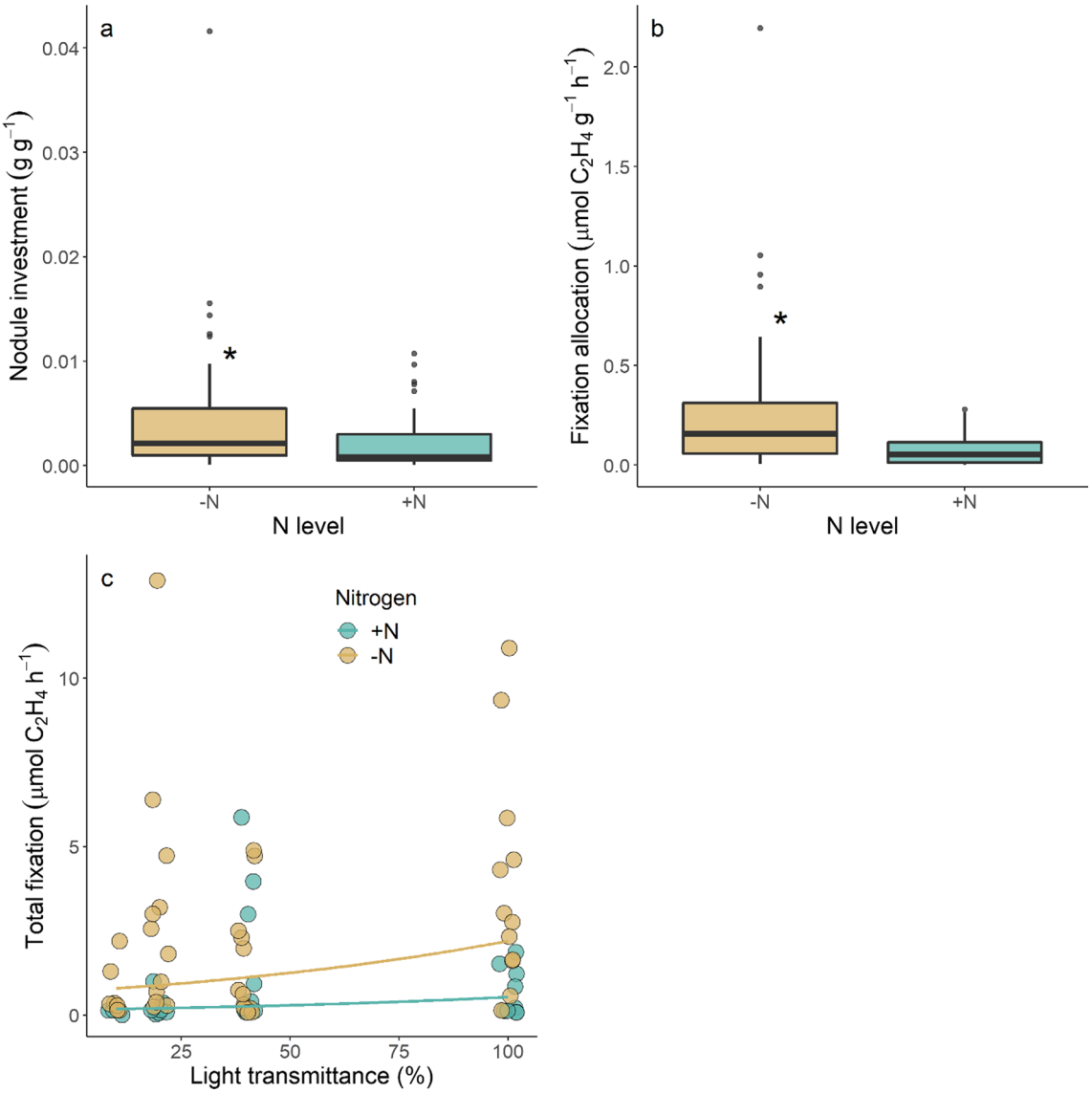

Fig. 3 Nodule investment, fixation allocation, and total nitrogen fixation rate of black locust seedlings, for **a** nodule investment (g nodule g^{-1} seedling), **b** fixation allocation (µmol C_2H_4 g^{-1} seedling h^{-1}), **c** total seedling nitrogen fixation (µmol C_2H_4 h^{-1}). Points are individual seedlings, jittered for better visualization, and lines show model predictions of significant relationships ($\alpha \leq 0.05$). Boxplots indicate the median and upper and lower quartile range, points represent outliers, and asterisks denote significant differences between nitrogen treatments ($\alpha \leq 0.05$). All predictions are back-transformed to be visualized at the original scale

mean nodule activity by treatment, which gave a mean of 10.3 (Online Resource 5).

Discussion

We investigated the fixation strategy of black locust by manipulating light and soil nitrogen availability and found that black locust was responsive to both resources. Of particular significance was our finding that only nitrogen addition reduced nodule biomass and fixation activity when normalized to seedling biomass. The sensitivity of black locust to nitrogen contrasts with the theoretical expectation that black locust has an obligate strategy, and our findings also reveal the additional physiological role of light on fixation.

Does black locust have a facultative strategy?

Despite theoretical evidence suggesting black locust would be obligate because it is a temperate forest nitrogen fixer (Sheffer et al. 2015), we found that the addition of nitrogen reduced both nodule investment and fixation allocation (Fig. 3a, b) while low light did not. Often, a nitrogen fixer's growth rate influences nitrogen demand and, therefore, rates

of fixation (Wurzburger and Hedin 2016), but because our seedlings reduced fixation rates on a per mass plant basis, it suggests that black locust can downregulate fixation like facultative fixers can, independent of the effect of nitrogen on plant size. Our findings bolster previous experimental evidence that black locust adjusts its fixation rate in response to the addition of nitrogen by lowering nodule investment and activity (Johnsen and Bongarten 1991; Wang et al. 2020). In our study, we also observed a few individuals to fully downregulate fixation by having no nodules (Fig. 2a), or no active nodules (Fig. 2c), and the probability of nodule presence decreased with nitrogen addition. With nitrogen addition, the probability of a plant producing active nodules ranged from 66 to 94%, from our lowest to highest light treatments. This finding suggests that nitrogen addition had a stronger negative effect on fixation under lower light, where seedlings tended to have smaller biomass and hence had a lower nitrogen demand, as has been observed with some tropical tree species (McCulloch and Porder 2021). Because nitrogen addition did not cause all replicates of black locust to downregulate fixation, it remains a possibility that our nitrogen addition levels did not fully alleviate seedling nitrogen demand. Therefore, our experiment may not be sufficient for definitively testing whether black locust has a completely facultative strategy. A study that investigates many levels of nitrogen from zero to fully saturating in high light conditions when nitrogen demand is highest would better test whether the strategy is completely facultative.

Fixation strategies for regulating SNF

Obligate and facultative strategies may represent only two of the potential strategies that nitrogen fixers use to regulate fixation (Menge et al. 2015). For example, some herbaceous legumes may only coarsely control SNF by excising nodule biomass. This may be an "intermediate" strategy of fixation between facultative and obligate, when nitrogen fixers cannot adjust both biomass and nitrogenase activity (Menge et al. 2015). In contrast, a tropical nitrogen-fixing tree and a Mediterranean shrub can suppress fixation by reducing nodule biomass and nitrogenase activity under high nitrogen addition, a phenomenon coined the "combined control of fixation" (Dovrat et al. 2018). Similarly, we found that black locust reduced its nodule investment and nitrogenase activity with the addition of nitrogen; however, some seedlings retained inactive nodules, which may indicate a further subdivision of the "combined control" strategy. Thus, it appears that black locust's control of fixation in response to available soil nitrogen begins with the initiation of nodule development, and proceeds to nodule maintenance or excision, to the activation of retained nodules, and lastly, to the magnitude of nitrogenase activity. Having hierarchical control on fixation may allow black locust to temporarily reduce carbon allocation to the symbiont while allowing for the possibility of reactivating nodules (Truchet and Dazzo 1982), which may be possible because of their indeterminate growth form (Patriarca et al. 2002). Such a strategy may allow black locust to increase its competitive ability in environments with temporal or spatial heterogeneity in soil available nitrogen.

Light effects on SNF

Unlike nitrogen, we found that light availability affected SNF by influencing seedling biomass, thereby resulting in a proportional change in nodule biomass across seedlings. We found that light had a significant positive effect on nodule presence, total nodule biomass, active nodule presence, active nodule biomass (Fig. 2b–d), and total seedling nitrogen fixation (Fig. 3c). However, when we normalized variables to seedling biomass, we found that light did not affect black locust's investment in nodules and allocation to nitrogen fixation (Fig. 3a, b). These findings are similar to those from a study manipulating light and nitrogen on tropical legumes (McCulloch and Porder 2021), where light had no effect on normalized metrics of nodule investment and total nitrogen fixation. However, our findings contrast with those from another tropical nitrogen-fixing tree species, where nodule investment increased with light and decreased with nitrogen addition (Taylor and Menge 2018). For black locust, the inability to adjust nitrogen fixation allocation as strongly under low light may explain patterns in this species' abundance in forests. Black locust frequently colonizes light gaps and recruits seedlings when there is sufficient canopy openness (Boring and Swank 1984a, b; Elliott et al. 2017), but it is rare under low light conditions. This may result from its inability to completely downregulate fixation under low light, which may reduce its competitive ability with non-fixing seedlings due to the tradeoff between carbon allocation to nitrogen fixation and growth (Menge et al. 2009).

In our study, while nodule activity (per unit nodule mass) was not influenced by light (Fig. 2e), it was correlated with instantaneous net photosynthesis (A_{net}) in the absence of nitrogen addition. This suggests an indirect effect of light on nodule activity where higher rates of carbon fixed via photosynthesis can be allocated to support nitrogen fixation. Similarly, in soybeans, Walsh et al. (1998) found a proportional decrease in the partitioning of photosynthate to the root system and nodules when photosynthesis declined with light, and Ursino et al. (1982) observed a positive correlation between leaf photosynthesis and nitrogen fixation rates. The source and sink dynamics between plant photosynthate and demands belowground likely explain the relationship we observed between instantaneous A_{net} and nitrogen fixation. Higher nodule activity combined with larger seedling

biomass under high light may explain the increase in total nitrogen fixation with increasing light (Fig. 3c).

Our study counters the theoretical expectation that temperate nitrogen fixers have an obligate fixation strategy. Black locust's strategy appears to be more similar to those of facultative fixers, which regulate SNF via nitrogenase activity and investment in nodule biomass in response to available soil nitrogen. However, light also had indirect effects on SNF via two pathways. First, light increased seedling growth, and hence, nitrogen demands, which resulted in proportional increases in nodule biomass. Second, light increased A_{net}, which then increased the activity of nodules in the absence of nitrogen addition. As a result of both, total plant nitrogen fixation was greater under high light. Perhaps most interestingly, nitrogen appeared to have stronger control over fixation than did light due to the persistence of nodulation and nodule activity in low light conditions, and the lack of response of nodule investment and fixation allocation to light. Because black locust is now distributed well outside its native range, it is critical to understand whether the patterns we observed in this study are consistent across diverse soil types and climates.

Our findings indicate that nitrogen fixation by black locust can be highly responsive to soil-available nitrogen and light, which suggests that ecosystem SNF rates may not always be directly proportional to the density of black locust biomass. While experimental manipulation of light and nitrogen on black locust of various ages would provide a more definitive assessment of its SNF strategy in forests, our results indicate the potential that black locust can maintain strong hierarchical control on SNF. These findings on the SNF strategy of black locust can help improve ecosystem predictions of ecosystem SNF under the changing light and nitrogen regimes of eastern deciduous forests.

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Author contribution statement SLO, CFM, and NW designed the study and contributed to greenhouse work. SLO conducted the laboratory analysis. SLO and NW analyzed the data. SLO wrote the article with contributions from NW and CFM.

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Data availability The datasets generated and analysed during the current study are available online through the Forest Service Research and Development Data Archive https://www.fs.usda.gov/rds/archive/.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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