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RESEARCH ARTICLE

A framework for scaling symbiotic nitrogen fixation using the most widespread nitrogen fixer in eastern deciduous forests of the United States

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Abstract

1. Carbon uptake by the terrestrial biosphere depends on supplies of new nitrogen (N) from symbiotic N fixation, but we lack a framework for scaling fixation accurately and for predicting its response to global change.
2. We scaled symbiotic N fixation from individual N fixers (i.e. plants that host N-fixing bacteria), by quantifying three key parameters—the abundance of N fixers, whether they are fixing N and their N fixation rates. We apply this framework to black locust, a widespread N-fixing tree in temperate forests of the eastern United States, and harness long-term data from southern Appalachian forests to scale fixation from trees to the landscape and over succession.
3. Symbiotic N fixation at the landscape scale peaked in the first decade following forest disturbance, and then declined. This pattern was due to the declining density and declining fixation rates of individual black locust trees over succession. Independent of forest succession, and coincident with chronic atmospheric N deposition, we have evidence suggesting that nodule biomass produced by black locust trees has declined by 83% over the last three decades. This difference in nodule biomass translates to a maximum fixation rate of 11 kg N ha^{-1} $year^{-1}$ and a landscape average of 1.5 kg N ha^{-1} $year^{-1}$ in contemporary forests.
4. *Synthesis*. We find key controls on symbiotic N fixation by black locust over space and time, suggesting lower fixation rates in eastern deciduous forests than previous estimates. Our scaling framework can be applied to other N fixers to aid predictions of symbiotic N fixation and ecosystem response to global change.

KEYWORDS
biogeochemistry, ecosystem ecology, global change, land use change, nitrogen deposition, nitrogen fixation, nutrient limitation, scaling

1 | INTRODUCTION

Carbon uptake by the terrestrial biosphere depends on the availability of N, making it critical to understand when, and how much, atmospheric N_2 enters terrestrial ecosystems through symbiotic N fixation (Levy-Varon et al., 2019; Meyerholt et al., 2020; Peng et al., 2020). At the core of this ecosystem process is a mutualistic symbiosis, where plants provide carbon to bacteria in exchange for the N they fix (Lambers et al., 2008). This direct supply of carbon to bacteria can fuel high N fixation rates, making symbiotic N fixation the most significant natural source of reactive N inputs to most ecosystems (Vitousek et al., 2013). Such a direct physiological relationship also makes symbiotic N fixation fully dependent on the host plant, or N fixer. As a result, the rate of symbiotic N fixation in an ecosystem depends on the abundance of N fixers, whether they are fixing N and their N fixation rates. In spite of this logic, symbiotic N fixation is rarely measured at the N fixer scale because of challenges with sampling nodule biomass (Barron et al., 2011), especially for large trees. In addition, symbiotic N fixation rates can be estimated from soil-based sampling (Winbourne et al., 2018), which does not require knowledge of individual N fixers. However, neglecting the N fixer limits our understanding of how fixation is governed, which prevents our ability to scale fixation accurately and predict fixation and ecosystem responses to global change.

Understanding N fixer presence and abundance across ecosystems would aid prediction, because symbiotic N fixation depends on the presence of an N fixer. N fixers are most abundant in early-successional tropical, temperate and boreal forests, when often N is limiting but light is abundant (Sheffer et al., 2015). While N fixers can persist into late succession in tropical forests (Gei et al., 2018), their abundance tends to decline with succession in temperate and boreal forests (Lu & Hedin, 2019). These patterns with succession and across biomes are driven by climate and disturbance effects on the N cycle, which determine whether the N-fixing or non-fixing strategy is more competitive amongst plants (Sheffer et al., 2015). However, we can poorly predict N fixer abundance across ecosystems. Not all early-successional forests contain N fixers, and those that do have widely varying densities (Menge et al., 2019). While the presence of N fixers depends, in part, on their geographic range distribution (Staccone, Liao, et al., 2020), local N fixer abundance depends on the seed bank, seedling recruitment and competition with non-fixers, which we poorly understand for many N fixer species.

N fixer presence alone does not guarantee symbiotic N fixation, however, making it necessary to understand when and where N fixers fix. In tropical forests, many N fixers appear to have a facultative strategy, meaning they can up- or downregulate the symbiosis based on soil N supply and plant N demand (Menge et al., 2009; Sheffer et al., 2015). For example, more N-fixing trees possess nodules in early succession versus late succession (Barron et al., 2011; Batterman et al., 2013), and, also, in tree-fall gaps versus interior, undisturbed mature forests (Barron et al., 2011; Wurzburger & Hedin, 2016). Yet, across mature tropical forests, nodule presence among N-fixing tress can range from 4% to 80% (Brookshire et al., 2019; Wong et al., 2020), indicating wide variation across sites and species. In temperate and boreal forests, theory predicts that N fixers have an obligate strategy, meaning that they hold fixation at a constant rate (Menge et al., 2009). But, because much of the empirical evidence for the fixation strategy of extra-tropical N fixers comes from studies of seedlings (Granhall et al., 1983; Johnsen & Bongarten, 1991; Röhm & Werner, 1991), and because manipulative studies on mature plants (Dovrat & Sheffer, 2019) are rare, we know little about ontogenetic effects on symbiotic fixation for relatively long-lived N-fixing trees. Thus, in spite of well-developed theory (Lu & Hedin, 2019; Menge et al., 2009; Sheffer et al., 2015), we still can poorly predict when and where N fixers fix.

For N fixers that are fixing, understanding the relationship between their fixation rate and growth is necessary for predicting symbiotic N fixation. In theoretical models, N fixers are most efficient when fixation combined with soil N uptake perfectly meet the plant's N demand for growth (Menge et al., 2009). In this case, N fixers adjust fixation via nodule biomass and nodule fixation activity, based on soil N availability, but only to the extent that light, water, phosphorus and other limiting resources can support their growth (Minucci et al., 2017; Ruess et al., 2013; Taylor & Menge, 2018; Wurzburger & Hedin, 2016; Wurzburger & Miniat, 2014). But whether fixation rates fulfil plant N demands depends on fixation strategy. Facultative N fixers can adjust fixation rates to account for deficits in available soil N, making them sensitive to the disturbance dynamics that drive the local N cycle (Sheffer et al., 2015). For example, the nodule biomass of tropical N fixers can decline over forest succession, as available soil N increases (Barron et al., 2011; Batterman et al., 2013). But such a relationship may not hold for obligate N fixers that support constant rates of fixation, or for N fixers with alternative strategies (Menge et al., 2015) that may under-fix relative to their N demand. Without an understanding of how N fixers regulate fixation, it remains tenuous to predict rates within and across ecosystems.

The parameters of symbiotic N fixation—the abundance of N fixers, whether they are fixing N and their N fixation rates—can illuminate the ecological processes that control fixation, and thus aid prediction (Figure 1). Seedbank size and seedling demography can determine differences in local N fixer recruitment immediately following disturbance (Figure 1a, left box), and mortality due to competition, herbivory or disease can reduce N fixer abundance over time (Figure 1a; Batterman et al., 2013; Liao & Menge, 2016; Menge et al., 2009; Ruess et al., 2009; Taylor et al., 2019). Increasing decomposition and N mineralization rates that lead to greater soil N availability may cause facultative N fixers to fully downregulate fixation (Figure 1b) or reduce their investment in nodule biomass and/or nodule fixation activity (Barron et al., 2011; Figure 1c). Ultimately, the ecosystem rate of symbiotic N fixation (Figure 1d) depends on these parameters of fixation. But, future predictions of these ecosystems depend on an understanding of the ecological processes that govern symbiotic N fixation, and how they respond to human disturbances (Peng et al., 2020). For example, elevated CO_2 may induce N limitation on ecosystem productivity, but whether fixation increases in response depends on what controls the abundance of N fixers, whether N fixers can upregulate fixation, and the relationship between fixation and growth. Thus, making accurate predictions of symbiotic N fixation at the global scale depends on the knowledge of how fixation is regulated at the N fixer scale.

A number of methods exist for quantifying symbiotic N fixation (Bormann & DeBell, 1981; Danso et al., 1995; Winbourne et al., 2018), but most of them either partially or fully circumvent studying the N fixer directly. One popular method that requires no knowledge of N fixer abundance or location is a soil-based

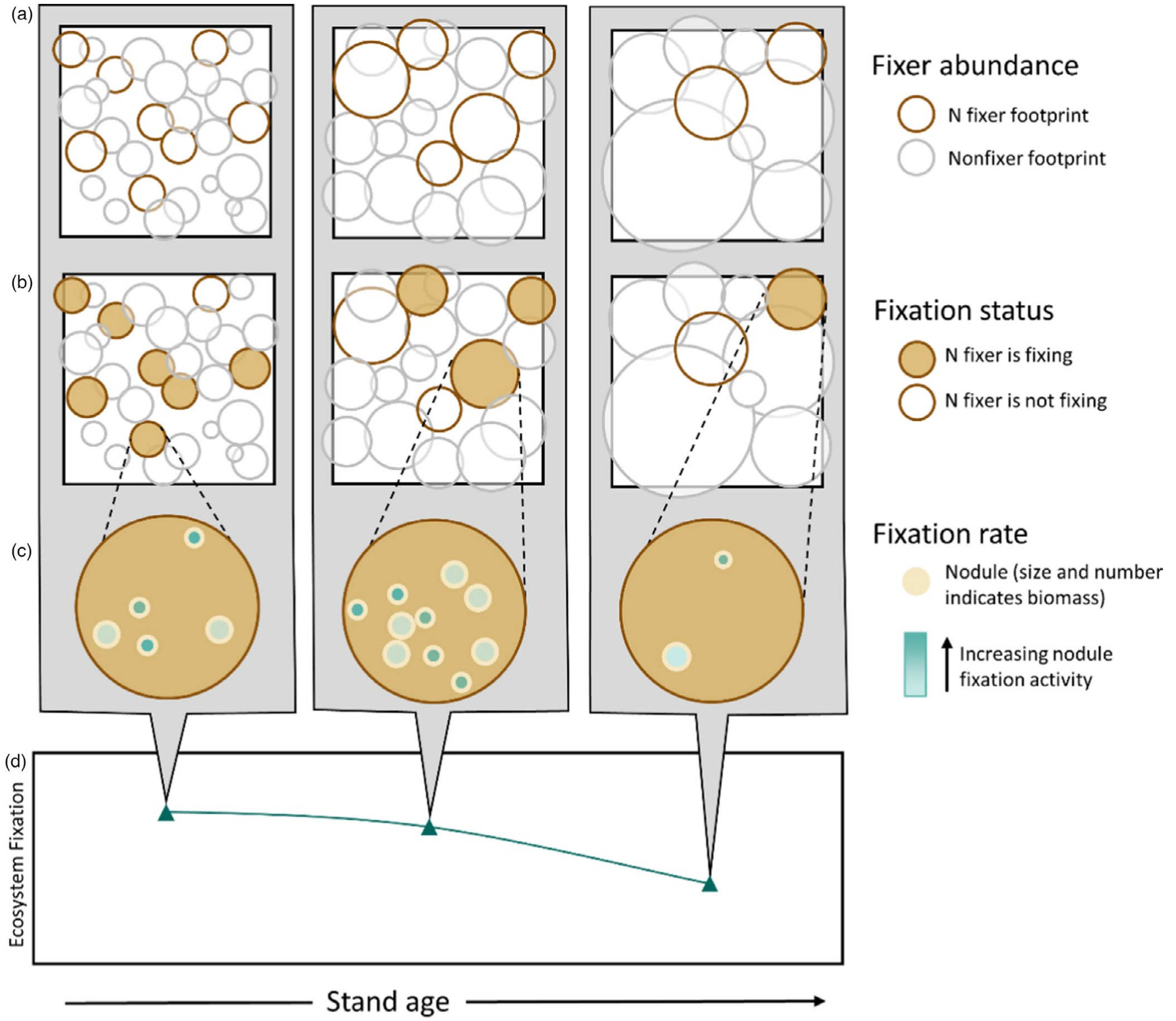

FIGURE 1 Conceptual example of the parameters of symbiotic N fixation in a forest ecosystem over stand age: (a) the abundance and size of N fixers relative to non-fixers, (b) the fraction of N fixers actively fixing and (c) the rate at which active N fixers are fixing, which reflects both nodule biomass and nodule fixation activity, can all vary over stand age. These parameters collectively give rise to (d) the rate of symbiotic N fixation in the ecosystem. In this example, symbiotic N fixation declines with stand age because of a decline in N fixer abundance and the fraction that are fixing N, even though fixation rate of active N fixers is highest in mid-aged stands. Circles in panels a and b represent the footprint, or the rooting zone, of each tree

approach, which provides area-based estimate of symbiotic N fixation (Winbourne et al., 2018). However, the intensive soil sampling required by this approach is rarely implemented by most studies (Winbourne et al., 2018). And because this approach does not consider the N fixer, it is not ideal for extending predictions outside the sampling area where N fixer abundance might differ. In contrast, methods that study the N fixer directly often rely on containerized seedlings (Johnsen & Bongarten, 1991; Röhm & Werner, 1991; but see Dovrat & Sheffer, 2019), making it unrealistic to extend fixation rates to the field where N fixers might change their fixation rate with ontogeny. Of the few studies that have quantified nodule presence, abundance and activity at the individual scale of N-fixing trees (Barron et al., 2011; Batterman et al., 2013; Taylor et al., 2019; Wurzburger & Hedin, 2016), even fewer attempt to apply fixation parameters to scale up rates to the ecosystem and these efforts are challenged by how to account for uncertainty appropriately.

Here, we present a framework for understanding and scaling the process of symbiotic N fixation. We quantify the parameters of symbiotic N fixation to scale from individual N fixers to the landscape and over succession using black locust *Robinia pseudoacacia* L., a temperate leguminous tree. Black locust is the most abundant N-fixing tree in eastern forests of the United States, and the most frequent N fixer in the coterminous United States (Staccone, Liao, et al., 2020). It can fix N at high rates (Boring & Swank, 1984a), locally increase the rates of soil N cycling and affect the growth of non-fixing trees (Minucci et al., 2019; Staccone, Kou-Giesbrecht, et al., 2020). However, we have limited fixation data for black locust in natural forest conditions. Thus, efforts to scale fixation rates of eastern forests and predict their response to global change are highly uncertain.

We illustrate how this framework for symbiotic N fixation can lead to ecosystem prediction by scaling fixation rates from individual N fixers to forest stands over succession, by quantifying: (a) tree fixation rates, and (b) tree biomass growth and the contribution of fixation to net N demand over tree age. In our study, and commonly in nature, tree age is approximately stand age, since black locust is often recruited following a stand-replacing disturbance; thus, we apply our framework to infer how fixation changes over both tree and stand age. We then (c) parameterize a simulation model of fixation rates for individual N fixers over tree age, and (d) estimate ecosystem fixation using point-density plots, selected for their high density of black locust, and landscape-density plots, which were stratified across the landscape, each representing over three decades of forest recovery. For both tree and landscape simulations, we have the unique opportunity to compare fixation estimates derived from our nodule data to those collected in the same location, but 37 years earlier. From this, we demonstrate dynamics in symbiotic N fixation at multiple scales, and that by accounting for the uncertainty in fixation parameters, black locust has a lower potential to fix N than suggested by previous estimates. Most importantly, our framework offers insight into how fixation is regulated, which is necessary for predicting responses to global change.

2 | MATERIALS AND METHODS

2.1 | Site description

All forest stands were located in western North Carolina, United States. These are mixed deciduous forests of the southern Appalachians that have a large component of oak *Quercus* spp., maple *Acer* spp. and tulip poplar *Liriodendron tulipifera* L. Mean annual temperature is 12.6°C and mean annual precipitation is 1,800 mm (Swift et al., 1988). Soils are deep sandy loams underlain by folded schist and gneiss (Thomas, 1996). Black locust is native to this region, is a disturbance-responding species that grows rapidly in high light, and thus, its age typically corresponds to the time of the last major disturbance (Boring & Swank, 1984b).

2.2 | Field sampling

To quantify fixation at the N fixer level over tree age, we sampled individuals of black locust from 11 forest stands in the Nantahala National Forest that ranged in time since disturbance from 3 to 75 years. Four of these stands were also located in the Coweeta Hydrologic Lab, in watersheds (WS) 7, 6, 28 and 3. Three of these (WS7, WS28 and WS3) were sampled for nodules in 1981 (Boring & Swank, 1984b). In each stand, we sought to sample black locust trees responding to the same prior disturbance. In recently cut stands (≤25 years), we selected trees in areas where tree cutting was uniform and individual black locust was similar in size. Older stands (>25 years) were recovering from clear-cut or agriculture, making it likely that all trees recruited shortly after the logging disturbance or agricultural abandonment. To verify our assumption, we sampled tree cores from the 41-year-old stand and the 75-year-old stand, and we confirmed that tree age at breast height was within 10 years of stand age.

2.3 | Sampling monoliths

At each stand, we quantified symbiotic nitrogen fixation on 7–20 black locust trees during the summer and fall of 2017 and 2018 (n = 118 trees). For each tree, we excavated at least five randomly distributed 20 cm × 20 cm × 25 cm (deep) soil volume monoliths within a 1-m radius of small trees (<10 cm diameter at 1.3 m above ground height, dbh) and within a 2-m radius for larger trees (>10 cm dbh). We increased the sampling to as many as nine monoliths for trees where no or few nodules were found. Although we did not systematically quantify nodule biomass with depth increments, we rarely observed nodules in the lower 15 cm of our monoliths. Our approach of sampling nodules within a radius from each tree was based on evidence that the maximum rooting extent of plants, and therefore the zone of potential nodulation, scales with canopy volume (Casper et al., 2003). Sampled monoliths represented an average of 5% of the sampling area—the area within the defined radius—for each tree, which targeted the area where the tree's nodules were concentrated, based on additional tests. We note that a sampling intensity of 5% is an order of magnitude higher than previous tree-based sampling efforts (Batterman et al., 2013; Taylor et al., 2019; Wurzburger & Hedin, 2016). Nodule biomass sampled from monoliths was scaled to the individual tree.

2.4 | Testing assumptions of nodule sampling

We tested our assumption that nodule biomass is concentrated near the base of trees in two ways. First, we extended nodule sampling to 2 m from the stem on three trees between 6 and 10 cm dbh, and out to 5 m from the stem on three trees >40 years old and between 16 and 40 cm dbh, while sampling at the same intensity (5%). We observed nodules on all six trees within the sampling radius. To quantify how nodule biomass declines with increasing distance from the tree, we fit models using nonlinear least squares, and selected the model structure with the lowest $\mathrm{AIC_c}$ value. Our model predicted that 92% (95% CI: 83%–100%) of the sampled nodule biomass occurred within our sampling radius (Figure S1), and it declined rapidly with increasing distance. Second, we also tested the difference in sampling nodules using both a tree-based (our study) versus a plot-based (Boring & Swank, 1984b) approach at four stands. We found that sampling four randomly located 0.05 m^2 cores in a 10 × 10 m plot captured only 72% (21%; *SE*) of nodule biomass of that detected at the tree level, for trees in the same plot.

2.5 | Nodule activity

All nodules were harvested from each monolith, quantified for acetylene reduction activity (ARA) and a subset was incubated with $^{15}N_2$ in parallel. We generated acetylene by reacting calcium carbide with water, and stored the gas in sampling bags for each day's use. To minimize the pitfalls of ARA assays (Freschet et al., 2021), nodules were carefully excavated within 15 min of sampling monoliths with at least 1 cm of root tissue attached and were placed in 250-ml gas-tight chambers with 10% of the headspace replaced with acetylene. Headspace from the chambers was sampled twice over 30 min and the concentration of ethylene was analysed on a gas chromatograph. To correct for potential contamination of ethylene, we conducted incubations with acetylene and no nodules, and with nodules and no acetylene, and we never detected ethylene. Incubations contained several nodules, with an average of 70-mg (dried) nodule biomass, and we ran duplicate or triplicate incubations (when possible) from each tree to determine a mean rate. In total, we conducted 120 incubations representing 55 trees. To convert the rate of acetylene reduced to N_2 fixed (C_2H_4:N_2 conversion factor), we conducted incubations with 10% headspace replaced with 99 atom% $^{15}N_2$ on nodule subsamples from the same tree. We conducted 14 $^{15}N_2$ incubations in parallel with ARA assays, representing 11 individual trees. All nodules were cleaned with DI water and paintbrushes, dried at 60°C for 48 hr and measured for biomass. We used the paired ARA nodule samples associated with each $^{15}N_2$ incubation to serve as the unenriched control, and ground all tissues to a fine powder, and for quantified $\delta^{15}N$ with isotopic ratio mass spectrometry.

2.6 | Modelling tree-based fixation

To construct a simulation model for estimating fixation at the N fixer level, we first determined the age relationship for nodule presence, nodule mass and nodule activity and fit models to estimate model coefficients and their associated error. We fit a logistic regression model to predict nodule presence or absence given tree age with a binomial distribution (*n* = 118). For nodule biomass (*n* = 68) and ARA (*n* = 55), we fit models using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1). We then used maximum likelihood estimation to assign a gamma-distributed error to these models (Bolker, 2020) and calculated 95% confidence intervals on model coefficients (Ripley, 2020). We found no age relationship with ARA, but activity did decline with increasing nodule biomass per tree (Table S1). Because older trees had fewer nodules, we could not resolve the relationship of C_2H_4:N_2 conversion factor with tree age. We fit a gamma distribution to our C_2H_4:N_2 conversion factor data (*n* = 14; Figure 2d).

We then constructed a Monte Carlo simulation model applying model coefficients and errors, or fitted distributions of the parameters of fixation. Because we used a combination of two approaches to quantify nodule activity—ARA and our empirically determined C_2H_4:N_2 conversion factor—we included both of these as parameters in our simulation model. For each tree in a given year, the model sampled from the binomial distribution to assign nodule presence or absence. If a given tree was assigned no nodules in a given year, its fixation rate was assigned as 0. For trees with nodules present at

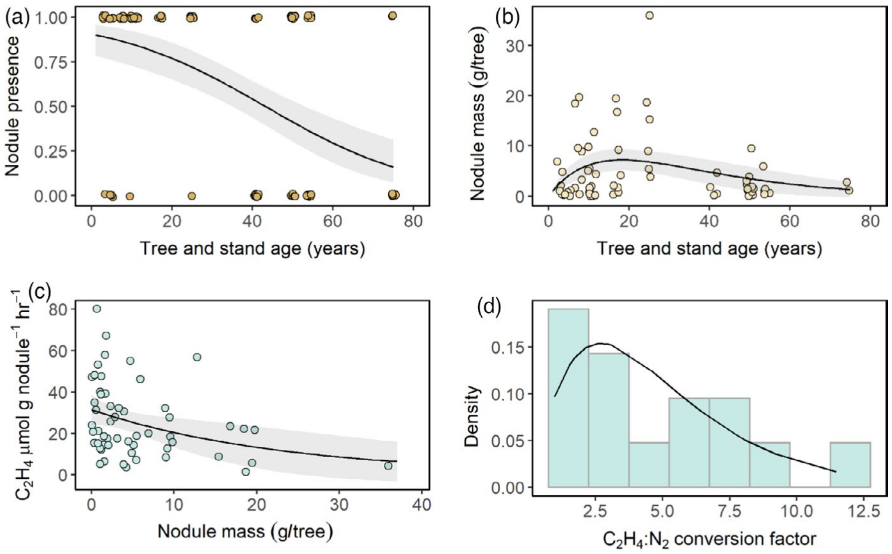

FIGURE 2 Fixation parameters of black locust: (a) The probability that trees have nodules declined with tree age, (b) nodule biomass per tree peaked at 18 years and then declined, (c) acetylene reduction activity, which declined with increasing nodule mass produced per tree and (d) the C_2H_4:N_2 conversion factor was gamma-distributed. Each point represents a tree, points are jittered in panels (a) and (b) for better visualization. Panels (a)–(c) show model fits with 95% confidence intervals (Tables S2 and S3). Panel (d) shows a histogram and density distribution

a given age, the model then sampled from the distribution of nodule biomass specific to the age of the tree. Because ARA did not vary with tree age, but did vary with total tree nodule biomass, the ARA of nodules was then estimated by sampling the distribution of ARA for the given value of nodule biomass. Last, the model sampled from our C_2H_4:N_2 conversion factor distribution to estimate N fixed (g tree^{-1} year^{-1}). We performed 100,000 iterations on the simulation model over 75 years of tree age, and fit the simulated data with a generalized additive model with a cubic regression spline (Table S4). Because our dataset represents our population, and we performed numerous iterations, our sample means estimate our population means.

2.7 | N fixer variables

To understand the relationship between N fixation and growth-related N demands, we estimated above-ground biomass (AGB, kg) from each tree's diameter (*n* = 118) using an allometric equation developed from the age and diameter (dbh, cm) of trees with similar diameters to those in our study (Scott et al., 2021):

$$\log_{10}(\text{AGB}) = 2.526 \times \log_{10}(\text{dbh}) - 1.067. \quad (1)$$

To estimate above-ground biomass growth, we returned to most trees (*n* = 91) 2 years later for a second measure of dbh. We then fit models to our biomass and growth data as a function of tree and stand age using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1).

For trees where we detected both N fixation and growth (*n* = 52), we calculated the fraction of net N demand supplied by fixation. Net above-ground N demand is the N associated with net biomass (foliage, branch or stem) gains. This calculation differs from total, or gross, above-ground N demand, which also includes the N needed to replace the foliar biomass pool, which turns over annually for deciduous tree species. We first quantified each tree's N fixation rate by multiplying nodule biomass by ARA, and dividing by the median value of the C_2H_4:N_2 conversion factor, assuming that nodules of black locust are active 6 months a year (Boring & Swank, 1984a). If nodule biomass was present but not sufficient to conduct an ARA, we applied a site-level ARA mean to those trees. To estimate net N demand associated with biomass growth, we first determined the relationship between %N of above-ground biomass and tree age from whole-tree harvests of black locust with foliage, branch and stem biomass proportions and their associated tissue nutrient concentrations (Elliott et al., 2002; Scott et al., 2021). We fit these data using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1; Figure S2). We applied these %N model predictions to estimate the N demand associated with biomass growth, and then determined the fraction of this N that was supplied by fixation. We fit models as a function of tree age using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1).

2.8 | Comparison of nodule biomass

We had the opportunity to compare our contemporary nodule biomass data (sampled across 2017–2018; hereafter 2017) with that collected in the same location, but 37 years earlier (sampled across 1980–1981; hereafter 1981; Boring & Swank, 1984b), and apply those nodule biomass coefficients to our simulation model. In both cases, sampling was conducted over different seasons and years, thereby capturing natural, short-term variability in biomass. Because Boring and Swank (1984b) sampled nodules from cores at the plot level, we normalized nodule biomass data from 1981 by the number of black locust trees in each of their plots (g/tree). As above, we fit models using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1) and used maximum likelihood estimation to assign a gamma-distributed error. We then compared estimates of fixation using two simulation models: one parameterized with 2017 nodule biomass coefficients and one parameterized with 1981 nodule biomass coefficients. We took 100,000 iterations over a tree age of 41 years; Boring and Swank's nodule data (Boring & Swank, 1984b) extend to 38 years, and our closest aged stand was 41. We fit the simulated data with a generalized additive model with a cubic regression spline (Table S1).

2.9 | Scaling fixation from trees to forests over time

We applied our simulation model to scale fixation rates to the landscape and over succession using tree density data from point-density plots or landscape-density plots of black locust over three decades of forest succession. First, we used a stand age gradient (ages 4, 17, 38 years) from Boring and Swank (1984b), where eight 100-m^2 plots were purposefully selected as points on the landscape due to their high density of black locust (henceforth point-density plots). These plots capture the highest potential rate of fixation. Second, we used density data from plots randomly stratified across a watershed and measured over 31 years of forest recovery following a forest clear-cut (WS7, henceforth landscape-density stands). These plots capture the natural variability of black locust density following a stand-replacing disturbance, including plots where it was absent. Therefore, the comparison between these plot types is one between points on the landscape versus the landscape average. These data represent six surveys of twenty-four 800 m^2 plots. In both cases, all black locust stems ≥2.5 cm dbh were measured and recorded (Boring et al., 2014; Boring & Swank, 1984b; Elliott et al., 1997). To predict stem density of black locust per year, we fit models using nonlinear least squares and selected the model structure resulting in the lowest AIC_c value (Table S1). The simulation model first took a random draw from stem density distribution for each year, which assigned the number of stems and thus, determined the number of draws from our parameters of fixation (nodule presence, nodule biomass, nodule activity). We summed tree level fixation for each plot per year and repeated the process for 1,000 iterations. We compared estimates from simulations parameterized by 1981 or 2017 nodule data and fit the simulated data with a generalized additive model with cubic regression spline.

We conducted uncertainty and sensitivity analyses on our models of fixation, comparing the two tree density datasets. To determine the effect of uncertainty on fixation estimates, we quantified the relative change in fixation using the upper and lower bound of 95% confidence intervals for each parameter coefficient, while holding others at their predicted value. For the conversion factor, we used the percentile method to calculate the interval on the median value of the gamma distribution. We then calculated the maximum and minimum % change in the fixation estimate for each fixation parameter over time. To determine the sensitivity of fixation estimates to changes in fixation parameters, we individually increased and decreased all parameter coefficients by 10%, while holding all others at their predicted value. We then calculated the maximum and minimum % change in the fixation estimate for each fixation parameter over time. All analyses were conducted in R statistical software version 4.0.2 (R Core Team, 2020).

3 | RESULTS

Our analyses revealed that most parameters of symbiotic fixation were highest in young trees of black locust. We found that the probability that trees possessed nodules declined rapidly with tree age, from 90% to 16% over 75 years (Figure 2a; Tables S2 and S3). Nodule biomass peaked at ~7 g/tree (95% CI: 5.1–9.3) at 18 years and then declined (Figure 2b; Tables S2 and S3). The best model did not change after removing the largest value of nodule biomass. Acetylene reduction activity was unrelated to tree age, but declined with increasing tree nodule biomass (Figure 2c; Tables S2 and S3). Trees with greater nodule biomass tended to have larger individual nodules, which may have lower mass-specific activity (Boring & Swank, 1984a).

The dynamics of fixation parameters over tree age were related to black locust biomass and growth. Tree above-ground biomass increased with tree age (Figure 3a; Tables S2 and S4), but biomass growth rate stabilized within 20 years after increasing initially (Figure 3b; Tables S2 and S4). We then calculated the N demand associated with net biomass growth, after accounting for declines in biomass N concentration with tree age (Tables S2 and S4; Figure S2). We found that fixation could supply as much as 23% (95% CI: 10%–36%) of a tree's above-ground net N demand, and this percentage declined as trees aged (Figure 3c; Tables S2 and S4).

Applying our simulation model to estimate fixation over tree age, we found an increasing rate in young trees, reaching a maximum rate of 4.7 g N year^{-1} tree^{-1} at 14 years (Figure 4b; Tables S2 and S4). This is similar to the above-ground biomass growth pattern in the first two decades following disturbance, suggesting a relationship between N demands and fixation rate during this time. However, individual tree fixation rates declined sharply with tree age, to below 1 g/year by 55 years post disturbance, while above-ground biomass growth rates remained high (Figure 3b), suggesting that older trees increasingly rely on soil N to meet biomass growth demands.

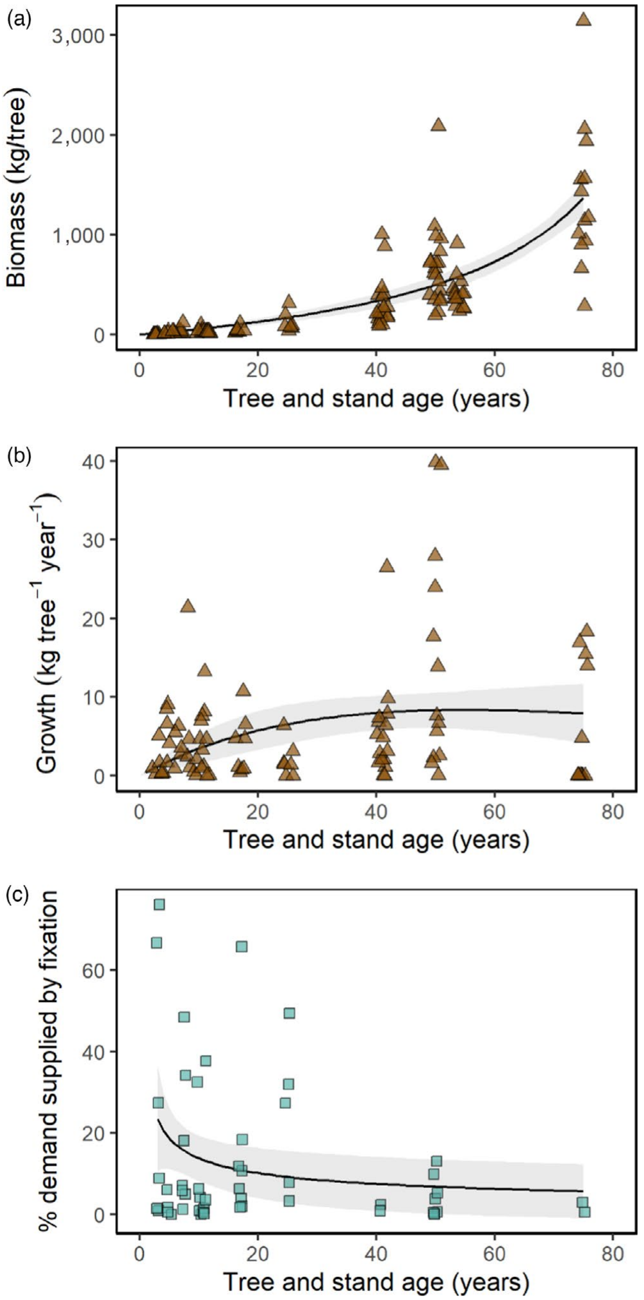

FIGURE 3 Variables associated with the N fixer, black locust and how they vary over tree and stand age, including: (a) above-ground biomass, which increased with age, (b) above-ground biomass growth rates, which stabilized by ~20 years and (c) the percent of N demand supplied by fixation, which declined with age. Each point represents a tree, and points are jittered for better visualization. Lines represent model fits with 95% confidence intervals (Tables S2 and S4)

We then estimated differences in individual tree fixation resulting from the 1981 and 2017 nodule biomass sampling. Maximum predicted nodule biomass in 2017 was 83% lower than it was in 1981 (Figure 4a; Tables S2 and S3). Using our contemporary nodule activity data—ARA and our empirically determined C_2H_4:N_2 conversion

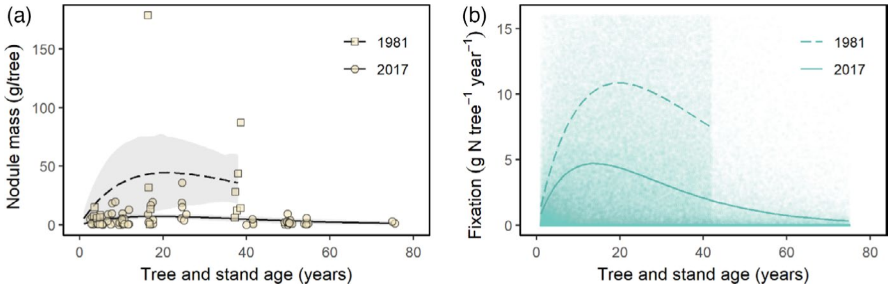

FIGURE 4 Estimates of symbiotic fixation by individual trees of black locust parameterized by two datasets of nodule biomass, where (a) nodule biomass per tree from 1981 (Boring & Swank, 1984b) or 2017 (this study), and (b) fixation estimates per tree from 100k iterations of a simulation model constrained by fixation parameters: nodule presence, nodule mass and nodule activity, over 41 or 75 years of tree and stand age. In panel (a), points represent plot data normalized to an individual tree basis (1981) or individual tree data (2017; this study) and lines are nonlinear least squares model fits with 95% confidence intervals. In panel (b), points are simulated data and lines are fits from generalized additive models (Tables S2–S4); some simulated data exceeded the *y*-axis limit

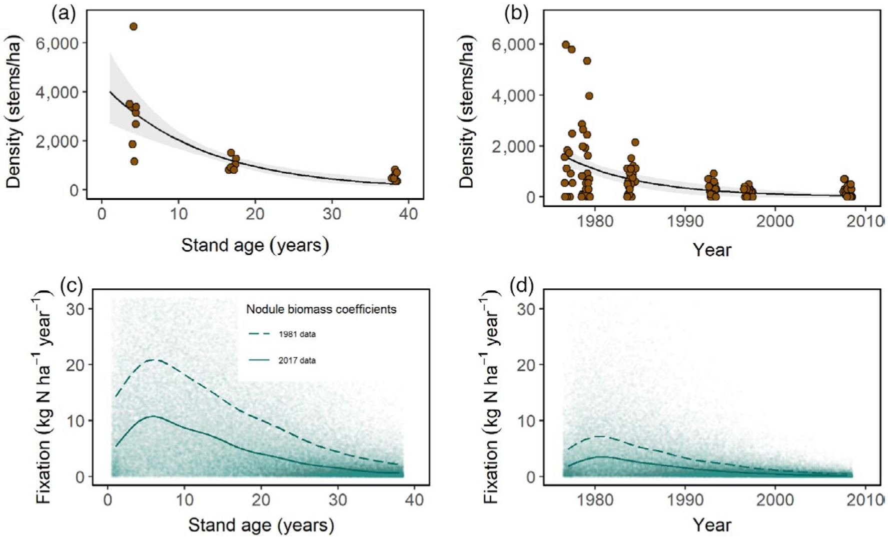

FIGURE 5 Black locust stem density and resulting landscape estimates of symbiotic N fixation over forest succession, including stem density data from (a) point-density plots (Boring & Swank, 1984b) or (b) landscape-density plots, and estimates of fixation using (c) point-density plots or (d) landscape-density plots. Fixation estimates generated from 1,000 interactions of simulation models parameterized by nodule biomass data from 1981 (Boring & Swank, 1984b) or 2017 (this study). In panels (a) and (b), points represent individual plots, jittered for better visualization and lines are nonlinear least squares model fits with 95% confidence intervals. In panels (c) and (d), points are simulated data and lines are fits from generalized additive models (Tables S2–S4); some simulated data exceed the *y*-axis limit

factor—the higher nodule biomass in 1981 translated to a predicted maximum rate of 10.8 g N tree^{-1} year^{-1} (Figure 4b; Tables S2 and S4), suggesting a 55% decline in maximum individual-tree fixation between 1981 and 2017 due to a reduction in nodule biomass. We acknowledge the possibility that nodule activity also differed between 1981 and 2017. Although we do not have ARA data from multiple tree and stand ages from 1981, ARA rates from a 4-year-old stand sampled in 1981 are similar to those that we sampled in 2017 (Boring & Swank, 1984a).

We scaled symbiotic fixation to the landscape and over succession by applying black locust densities from point-density and landscape-density plots to our simulation model (Figure 5a,b; Tables S2 and S4). Here, fixation estimates from point-density plots represent points on the landscape with highest density of black locust, while those from landscape-density plots represent the landscape average. We found that point-density plots had a maximum and mean (over 38 years) symbiotic N fixation rate of 22 and 11 kg ha^{-1} year^{-1} using 1981 nodule coefficients or a maximum and mean rate of 11 and 4.8 kg ha^{-1} year^{-1} using 2017 nodule coefficients (Figure 5c; Tables S2 and S4). In contrast, we found much lower estimates of symbiotic N fixation in the landscape-density plots, where maximum and mean fixation rates were 7.2 and 3.2 kg ha^{-1} year^{-1} or 3.5 and 1.5 kg ha^{-1} year^{-1} using 1981 or 2017 nodule coefficients respectively (Figure 5d; Tables S2 and S4). Thus, using landscape-density plots, which more realistically capture black locust densities at a landscape scale, lowered our estimates of landscape fixation by 65%–70%.

Uncertainty in tree density had the largest relative effect on modelled estimates of fixation, with as much as a 369% relative change for the landscape-density plots, and as much as 200% for the point-density plots. For both, the greatest relative effects were in stands older than 20 years (Figure S3a). We then conducted a sensitivity analysis of our model and found that landscape fixation was most sensitive to changes in the parameter of tree density. Changing the parameter coefficients of tree density by ±10% resulted in as much as a 43% change in landscape fixation in older stands (Figure S3b).

4 | DISCUSSION

Future rates of carbon uptake by the terrestrial biosphere will depend on symbiotic N fixation (Meyerholt et al., 2020), but we can poorly predict the spatial extent and magnitude of these N inputs. Using black locust, a common and widespread N-fixing tree in eastern forests of the United States, we present a new framework for scaling fixation from trees to the landscape. We found that black locust density gradually declined over four decades following disturbance, while individual tree fixation rates peaked within the second decade following disturbance. Together, these dynamics of tree density and tree fixation rate resulted in a peak in ecosystem symbiotic N fixation within the first decade following disturbance and a gradual decline over time. Independent of this pattern over succession, we also found evidence that black locust reduced its individual tree fixation rate over the last three decades, a period coinciding with dramatic, human-induced changes to the N cycle (Gruber & Galloway, 2008). Because we find that fixation is dynamic on multiple temporal and spatial scales, using a framework based on individual N fixers is necessary for accurately predicting the fixation and ecosystem response of eastern forests to global change.

We found that black locust trees reduced their fixation rate over tree and stand age (Figure 4b), reflecting the decline in both nodule presence (Figure 2a) and biomass (Figure 2b) with age. The decline in nodule presence is counter to the theoretical prediction that temperate forest N fixers have an obligate strategy (Menge et al., 2009; Sheffer et al., 2015). Such a response suggests that black locust is facultative, and downregulates fixation in response to increasing available soil N over succession (Boring & Swank, 1984b). While black locust's fixation strategy has yet to be rigorously tested, it is in the Leguminosae family and phylogenetically related to tropical N fixers (Pennington et al., 2009) that can downregulate fixation in response to available N (Nygren et al., 2000; Thomas et al., 2000) but remain abundant even in late succession (Gei et al., 2018). This is in contrast to other common temperate N fixers, such alder (*Alnus* spp.; Betulaceae), that fix at high rates over stand age, in spite of increasing soil N availability (Binkley et al., 1992), but decline in density over time (Liao & Menge, 2016). However, it remains a possibility that black locust's fixation strategy and fixation rate change with ontogeny (Dovrat & Sheffer, 2019) independent of changes in N availability. Because tree age and stand age did not vary independently in our study, we cannot disentangle the two. However, most young trees fixed N (Figure 2a) and seedlings tend to reduce, but not cease, fixation in response to experimental increases of available N (Johnsen & Bongarten, 1991; Röhm & Werner, 1991). In contrast, older trees generally did not fix N (Figure 2a), but it is unknown if black locust is capable of upregulating fixation in response to local conditions, as tropical N fixers can. Furthermore, older black locust trees fixed N at a declining rate, in spite of stable biomass growth rates (Figure 3b), suggesting an increasing reliance on soil N as trees age (Figure 3c). Whether driven by N dynamics, ontogeny or another unmeasured factor such as bacterial symbiont identity, the decline in black locust fixation with stand age suggests low fixation rates in eastern forests broadly, because most stands are >20 years old (Pan et al., 2011).

N fixer density had a large impact on our scaled estimates of fixation, pointing to the importance of understanding N fixer presence, abundance and its decline over succession. The decline of black locust density over stand age is similar to observations of other temperate forest N fixers (Liao & Menge, 2016), but contrasts to tropical N fixers, which can remain abundant into late succession (Gei et al., 2018). All plots in our analysis were recovering from a stand-replacing disturbance, which typically stimulates black locust recruitment (Elliott et al., 2017), yet our fixation estimates were dramatically lower when scaled from landscape-density plots versus point-density plots. And in both cases, variation in black locust density among plots had a greater relative impact on scaled fixation estimates than any other parameter (Figure S3). Such uncertainty may be related to spatial heterogeneity in the local seedbank or root stock from which it can sprout, or that black locust is most competitive with non-fixing tree species under certain light or moisture conditions (Minucci et al., 2019). Thus, our findings reveal a critical weakness in our current understanding of symbiotic N fixation—we often study fixation where N fixers are most abundant, but we poorly understand where and why N fixers are rare or absent. This underscores the risk of overestimating fixation when upscaling rates without considering temporal and spatial heterogeneity in N fixer density.

One intriguing finding that deserves more attention is that nodule biomass appears to have declined between 1981 and 2017, independent of tree and stand age, which translates to a 55% reduction in our estimates of landscape fixation (Figure 5c,d). We acknowledge that differences in nodule sampling methods between sampling dates may have contributed to this difference in nodule biomass. First, nodules were sampled to different depths in 1981 and 2017 (0–30 cm vs. 0–25 cm respectively). It remains a possibility that we undersampled nodule biomass in 2017—nodules of other N-fixing plant species can persist well below 50 cm (Li et al., 2012)—however, we rarely observed nodules below the top 10 cm of our monoliths, and root biomass declines exponentially with soil depth in these forests (Hales et al., 2009). Second, nodules were horizontally sampled in different ways in 1981 and 2017 (a plot-based vs. tree-based approach respectively). However, in a comparison of methods (see Section 2.8), we found that the plot-based approach resulted in lower, not higher, estimates of per-tree nodule biomass. If the decline in nodule biomass between 1981 and 2017 years is real, we consider potential reasons for this change. Over these 37 years, atmospheric CO_2 has increased by 65 ppm and soil temperature by 1°C (Dlugokencky & Tans, n.d.; Knoepp et al., 2018), both of which may increase fixation potential by black locust (Houlton et al., 2008; Norby, 1987; Olesniewicz & Thomas, 1999). Yet at our study site, a total of 200 kg N/ha was deposited from the atmosphere over that time (National Atmospheric Deposition Program, 2019), and fire has been excluded since the turn of the 20th century; both of these, in contrast, have the potential to decrease fixation potential through increasing soil N pools (Carpenter et al., 2021; Veerman et al., 2020). In fact, the possibility of higher soil N availability at our study site is supported by rising litter N concentrations over the last few decades (Knoepp et al., 2018). Thus, it is possible that black locust has reduced its investment in nodule biomass in response to these disturbances that increase soil N. Such a sensitivity to N would be consistent with our observation that fixation declines with tree and stand age despite a warmer and higher CO_2 environment. The reduction in the individual fixation rate of black locust may reveal how human disturbances diminish the ecological importance of, and need for, symbiotic N fixation. Based on our scaled estimates, just over three decades ago, average annual atmospheric N deposition (5.8 kg ha^{-1} $year^{-1}$) at our field site was half of landscape fixation inputs, whereas currently, deposition exceeds fixation threefold.

Not only does symbiotic N fixation change with temporal scales but also a key challenge is to quantify how it changes spatially. Our framework for symbiotic fixation addresses this challenge by scaling fixation from individual N fixers. But this approach depends on accurate sampling of N fixer density, nodule presence and nodule biomass. Quantifying N fixer density is relatively straightforward in ecosystems where there is only one N-fixing species; but in many tropical forests, legumes are diverse, are difficult to identify or have yet to be tested for their ability to fix (Afkhami et al., 2018). Furthermore, we know little about what controls the local rarity or absence of N fixers within their geographic range. Another challenge involves sampling nodule biomass, because it is exceedingly small even relative to the below-ground biomass of N-fixing trees. Yet, assuming that nodules are more likely found near the base of N fixers where most of their root biomass occurs is logical and supported by prior studies (Barron et al., 2011; Ruess et al., 2009), which can aid sampling efforts. We found that nodule biomass was concentrated near the stem of black locust trees (Figure S1), and that plot-based relative to tree-based sampling underestimated nodule biomass. Approaches to best sample nodules may vary across ecosystems where N fixers and their roots have different spatial distributions (Casper et al., 2003)—a combination of targeted tree and stratified soil sampling (Winbourne et al., 2018) may be the most rigorous. Most importantly, in the framework we present, attributing nodule biomass to individual N fixers is critical for illuminating controls on fixation.

Our framework for understanding symbiotic N fixation reveals three potential constraints on predictions of fixation by black locust. First, our estimates show that fixation by individual black locust trees peaks within two decades of disturbance and then declines over tree and stand age. Second, landscape fixation by black locust is low after accounting for the rarity or absence of black locust across the landscape. These two findings imply a binary pattern of fixation—stands are either recently disturbed and have a high potential to fix N, or are mature and have a low potential to fix N. Third, black locust fixation rates appear to have declined over time, independent of tree and stand age, coinciding with human-derived N inputs that now dwarf natural inputs from symbiotic N fixation. If this pattern extends across the geographic range of black locust, it suggests that eastern forests have a much lower, and potentially declining, capacity to fix N than was previously estimated (Staccone, Liao, et al., 2020). Whether N fixers in other ecosystems display similar dynamics over succession and with human disturbance not only depends on their fixation strategies, which may be diverse (Afkhami et al., 2018; Menge et al., 2015), but also on how these disturbances directly affect their abundance. This underscores the importance of studying symbiotic N fixation at the N fixer scale, to illuminate such patterns and improve our prediction of fixation and ecosystem response to global change.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. Nina Wurzburger is an Associate Editor of *Journal of Ecology*, but took no part in the peer review and decision-making processes for this paper.

AUTHORS' CONTRIBUTIONS

N.W. and C.F.M. were involved in the conceptualization of the study; J.I.M., N.W. and C.F.M. investigated the study; N.W. and J.I.M. were involved in formal analysis; N.W. was involved in writing-original draft; N.W., J.I.M. and C.F.M. were involved in writing-review & editing.

DATA AVAILABILITY STATEMENT

Data are available at https://www.fs.usda.gov/rds/archive/Catalog/RDS-2021-0099 (Wurzburger et al., 2021).

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