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ARTICLE

Tree symbioses sustain nitrogen fixation despite excess nitrogen supply

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Abstract

Symbiotic nitrogen fixation (SNF) is a key ecological process whose impact depends on the strategy of SNF regulation—the degree to which rates of SNF change in response to limitation by N versus other resources. SNF that is obligate or exhibits incomplete downregulation can result in excess N fixation, whereas a facultative SNF strategy does not. We hypothesized that tree-based SNF strategies differed by latitude (tropical vs. temperate) and symbiotic type (actinorhizal vs. rhizobial). Specifically, we expected tropical rhizobial symbioses to display strongly facultative SNF as an explanation of their success in low-latitude forests. In this study we used ^{15}N isotope dilution field experiments in New York, Oregon, and Hawaii to determine SNF strategies in six N-fixing tree symbioses. Nitrogen fertilization with +10 and +15 g N m^{-2} $year^{-1}$ for 4–5 years alleviated N limitation in all taxa, paving the way to determine SNF strategies. Contrary to our hypothesis, all six of the symbioses we studied sustained SNF even at high N. *Robinia pseudoacacia* (temperate rhizobial) fixed 91% of its N (%N_{dfa}) in controls, compared to 64% and 59% in the +10 and +15 g N m^{-2} $year^{-1}$ treatments. For *Alnus rubra* (temperate actinorhizal), %N_{dfa} was 95%, 70%, and 60%. For the tropical species, %N_{dfa} was 86%, 80%, and 82% for *Gliricidia sepium* (rhizobial); 79%, 69%, and 67% for *Casuarina equisetifolia* (actinorhizal); 91%, 42%, and 67% for *Acacia koa* (rhizobial); and 60%, 51%, and 19% for *Morella faya* (actinorhizal). Fertilization with phosphorus did not stimulate tree growth or SNF. These results suggest that the latitudinal abundance distribution of N-fixing trees is not caused by a shift in SNF strategy. They also help explain the excess N in many forests where N fixers are common.

KEYWORDS
Acacia, *Alnus*, biogeochemistry, *Casuarina*, ecosystem theory, facultative, *Gliricidia*, *Morella*, obligate, *Robinia*, symbiosis, symbiotic nitrogen fixation

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INTRODUCTION

Introduction to nitrogen fixation and the dynamics of nitrogen fixation

Biological nitrogen (N) fixation, the conversion of atmospheric N_2 gas to plant-usable N, is the primary natural source of N in ecosystems (Fowler et al., 2013). Given that N is an essential component of all living tissues (Chapin et al., 2011), N fixation is essential for life as we know it. Symbioses between bacteria, which perform the actual N fixation, and vascular plants, which house the bacteria in specialized root structures called nodules, have the highest capacity for N fixation, bringing in upwards of 100 kg N ha^{-1} $year^{-1}$ (10 g N m^{-2} $year^{-1}$) in some ecosystems (Binkley et al., 1994; Ruess et al., 2009).

In addition to the quantity of N it can provide, symbiotic N fixation (SNF) generates the potential for dynamic feedbacks within the N cycle (Vitousek et al., 2002). In much the same way that a thermostat turns a heater on or off when a room is too cold or hot, SNF might act as a "nitrostat" (Menge & Hedin, 2009), turning on when N is in short supply and off when N supply is sufficient. A nitrostat could occur at the scale of individual symbioses, adjusting the quantity or activity of nodules within a plant's root system, or at the scale of plant communities via competitive dynamics that favor or disfavor N-fixing symbioses (hereafter, "N fixers"). Why might SNF turn off or be disfavored when N supply is sufficient? Why would SNF not simply substitute for N uptake from the soil? One clear answer is that SNF is more expensive than soil N uptake when soil N is plentiful because of both the metabolic cost of breaking the triple bond of N_2 and the structural cost of building and maintaining nodules (Gutschick, 1981). Indeed, if SNF were consistently cheaper for plants than soil N uptake, natural selection might have favored only N fixers.

Following previous studies (e.g., Barron et al., 2011; Bauters et al., 2016; Hedin et al., 2009; Menge et al., 2009; Menge & Hedin, 2009; Sheffer et al., 2015), we use the following nomenclature to distinguish the different individual-scale SNF regulation strategies (see also Figure 1). "Obligate" SNF refers to symbioses that maintain a similar level of SNF per plant biomass across an ecologically relevant range of N supply, regardless of whether plant growth is limited by N. When SNF is obligate, nitrostat dynamics can only play out at the community scale. "Facultative" SNF refers to the opposite end of the spectrum: symbioses that adjust SNF to maintain colimitation between N and whichever other resource(s) is (are) limiting; that is, an individual-level nitrostat. Crucially, a facultative strategy means that the plant shuts SNF off entirely when soil N supply meets or exceeds the plant's N demand. Myriad strategies fall in between the obligate and facultative ends of the spectrum, but here we mention only one, "incomplete downregulation," which is similar to facultative SNF except

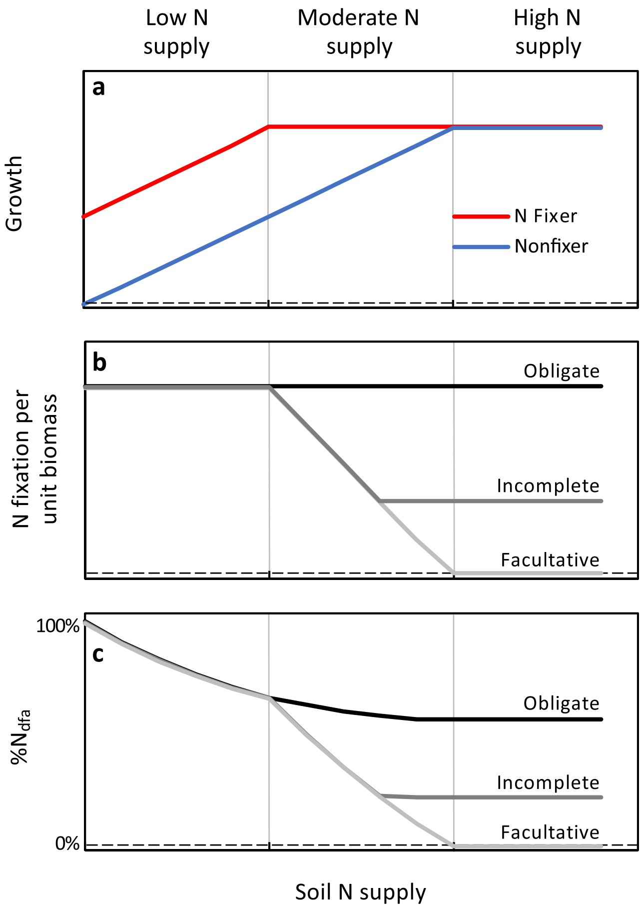


FIGURE 1 Theoretical predictions for different nitrogen (N) fixation strategies. (a) Growth of (red) N-fixing trees and (blue) nonfixing trees, (b) N fixation rates per unit biomass, and (c) percentage of N derived from fixation (%N_{dfa}) for N-fixing trees are shown as functions of soil N supply for three strategies: obligate N fixation, facultative N fixation, and incomplete downregulation. Three regions are indicated. In the low N supply region, both N-fixing and nonfixing trees are N limited, and all three N-fixing strategies fix N at the same rate. In the moderate N supply region, nonfixing trees are N limited, but N-fixing trees can fix enough N to overcome N limitation; all three N-fixing strategies fix N, though the fixation rates of incomplete and particularly facultative strategies decline with soil N supply. In the high N supply region, soil N supply is sufficient to overcome N limitation, so neither the N-fixing nor nonfixing trees are N limited. The high N supply region distinguishes most clearly between perfectly facultative N fixation (zero %N_{dfa} and zero N fixation) and obligate or incomplete strategies (nonzero %N_{dfa} and nonzero N fixation). The distinction between obligate and incomplete downregulation is that the N fixation rate per unit biomass decreases from the moderate to the high N supply regions for incomplete downregulation but not for obligate N fixation. Note that a decline in %N_{dfa} does not reject obligate N fixation because %N_{dfa} can decrease due to more N uptake from the soil as well as decreased N fixation per unit biomass.

that some SNF continues even when N supply exceeds the plant's N demand (Menge et al., 2015).

Theoretical studies have explored the consequences of different SNF strategies on communities and ecosystems (Bytnerowicz & Menge, 2021; Hedin et al., 2009; Kou-Giesbrecht & Menge, 2019; Lu & Hedin, 2019; Menge et al., 2009, 2015; Sheffer et al., 2015). At the community level, theory suggests that different strategies can lead to qualitatively distinct outcomes of competition (competitive exclusion vs. coexistence vs. priority effects) as well as different quantitative abundance levels within each qualitative outcome (Bytnerowicz & Menge, 2021; Menge et al., 2015). At the ecosystem level, theory suggests that different SNF strategies have different effects on soil N enrichment and, ultimately, on N export (Hedin et al., 2009; Kou-Giesbrecht & Menge, 2019; Menge et al., 2009, 2015).

The community effects of different SNF strategies have implications for global biogeography. N-fixing trees are 10 times more abundant at lower (<35° N) than higher (>35° N) latitudes in the Americas (Menge et al., 2014, 2019; Menge, Batterman, Liao, et al., 2017; Steidinger et al., 2019; ter Steege et al., 2006). This pattern has long fascinated ecologists (Crews, 1999; Jenny, 1950; Rundel, 1989; Vitousek & Howarth, 1991). Based on theoretical predictions, the "differential regulation hypothesis" states that a difference in the SNF strategies at different latitudes—facultative SNF at low latitudes versus obligate SNF at higher latitudes—could explain the abrupt transition in N-fixing tree abundance (Lu & Hedin, 2019; Menge et al., 2014; Menge, Batterman, Hedin, et al., 2017; Sheffer et al., 2015). The differential regulation hypothesis also aligns nicely with the transition between two different taxonomic groups of N-fixing trees. Rhizobial symbioses (predominantly legume plants that associate with *Rhizobia*-type bacteria; see Sprent, 2009) are the primary N-fixing tree symbioses below 35° N, whereas actinorhizal symbioses (plants from eight other families that associate with *Frankia* bacteria; see Huss-Danell, 1997) are the primary N-fixing tree symbioses above 35° N (Menge et al., 2014; Menge, Batterman, Liao, et al., 2017). Furthermore, the differential regulation hypothesis predicts the different successional trajectories of N-fixing trees in different biomes. N-fixing trees are primarily early successional in temperate North America (Menge et al., 2010), as predicted for obligate N fixers, whereas N-fixing trees are abundant throughout succession in the tropical Americas (Gei et al., 2018), as predicted for facultative N fixers.

Evidence for N-fixation strategies

Although the previously discussed theoretical work demonstrated that SNF strategies affect many community and ecosystem processes, it is not clear what strategies N fixers actually use. N fixation is a notoriously difficult process to quantify, particularly in trees and particularly in the field (Soper et al., 2021; Winbourne, Harrison, et al., 2018). Because of the relative lack of data on SNF strategies in trees and forests, we focus here on field-based measurements of trees rather than greenhouse measurements or herbs. The ideal way to determine SNF strategies is to conduct experiments that manipulate resource supply to alter N limitation of plant growth while also measuring SNF rates (Figure 1, Menge et al., 2015). Such experiments are rare, though, so existing field data are primarily observational.

Observations of high rates of SNF (often exceeding 100 kg N ha^{-1} year^{-1} and 98% of the plant's N from SNF) in various temperate and boreal species of the actinorhizal genus *Alnus* (e.g., Binkley et al., 1994; Mead & Preston, 1992; Ruess et al., 2009; Tang, 1997) are consistent with obligate SNF or incomplete downregulation. Observations that other temperate actinorhizal species exhibit high SNF (Chaia & Myrold, 2010) or similar SNF across a range of soil N (Menge & Hedin, 2009) are also consistent with obligate SNF or incomplete downregulation. Some of these observations of high SNF come from sites where the concentration or export of soil nitrate is high (e.g., *Alnus rubra* in Binkley et al., 1992, 1994), providing stronger evidence for the idea that *Alnus* exhibits obligate or incomplete SNF, but observational evidence alone cannot diagnose a SNF strategy. Without experimental evidence of whether N limits plant growth, observations of high SNF might simply reflect a high demand for N that is unmet by soil N supply. Binkley et al. (1994), summarizing a host of greenhouse experiments, noted that moderate to high soil N additions *stimulated* SNF in many greenhouse studies, which would be further support for obligate or incomplete SNF, though it is unclear how well seedlings in pots reflect mature trees in forests. Binkley et al. (1994) did note that exceptionally high levels of soil N (above natural concentrations) in some greenhouse studies inhibited SNF.

A variety of observational studies in tropical forests have detected variable or low SNF by trees, which have typically been interpreted as facultative SNF. For example, *Inga* species in Panama had many more nodules (corresponding to higher SNF) in secondary forests and shoreline sites than in mature forests (Barron et al., 2011). Nodule abundance in the mature forest was low, which, in combination with a negative relationship between nodule abundance and soil nitrate across the sites, was consistent with facultative SNF (Barron et al., 2011). Other studies in tropical forests have found low or variable nodule counts or activity across succession (Batterman, Hedin, et al., 2013; Bauters et al., 2016; Sullivan et al., 2014; Taylor et al., 2019), seasons (Gei & Powers, 2015; Wong et al., 2020), or sites

(Wurzburger & Hedin, 2016), which is usually interpreted as facultative SNF.

However, there are a number of reasons why these studies do not provide ironclad evidence of facultative SNF in tropical tree symbioses. First, there are some exceptions where SNF in tropical forests was relatively high and invariant across the range of successional ages (Winbourne, Feng, et al., 2018) or soil N availability (Brookshire et al., 2019). Second, even in the studies that show wide variation, SNF is rarely zero, and according to the theory described earlier, incomplete downregulation has starkly different effects on ecosystems than perfectly facultative SNF. Third, these studies were ill-suited to diagnose SNF strategies due to both the methods used and the observational nature of the studies. Counting nodules, while arguably the best strategy for detecting SNF rates in many situations (Soper et al., 2021), has a number of issues (Winbourne, Harrison, et al., 2018). Most relevant to the present work is the fact that finding zero nodules does not mean zero SNF unless entire trees or forests are excavated. The lack of experimentation also makes it difficult to diagnose SNF strategies, given the lack of information about which successional stages or sites, for example, are N limited. For these reasons, despite tremendous progress, SNF strategies are still poorly known.

Questions and study design

Here we used a field experiment to study the SNF strategies of six tree species in temperate and tropical locations. One motivation was to test the differential regulation hypothesis as an explanation for the 10-fold greater abundance of N-fixing trees in lower compared to higher latitudes in the Americas, so we targeted symbiotic N-fixing tree species from temperate and tropical regions. Given the alternative hypothesis that a difference in SNF strategies might be driven by phylogeny rather than region of origin, we studied both rhizobial and actinorhizal symbioses. The six species we used, for which we provide more details in the *Methods* section, were *Robinia pseudoacacia* (temperate rhizobial), *Alnus rubra* (temperate actinorhizal), *Gliricidia sepium* (tropical rhizobial), *Acacia koa* (tropical rhizobial), *Casuarina equisetifolia* (tropical actinorhizal), and *Morella faya* (tropical actinorhizal). We planted trees as seedlings and measured them for several years (at the conclusion of the experiment, trees were as tall as 8 m and as wide as 19-cm basal diameter) to capture some of the nuances of trees in the field while maintaining experimental tractability. We used isotopic soil enrichment (Chalk, 1985; Marron et al., 2018; Mead & Preston, 1992; Yelenik et al., 2013) to measure SNF. This isotopic enrichment method is better suited to detecting zeros than counting nodules because field sampling may fail to locate nodules or may locate nodules that appear healthy but do not support SNF (Menge et al., 2015).

To capture SNF regulation across a range of realistic soil N supply levels while following theoretical guidance (Figure 1; Menge et al., 2015), we grew each symbiosis across a range of N supply. To establish whether our N additions exceeded plant N demand even in the absence of SNF (i.e., whether our N additions relieved N limitation), we grew the N-fixing symbioses alongside nonfixing tree species and added N at two levels above the control. If our highest N addition level stimulated the growth of the nonfixing species more than the intermediate level (i.e., if the intermediate treatment is in the low or moderate N supply region in Figure 1), we would not know whether plants in the highest N addition level were N limited, so we would not be able to distinguish between the different SNF strategies. If, on the other hand, our intermediate level satisfied N demand of the nonfixers (i.e., if the intermediate treatment was in a high-N-supply region in Figure 1), then facultative SNF would shut off entirely at both the intermediate and high-N-addition levels, providing a clear way to distinguish facultative (zero SNF when N demand is saturated) from obligate SNF or incomplete downregulation (greater than zero SNF when N demand is saturated; Figure 1b,c). To distinguish obligate SNF from incomplete downregulation, we would look for a decrease in SNF per plant biomass from the control to the N addition treatments (Figure 1b). Importantly, a decrease in the percentage of N derived from SNF, denoted by $\%N_{dfa}$, would not on its own indicate downregulation because $\%N_{dfa}$ decreases with N addition in obligate SNF due to increased N uptake “diluting” the same quantity of fixed N (Figure 1c). Given the myriad ideas about the relationship between SNF and phosphorus (e.g., Crews, 1993; Nasto et al., 2014; Png et al., 2017; Vitousek & Howarth, 1991; Wang et al., 2007), we also added a treatment with high N and phosphorus (P) to assess whether P acted as a secondary limiting nutrient and how it affected SNF.

Our primary objective was to answer the following question: What are the N fixation strategies of these six symbioses in our sites? Following the differential regulation hypothesis, we hypothesized that tropical rhizobial symbioses (*Gliricidia* and *Acacia*) would be facultative and that the temperate actinorhizal symbiosis (*Alnus*) would be obligate. We did not have a clear hypothesis for tropical actinorhizal symbioses (*Casuarina* and *Morella*) or temperate rhizobial (*Robinia*) symbioses, but including these groups allowed us to better understand environmental versus taxonomic controls over SNF.

METHODS

Study sites and species

We examined rhizobial and actinorhizal tree symbioses from tropical and temperate regions. There were two obvious choices for temperate forest taxa: the rhizobial *R. pseudoacacia* and the actinorhizal *A. rubra*. *Robinia* and *Alnus* (we refer to both the plant taxa and their symbioses by the plant genus name after the first mention in the *Methods* section) account for 64% and 24%, respectively, of tree-associated SNF in the coterminous USA (Staccone et al., 2020). *Robinia* is also common throughout Eurasia (Cierjacks et al., 2013), as are various species of *Alnus* (Benson & Dawson, 2007). We studied *Robinia* at Black Rock Forest (Mitchell, 1936; Schuster et al., 2008; Stout, 1956) in New York State (see Table 1 for site characteristics), which is in the northern part of its range (Staccone et al., 2020). We studied *Alnus* in privately owned Starker Forest in the Coast Range of Oregon (Table 1), in the heart of its range (Staccone et al., 2020).

In contrast to temperate SNF, tropical SNF is not dominated by a few tree species. The Neotropics contain a great diversity of N-fixing trees (Jenny, 1950; Menge et al., 2019; Menge, Batterman, Liao, et al., 2017; Sprent, 2009; ter Steege et al., 2006). For rhizobial trees we chose *G. sepium*, a common rhizobial tree throughout its native range in Mesoamerica as well as pantropically (Stewart et al., 1996), and *A. koa*, a rhizobial tree endemic to the Hawaiian Islands (Baker et al., 2009; Elevitch et al., 2006). We chose two actinorhizal species: *C. equisetifolia*, which is common throughout its native range in the Pacific, Southeast Asia, and Australia and is invasive across tropical areas around the world (Parrotta, 1993); and *M. faya* (formerly known as *Myrica faya*), which is native to Macaronesia and highly invasive in Hawaii, where it poses a threat to local flora and alters biogeochemistry (Aplet, 1990; Vitousek et al., 1987; Vitousek & Walker, 1989). Given that these four taxa do not all co-occur in the same habitat, we chose two tropical sites on the Big Island of Hawaii: the lower elevation Waiakea Research Station for *Gliricidia* and *Casuarina* and the higher elevation Volcano Research Station for *Acacia* and *Morella*.

For reference species we chose trees that co-occurred with and that had characteristics similar to those of N-fixing trees. We chose *Betula nigra* as a reference for *Robinia*, as both share similar ranges, are relatively fast growing, and are shade intolerant. For *Alnus* we chose *Pseudotsuga menziesii*, which frequently co-occurs with *Alnus* in early succession. At Waiakea we used *Psidium cattleianum* to pair with both *Gliricidia* and *Casuarina* in triads. Similarly, at Volcano we chose *Dodonaea viscosa* to grow in triads with *Acacia* and *Morella*.

Experimental design

We planted all trees as seedlings, with the exception of *Pseudotsuga*, which had been planted 2 years before our experiment began. *Alnus* grows faster than *Pseudotsuga*, and we wanted them to be similar in size at the end of the experiment. The planting dates for the non-*Pseudotsuga* species ranged from May 2015 to May 2016 (see Appendix S1: Table S1 for planting dates and sizes at time of planting). Because of the different years of planting, we refer to years hereafter as the year of the experiment rather than the calendar year. We replaced trees when there was mortality within the first year of the experiment.

In each site we sought to minimize herbivory damage, prevent competition from ground-layer plants, and ensure even access to the ^{15}N label and the fertilizer across individuals within a treatment. Competition from other species and other threats to survival of the trees were different at each site, so our upkeep methods differed. In each site we installed wire fences (approximately 1 m radius) around each seedling to reduce damage from large mammals (deer in New York, deer

TABLE 1 Site characteristics.

Site	Latitude (°)	Longitude (°)	Elevation (m)	MAT (°C)	MAP (mm year^{-1})	Nitrogen-fixing tree	Reference tree
New York	41.42	−74.02	195	9.5	1248	*Robinia pseudoacacia*^{R}	*Betula nigra*
Oregon	44.56	−123.60	373	10.5	2166	*Alnus rubra*^{A}	*Pseudotsuga menziesii*
Waiakea	19.64	−155.08	196	23.3	4318	*Gliricidia sepium*^{R}, *Casuarina equisetifolia*^{A}	*Psidium cattleianum*
Volcano	19.47	−155.26	1249	16.3	3048	*Acacia koa*^{R}, *Morella faya*^{A}	*Dodonaea viscosa*

Note: Superscripts “A” and “R” indicate actinorhizal and rhizobial symbioses, respectively. Mean annual temperature (MAT) and mean annual precipitation (MAP) for New York and Oregon are averages from 1961 to 1990 from PRISM. MAT and MAP for Hawaii are University of Hawaii field station data.

and elk in Oregon, and pigs in Hawaii). We removed the fences a few years later as the plants grew, before the cages affected further growth. In New York and Oregon, where competition from ground-layer plants was a major concern, we applied glyphosate as an herbicide for the first 4 and 3 years (respectively) in a 5-m-radius circle around each plant. In Hawaii, where ground-layer competition was also a concern, we applied glyphosate in the first year but switched to mowing the 5-m ring (and weeding right near the plant) in subsequent years because the glyphosate killed some focal plants in the first year. Glyphosate adds some P, some of which can become available after a lag, though the amount it adds is a couple orders of magnitude less than typical fertilizer applications (Hébert et al., 2019).

Our trees were arranged in N-fixer–nonfixer pairs (New York and Oregon) or linear triads (Waiakea and Volcano) that shared the same fertilization treatment (Figure 2). Trees within pairs or triads were 5 m from each other. Each tree was at least 12 m from all trees in other pairs or triads. We applied ^{15}N within a 2-m ring around each tree to assess SNF. For noncontrol treatments, we applied the unlabeled fertilizers to all the ground area within 5 m of each tree. These distances ensured that the majority of each tree's roots were within the area that received the tree's assigned fertilizer amount while also meeting budget constraints for ^{15}N labeling of 320 individual tree-based plots. The total amount of N in the isotopic label was small (see following paragraphs), so the total N supply was consistent across the ^{15}N-labeled 2-m ring and the surrounding 5-m ring.

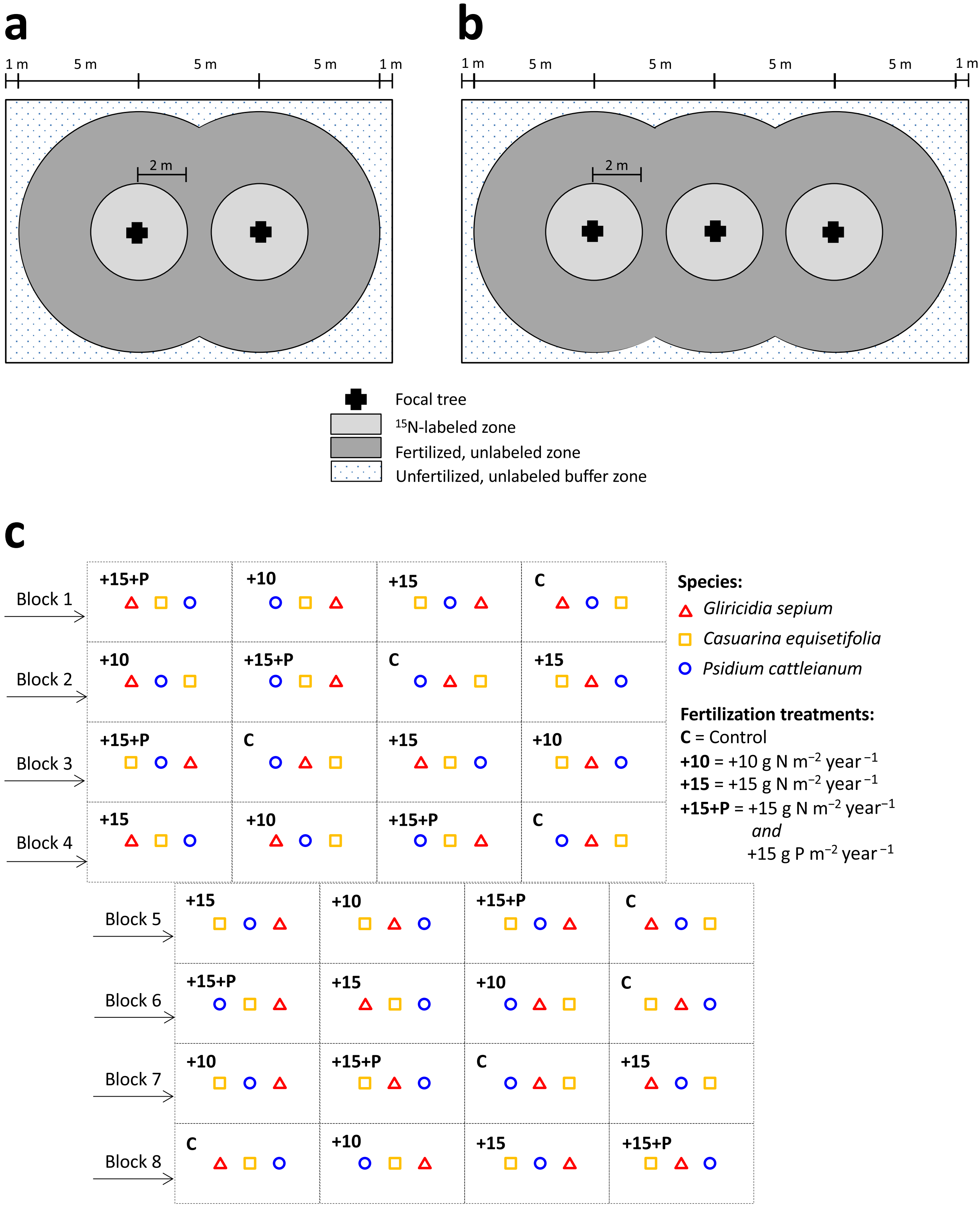
Our goals for fertilization were to add enough N to saturate N demand and to add enough isotopic label to distinguish fixed N from N taken from the soil. We had four fertilization treatments. Our control treatment ("C" in figures) received no unlabeled fertilizer and 0.1 g N m^{-2} year^{-1} of ^{15}N label in 2 years (see following paragraph). Our fertilization treatments were +10 g N m^{-2} year^{-1} (total amount, including both labeled and unlabeled fertilizer; "10" in figures), +15 g N m^{-2} year^{-1} ("15" in figures), and +15 g N m^{-2} year^{-1} + 15 g P m^{-2} year^{-1} ("15+P" in figures). We did not include +P only or +10+P treatments due to logistical constraints. In Oregon and New York, the unlabeled N fertilizer was applied as ammonium nitrate until ammonium nitrate became prohibitively difficult to purchase, at which point we switched to urea (Year 4 in New York, Year 3 in Oregon). In Hawaii, the unlabeled N fertilizer was applied as dolomite-coated ammonium nitrate (CAN 27; Yara North America, Tampa, FL); dolomite coating helps stabilize the explosive potential of ammonium nitrate. Because of the potential effects of dolomite on soil pH, dolomite (#Ag 65 Dolomite; Lhoist, Fort Worth, TX) was added to all the control and +10 treatments in Hawaii such that these plots received as much dolomite (via a combination of pure dolomite and the ammonium nitrate coating) as the +15 treatments (in the ammonium nitrate coating alone). Phosphorus was applied as monosodium phosphate. All unlabeled fertilizers were hand broadcast three or four times per year during the growing season (approximately March to October in NY and OR, year-round in Hawaii).

We added doubly labeled 98 atom % ammonium nitrate (^{15}NH$_4$^{15}NO$_3$; Isotec, Miamisburg, OH) in Years 2 and 3 at each site. To ensure sufficient labeling, we added more ^{15}N to the higher N treatments: 0.1 g N m^{-2} year^{-1} to the control treatment, 0.25 g N m^{-2} year^{-1} to the +10 treatment, and 0.375 g N m^{-2} year^{-1} to the +15 and +15+P treatments. We dissolved the ^{15}N label in water and added it evenly across the 2 m ring around each tree using a backpack sprayer. Similar isotope dilution techniques have been used for measuring SNF in *Alnus* (Mead & Preston, 1992; Tang, 1997), *Robinia* (Marron et al., 2018), and other taxa (e.g., Yelenik et al., 2013).

Our initial sample size was eight trees per species per fertilization treatment, for a total of 320 trees across all the sites, arranged in treatment-based blocks. Our sample sizes dwindled across the years, as reflected in the figures. There was some mortality and morbidity during the experiment, from deer damage (New York), pig damage (Waiakea and Volcano), pests (New York, Waiakea, and Volcano), and volcanic gases (the aptly named Volcano). Dead trees and trees with major damage (e.g., broken main stems) were not used in the analysis.

Tree size and growth

We assessed nutrient limitation by measuring how nutrient additions affected changes in tree biomass growth. Each year we measured the basal diameter of each tree with calipers and the height of each tree with a tape measure attached to a pruning pole. When trees were at least 1.3 m in height, we also measured the diameter at 1.3 m (dbh) in New York and Oregon. For diameter measurements, when the stems were noncircular we took two perpendicular measurements and used the geometric mean as the diameter. Starting in Year 3 we also measured canopy size as two perpendicular horizontal distances (length and width). We estimated biomass in all years from our measurements of diameters, heights, and canopy dimensions. Well-developed allometric equations exist for two (*Alnus* and *Pseudotsuga*) of our study species, but we needed to develop our own allometric equations for the remaining eight species.



Block 1	**+15+P** △ □ ○	**+10** ○ □ △	**+15** □ ○ △	**C** △ ○ □
Block 2	**+10** △ ○ □	**+15+P** ○ □ △	**C** ○ △ □	**+15** □ △ ○
Block 3	**+15+P** □ ○ △	**C** ○ △ □	**+15** △ □ ○	**+10** □ △ ○
Block 4	**+15** △ □ ○	**+10** △ ○ □	**+15+P** ○ □ △	**C** ○ △ □
Block 5	**+15** □ ○ △	**+10** □ △ ○	**+15+P** □ ○ △	**C** △ ○ □
Block 6	**+15+P** ○ □ △	**+15** △ □ ○	**+10** ○ △ □	**C** □ △ ○
Block 7	**+10** □ ○ △	**+15+P** □ △ ○	**C** ○ △ □	**+15** △ ○ □
Block 8	**C** △ □ ○	**+10** ○ □ △	**+15** □ ○ △	**+15+P** □ △ ○

FIGURE 2 Experimental setup. Diagram of spatial layout of (a) pairs and (b) triads of trees, indicating spacing of trees, unlabeled fertilizer, and labeled fertilizer. (c) Layout of plots at Waiakea as an example of spatial arrangement of blocks, species, and treatments.

We harvested trees in New York at the end of Year 5, as described in detail in Carreras Pereira et al. (2023). For aboveground biomass we felled each tree at its base, then separated tissues into main stem, secondary stem (≥1 cm diameter), and twigs and leaves. We determined the twig-to-leaf ratio via subsampling. For each tissue type we measured wet mass in the field, then took representative subsamples of each tissue type back to the lab, which we dried in a drying oven at 65°C for at least 2 days before measuring their mass.

For belowground harvesting in New York, we excavated entire rooting systems with a mini excavator, which was effective at isolating medium to large roots but likely missed many fine roots. We brought the entire excavated rooting systems back to the lab, removed the soil, recorded their wet masses, then dried subsamples and measured their masses as for the aboveground tissues.

At the end of Year 4, we harvested aboveground but not belowground biomass in Waiakea and Volcano. We used the same method as in New York, except that in Waiakea and Volcano we divided aboveground tissues into their component parts (main stem, secondary stem, and twigs and leaves) for a representative subset of the trees rather than for all harvested trees.

In Appendix S2 we provide details of the allometric equations we used. Briefly, we used AIC to compare a variety of candidate models that used different input variables (basal diameter, height, canopy width times canopy height, and combinations of these variables) to predict the final harvested biomass (Carreras Pereira et al., 2023). We used these equations to predict biomass of the trees at all time periods where we had size measurements.

To estimate tree growth, we calculated the change in aboveground (for all sites) and total (for New York and Oregon) biomass over time. For each year t starting in Year 2 we calculated the absolute growth rate (AGR) for the biomass or aboveground biomass B of tree i as

$$\mathrm{AGR}_{i,t} = \frac{B_{i,t} - B_{i,t-\Delta t}}{\Delta t}, \tag{1}$$

where Δt is the years since the previous measurement (close to but not exactly 1 because trees were measured on different days each year). We also calculated the relative growth rate (Condit et al., 2006) as

$$\mathrm{RGR}_{i,t} = \frac{\ln(B_{i,t}) - \ln(B_{i,t-\Delta t})}{\Delta t}. \tag{2}$$

Foliar chemistry

At the end of each growing season in New York and Oregon, and annually in Waiakea and Volcano, we collected fully expanded sun leaves (or leaflets in the case of *Robinia* and *Gliricidia*) from each healthy tree. We dried the leaves at 65°C, ground them in a Wiley mill or, if they were too small for the Wiley, a ball mill, then sent subsamples of homogenized tissues to the University of California, Davis isotope lab for analysis of atom $\%^{15}$N and N concentration. To avoid confusion with $\%\mathrm{N_{dfa}}$ and atom $\%^{15}$N, we report N concentration in mg N g leaf^{-1}. rather than %N.

Soil chemistry

At the same time that we measured tree size in 2019, as well as in 2018 in Oregon, we cored soils to measure some aspects of soil chemistry. We sampled soil from 0 to 10 cm in depth; 5-cm-diameter cores were taken 0.5 m from the base of each tree (one core per tree). Samples were immediately stored in a cooler with ice; samples were extracted on the same day as coring (5-g soil subsamples in 0.03 L of 2.0 M KCl). We analyzed the extracts for $\mathrm{NO_3}$-N and $\mathrm{NH_4}$-N on a discrete analyzer (SmartChem 170, Unity Scientific, Milford, MA, USA) and expressed N content per mass dry soil. Soil pH was measured on the Hawaii soils at the University of Hawaii-Hilo analytical lab, using a 1:1 soil-to-water mixture (Jones, 2001).

N fixation

N fixation can be measured in a variety of ways. Here, our two primary N-fixation-related response variables are $\%\mathrm{N_{dfa}}$, which is the fraction of a plant's N it acquires from SNF, and total $\mathrm{N_{dfa}}$, the total amount of N a plant acquires via SNF. We calculated total $\mathrm{N_{dfa}}$ by multiplying $\%\mathrm{N_{dfa}}$ by the amount of N in each plant. Given the importance of these metrics to our question, we calculated them in a number of ways to assess the robustness of our results. We note that these methods can produce negative values of $\%\mathrm{N_{dfa}}$ (and, thus, negative $\mathrm{N_{dfa}}$ values), which occur when the reference trees are less ^{15}N-enriched than the N fixers. Negative SNF is biologically meaningless, but we leave the values as negatives because of our focus on whether SNF is downregulated to zero. If we were to assign zero values to all negative $\%\mathrm{N_{dfa}}$ data, it would bias the results toward finding positive SNF.

$\%\mathrm{N_{dfa}}$: Reference plant method

The standard calculation of $\%\mathrm{N_{dfa}}$ by isotope dilution (Shearer & Kohl, 1986) is a two-end-member mixing model where a fraction of the plant's N comes from fixation ($\%\mathrm{N_{dfa}}$), and the remainder ($1 - \%\mathrm{N_{dfa}}$) comes from the soil:

$$\%\mathrm{N_{dfa}} = 100 \times \frac{\%^{15}\mathrm{N_{reference}} - \%^{15}\mathrm{N_{fixer\ foliage}}}{\%^{15}\mathrm{N_{reference}} - \%^{15}\mathrm{N_{fixation}}}. \tag{3}$$

Each of the terms in the numerator and denominator is the atom $\%^{15}$N in a given pool. The last term, $\%^{15}\mathrm{N_{fixation}}$, is a constant (0.3663%) as it represents the atom $\%^{15}$N of fixed N, which is assumed to be equivalent to the atom $\%^{15}$N of atmospheric N_2 gas. While the error around this assumption may be significant for natural-abundance ^{15}N methods for examining SNF, it is trivial for the enriched ^{15}N levels we use in this experiment. The term $\%^{15}\mathrm{N_{fixer\ foliage}}$ is the foliar atom $\%^{15}$N in an individual N-fixing tree, which we calculated independently for each year when the isotopic label was sufficiently strong (Years 3–5). The final term, $\%^{15}\mathrm{N_{reference}}$, represents the atom $\%^{15}$N of the N acquired from the soil by the N-fixing tree. It is impossible to measure that directly from the focal N-fixing tree, given that its N represents a mix of fixed N and soil N. Measuring soil N itself is not sufficient because it is unclear which soil N pool(s) the focal plant is accessing. The standard approach to calculating this term, which we use here, is to use paired nonfixing "reference" trees to gauge the isotopic composition of the soil N in the vicinity of the focal N-fixing tree.

There are a number of choices and assumptions involved in the reference tree method. For example, using the nonfixing tree in a pair or triad with a focal N-fixing tree gives a good estimate of the local soil environment because it is close to (5 m) the focal tree. However, using individual paired trees means that the vagaries of each nonfixing reference tree have an outsized effect. Averaging across all the nonfixing reference trees in a treatment at a given site would cut through this noise but, conversely, would fail to capture any plot-to-plot differences that may exist within a site. As another example, we chose to isotopically label the soil 2 m around each tree, balancing the needs for sufficiently large area of soil labeled and sufficiently large sample size. If the large majority of N uptake occurs within 2 m, or if similar fractions of N uptake occur within 2 m for the focal N-fixer and its reference nonfixer, our assumption that the reference tree represents the soil N available to the focal N fixer is valid. If the N fixer takes up substantially more N from outside the 2-m radius than the nonfixer does, however, then the reference tree would not be a good indicator of the soil N available to the N-fixing tree. As a final example, the reference tree method implicitly assumes that the fraction of N from storage (previous years' resorption) is similar for the focal N fixer and its reference tree. To assess the robustness of our results to these assumptions and choices, we calculated $\%\mathrm{N_{dfa}}$ in a variety of ways.

For our best estimate ("base case") we used the classic individual reference tree method. Specifically, we used foliage from the nonfixing trees from the same pair or triad as the focal tree, taken on the same day. In the few cases where the reference tree had died or was unhealthy, we used the arithmetic mean of the healthy nonfixing trees from the same treatment at the same site.

We conducted three sensitivity checks. Our first sensitivity check ("mean of reference trees in treatment") was to use the arithmetic mean of the healthy nonfixing trees within each treatment and site as the reference value for all N fixers in that treatment and site. Our second sensitivity check ("modified retranslocation") tested the assumption that N-fixing trees and their reference nonfixers used the same fraction of stored N. Specifically, we calculated $\%\mathrm{N_{dfa}}$ under the alternative assumption that the nonfixing reference trees used more N from storage (previously acquired N) than the N-fixing trees, as described in detail in Appendix S3. Our third and final sensitivity check ("rooting extent") examined our assumption that N-fixers and paired nonfixer species had the same proportion of their roots within the 2-m (^{15}N-labeled) radius. At harvest, *Robinia* typically had greater aboveground biomass than *Betula*, and a few *Gliricidia* and *Acacia* trees were noticeably larger than their paired *Psidium* and *Dodonaea*, respectively (Appendix S1: Figure S1). Larger biomass could translate into greater lateral root extent for these N fixers compared to their paired nonfixers. If the lateral rooting extent of N fixers substantially exceeded 2 m, N fixers might take more soil N from outside the ^{15}N-labeled soil area than nonfixers. When we harvested belowground biomass in New York, roots of some of the larger *Robinia* trees extended more than 2 m from the stem, whereas *Betula* (our paired nonfixer) had almost no roots outside 2 m (Akana, 2022). We assessed the robustness of our assumption that similar fractions of N uptake (or no N uptake) occur outside 2 m by calculating the fraction of each tree's N uptake that would need to have occurred outside 2 m to give a $\%\mathrm{N_{dfa}}$ value of 0. This calculation is a mixing model between soil N within the 2-m radius and soil N outside the 2 m radius (assumed to be at natural abundance levels), assuming that there is no N fixation. The calculation estimates soil N within the 2-m radius from the reference tree, assuming, conservatively, that all its N uptake is within 2 m. The solution to this mixing model is the same as the solution for $\%\mathrm{N_{dfa}}$ (Equation 3), but with different interpretations:

$$\%\mathrm{N_{outside\ 2\ m,\ assuming\ no\ SNF}} = 100 \times \frac{\%^{15}\mathrm{N_{reference}} - \%^{15}\mathrm{N_{fixer\ foliage}}}{\%^{15}\mathrm{N_{reference}} - \%^{15}\mathrm{N_{outside\ 2\ m}}}. \tag{4}$$

Therefore, if our base calculation yields a $\%\mathrm{N_{dfa}}$ of 90%, it also indicates that 90% of soil N uptake would have to be outside 2 m if it were not fixing.

%N_{dfa}: Cellulose disk method

In addition to the reference plant method and the robustness checks thereon, we also attempted a separate method that uses cellulose disks to assess the isotopic content of plant-available soil N. This method has been effective in pot studies (Hendricks et al., 2004) but to our knowledge has not been used in the field. Unfortunately, it did not give reliable estimates, so we did not use it in our main interpretation. Details of the disk method and results are in Appendix S4.

Total N_{dfa}

To calculate total N_{dfa}, we multiplied the base case of our %N_{dfa} estimates by the total amount of N in an N-fixing plant. We calculated total N_{dfa} for each time point in which we had %N_{dfa} data. Our approach is not a perfect estimate of total N_{dfa} since it misses some N that was in the plant but has since been lost (e.g., through litterfall, root turnover, loss to herbivory) and it misses some N currently in the plant (belowground biomass in Waiakea and Volcano, which we did not measure). However, our main goal in calculating total N_{dfa} was to assess how total N_{dfa} changed across treatments within a site, rather than to conduct an N budget, so we felt that omitting these other sources served our goals better than making assumptions about N turnover and belowground biomass.

For each N-fixing tree at each time point we calculated total N as the sum of biomass N (biomass multiplied by N concentration) in each tissue type. We estimated the biomass in each tissue type based on the allometric equations developed by Carreras Pereira et al. (2023) for our New York, Waiakea, and Volcano sites and the allometric equations from Cooke (1987), Helgerson (1981), and Tang (1997) for our Oregon site. We measured the N concentration in foliage in each of our trees, as described earlier. For other tissue types in New York, Waiakea, and Volcano, we measured the N concentration in a subset (approximately one third) of our trees for each species–treatment combination, then used species-, treatment-, and tissue type-specific arithmetic means to estimate the others. For the other tissue types in *Alnus*, which we did not harvest, we measured the N concentration in tissues of three trees adjacent to our plots. We used these as estimates of the N content in the control plots, then used the ratios of each fertilized treatment to the control for each tissue type in *Robinia* to estimate N concentrations in the other tissue types for *Alnus*.

Nodule counting

Because SNF is commonly estimated via counting nodules and measuring their masses, we used this method in one site (New York) to assess how well the isotope dilution method compared to nodule counting in our setting. Following the tree-based methods commonly used in tropical forests (Barron et al., 2011; Batterman, Hedin, et al., 2013; Wurzburger & Hedin, 2016), we took soil cores immediately prior to harvesting the trees in October 2019. The cores were 7.3 cm in diameter and 10 cm deep. The number of cores and the area from which they were taken varied with the size of the tree: We took three cores within a 1-m radius for trees <50 mm in basal diameter, four cores within a 1.5-m radius for trees 50–100 mm in basal diameter, and five cores within a 2-m radius for trees whose basal diameter was >100 mm. We then took the cores back to the lab, sorted the nodules, dried them, and measured their masses to determine dry nodule biomass per area ground.

Statistics

The basic design of our experiment is a set of categorical fertilization treatments on multiple species, with repeated annual measurements on individuals for most metrics. For the biomass growth measures, we used a linear mixed-effects model for each species (lme; Pinheiro et al., 2020). All analyses were done in R version 4.0.0 (R Core Team, 2020). The response variables were absolute annual growth rate (log-transformed for normality and homoscedasticity) and relative annual growth rate (untransformed) for aboveground biomass for all sites and for total biomass for New York and Oregon. The mixed effects were fertilization treatment within a site as a fixed effect, biomass (or aboveground biomass) as a fixed covariate, and individual tree as a random effect.

For N-fixation measurements we had three different response variables: %N_{dfa}, total N_{dfa} per biomass (g N fixed kg tree biomass^{-1} year^{-1}), and total N_{dfa} per tree (g N fixed tree^{-1} year^{-1}). For %N_{dfa} we calculated N fixation in three separate ways (see previous discussion) to assess the robustness of our results to the assumptions of the calculations. For each of these response variables we used a linear mixed-effects model with fertilization treatment within a site as a fixed effect and individual tree as a random effect. For foliar N content and for soil N in Oregon (where we had two or more years of measurements), we used a linear mixed-effects model with fertilization treatment and species within a site as fixed effects and individual tree as a random effect.

For soil N in New York and Hawaii and soil pH in Hawaii, where we had a single year of measurement, we used a linear regression with fertilization treatment and species within a site as fixed effects. For all post hoc pairwise comparisons we used emmeans (Lenth, 2022). To assess whether N fixation values differed from 0, we used 95% confidence intervals.

We also ran statistical analyses to determine how $\%N_{dfa}$ affected absolute aboveground biomass growth (log-transformed) and foliar N. For log-transformed absolute aboveground biomass growth, we used a linear mixed-effects model with $\%N_{dfa}$ and aboveground biomass as fixed effects and individual tree as a random effect. For foliar N, we used a mixed-effects model with $\%N_{dfa}$ as a fixed effect and individual tree as a random effect. For both models, we also tried additional models that included fixed effects of treatment, which gave identical qualitative results.

To see how well nodule biomass predicted total N_{dfa} (in units of g N fixed $tree^{-1}$ $year^{-1}$), we used a linear regression. We also used a linear regression to determine how well cellulose disk isotopic composition predicted the foliar N isotopic composition of nonfixers.

RESULTS

Overview

Our primary objective was to answer the following question: What are the SNF strategies of these six symbioses? Our experimental N additions, which alleviated N limitation for all six tree species (Figure 3, Appendix S1: Figures S2–S4), allowed us to answer this question.

To further explore the effects of our treatments, we assessed whether N fertilization over several years increased the N content in foliage and soil extractable inorganic N. As expected, adding N increased, or tended to increase, the N content of nonfixing tree foliage (Table 2). N-fixing trees, for the most part, did not have higher foliar N content when fertilized (Table 2). Adding N increased soil extractable inorganic N dramatically in the temperate sites, but marginally or not at all in Hawaii (Tables 3 and 4, Appendix S1: Table S2). Despite the small effect of fertilization on soil extractable inorganic N in Hawaii, the lack of a growth response to N addition over years clearly demonstrates a lack of N limitation. Soil extractable inorganic N merely gives a snapshot of a dynamic pool, and we suspect that our soil extractable inorganic N measurements in Hawaii, which were taken 3 months after the final fertilization, were at the wrong time to detect the effect of fertilization. Elsewhere in Hawaii, N fertilization in a non-N-limited site resulted in a rise in extractable ammonium and nitrate over a few weeks, but by 3 months the fertilized plots had similar extractable ammonium and nitrate pools as the control plots (Hall & Matson, 2003). We further detail our interpretations of limitation in Appendix S5.

Finally, to answer our primary question, we determined the SNF strategies of each species (Figures 4–6). For all species, $\%N_{dfa}$ was relatively high in our control treatment and remained above zero in our fertilized treatments (with the exception of one treatment in one species), suggesting that none of these species is perfectly facultative (Figure 5). Some species downregulated SNF partially in the fertilized treatments, indicating incomplete downregulation or obligate SNF for all species (Figure 6). In the following sections we provide the details of these results.

Tree growth and size

We addressed N limitation and combined N and P limitation of absolute (Figure 3) and relative (Appendix S1: Figure S2) growth rates for aboveground biomass for each species and for total biomass (Appendix S1: Figures S3 and S4) for the four species in New York and in Oregon. In New York, *Betula* was N limited in the control treatment but not in the fertilized treatments, according to all metrics (Figure 3a–e, Appendix S1: Figures S2–S4a–e). *Betula* did not respond to P addition (Figure 3a–e, Appendix S1: Figures S2–S4a–e). *Robinia* was not limited by N or the combination of N and P in any treatment (Figure 3a–e, Appendix S1: Figures S2–S4a–e). Overall, the limitation scenario in New York matches the “moderate N supply” region in Figure 1 for the control treatment (nonfixer grows more with additional N supply but the N fixer does not) and the “high N supply” region for the +10 and +15 treatments (both the nonfixer and the N fixer have sufficient N). Given this limitation scenario, the way to distinguish between different N fixation strategies is to examine $\%N_{dfa}$ and total N_{dfa} in these regions. Specifically, nonzero $\%N_{dfa}$ and nonzero total N_{dfa} in the +10 and +15 treatments would be evidence against perfectly facultative SNF, and a decline in total N_{dfa} from the control to the N-fertilized treatments would indicate either incomplete downregulation or perfectly facultative SNF.

In Oregon (Figure 3f–j, Appendix S1: Figures S2–S4f–j), Waiakea (Figure 3k–n, Appendix S1: Figure S2k–n), and Volcano (Figure 3o–r, Appendix S1: Figure S2o–r), we detected no N or P limitation in any of the nonfixing or N-fixing species. The only differences that were statistically significantly were that the relative growth rates of aboveground biomass in the +15 g N m^{-2} $year^{-1}$ treatment were lower than the +15 g N m^{-2} $year^{-1}$ +15 g P m^{-2} $year^{-1}$

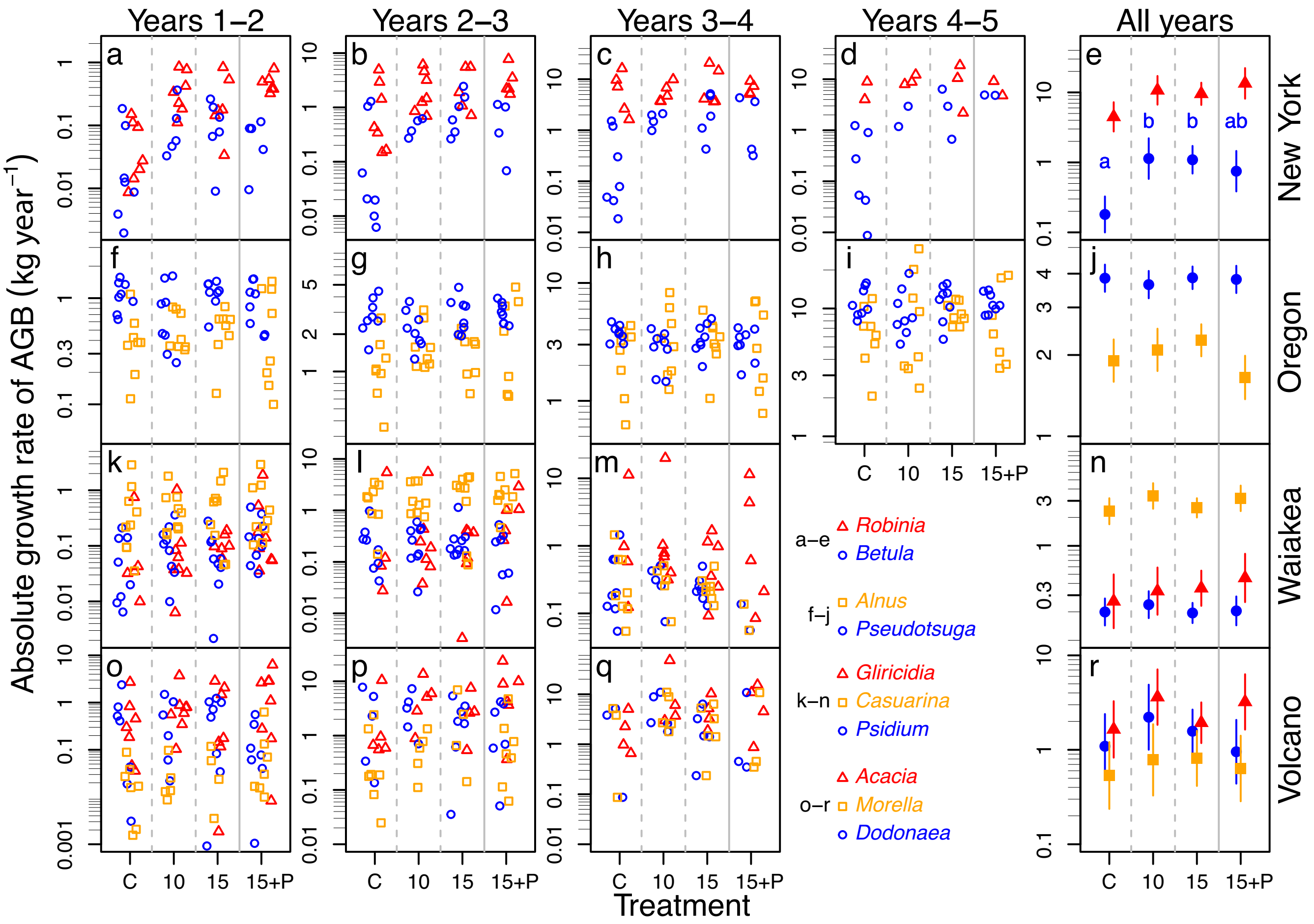


FIGURE 3 Aboveground biomass absolute growth rate (AGR) response to fertilization. The treatments are control (+≤0.1 g N m^{-2} year^{-1}; "C"), +10 g N m^{-2} year^{-1} ("10"), +15 g N m^{-2} year^{-1} ("15"), and +15 g N m^{-2} year^{-1} + 15 g P m^{-2} year^{-1} ("15+P"). Each point in (a)–(d), (f)–(i), (k)–(m), and (o)–(q) represents the change in one tree's aboveground biomass from one year's census to the next, for years 1–2 (a, f, k, o), 2–3 (b, g, l, p), 3–4 (c, h, m, q), and 4–5 (d, i). Points are jittered horizontally; gray vertical lines separate the treatments. The sample size is ≤8, as some trees died or were unhealthy. The genera for New York (a–e), Oregon (f–j), Waiakea (k–n), and Volcano (o–r) are indicated in the blank spaces below panel (i). Colors and symbols represent fixer types: rhizobial nitrogen-fixing trees (red triangles), actinorhizal nitrogen-fixing trees (orange squares), and nonfixing trees (blue circles). Points and error bars in (e), (j), (n), and (r) represent parameter estimates ± SE from a mixed-effects model fit to the data for each species, with fixed effects for treatment and aboveground biomass and a random effect for individual trees. Because the AGR varies greatly with size, the parameters in (e), (j), (n), and (r) are plotted for a tree of average size for the species in 2018. Parameter estimates with the same letter are not significantly different within a species (color for a given panel). Letters are not shown when there are no significant differences (j, n, r, and *Robinia* for e).

treatment for *Psidium* (nonfixer in Waiakea; Appendix S1: Figure S2n) and higher than the control and +15 g N m^{-2} year^{-1} +15 g P m^{-2} year^{-1} treatments for *Morella* (actinorhizal N-fixer in Volcano; Appendix S1: Figure S2r). Both of these trends were driven by the initial year's measurements (Appendix S1: Figure S2k,o), where small differences in the initial size had an outsized importance given that relative growth was calculated as a difference of logarithms. Overall, the limitation scenarios in Oregon, Waiakea, and Volcano match the "high N supply" region in Figure 1 for all treatments, which means that nonzero fixation (%N$_{\text{dfa}}$ or total N$_{\text{dfa}}$) in any treatment would be consistent with either obligate or incomplete downregulation. A decline in N fixation (total N$_{\text{dfa}}$, not %N$_{\text{dfa}}$) at higher fertilization would indicate incomplete downregulation, but constant N fixation (total N$_{\text{dfa}}$) across fertilization treatments cannot distinguish between obligate and incomplete downregulation.

Foliar N

As expected, adding N increased (statistically significant difference), or tended to increase (no statistically significant difference, but means changed by at least 10%), the N concentration of foliage in most of the nonfixing trees

TABLE 2 Foliar nitrogen (mg N g^{-1}) across treatments.

Site	Species	Control	+10 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$ +15 g P m^{-2} $year^{-1}$
New York	*Robinia*^{R}	32.0 (30.9–33.1)^{c}	33.4 (32.3–34.5)^{c}	33.9 (32.7–35.1)^{c}	36.1 (34.8–37.3)^{c}
New York	*Betula*	18.4 (17.3–19.4)^{a}	21.0 (19.6–22.3)^{ab}	25.0 (23.8–26.2)^{b}	23.8 (22.5–25.1)^{b}
Oregon	*Alnus*^{A}	25.8 (25.1–26.5)^{c}	26.1 (25.4–26.8)^{c}	25.5 (24.8–26.3)^{c}	25.1 (24.4–25.9)^{c}
Oregon	*Pseudotsuga*	15.5 (14.7–16.2)^{a}	17.0 (16.3–17.7)^{ab}	17.7 (17.0–18.4)^{ab}	19.6 (18.8–20.3)^{b}
Waiakea	*Gliricidia*^{R}	32.3 (31.0–33.6)^{d}	33.5 (32.5–34.5)^{d}	34.3 (33.3–35.4)^{de}	39.0 (37.9–40.2)^{e}
Waiakea	*Casuarina*^{A}	17.2 (16.3–18.1)^{bc}	17.6 (16.7–18.5)^{bc}	18.0 (17.1–18.9)^{bc}	18.8 (18.0–19.7)^{c}
Waiakea	*Psidium*	11.6 (10.7–12.5)^{a}	13.8 (12.9–14.8)^{ab}	14.3 (13.4–15.3)^{ab}	16.6 (15.4–17.7)^{bc}
Volcano	*Acacia*^{R}	19.7 (18.1–21.4)^{a}	21.3 (19.7–22.8)^{a}	22.4 (20.8–24.1)^{a}	20.1 (18.7–21.6)^{a}
Volcano	*Morella*^{A}	18.1 (16.8–19.5)^{a}	17.5 (16.1–19.0)^{a}	16.8 (15.1–18.4)^{a}	21.2 (20.0–22.5)^{a}
Volcano	*Dodonaea*	20.0 (18.2–21.7)^{a}	20.2 (18.7–21.7)^{a}	21.9 (20.6–23.2)^{a}	24.6 (23.0–26.2)^{a}

Note: Mean parameter values ± SE are shown. The parameters are mixed-model parameter estimates across all years for which we had data. Comparing within sites, treatments with the same letter are not significantly different. Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively.

TABLE 3 Extractable soil nitrate (µg (NO_2 + NO_3)-N g $soil^{-1}$) across treatments.

Site	Species	Control	+10 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$ +15 g P m^{-2} $year^{-1}$
New York	*Robinia*^{R}	0.25 (0.11–0.55)^{a}	32.6 (17.0–62.5)^{b}	65.0 (33.8–124.7)^{b}	41.2 (18.5–91.6)^{b}
New York	*Betula*	0.13 (0.08–0.21)^{a}	12.9 (5.8–28.7)^{b}	14.1 (7.3–27.0)^{b}	24.4 (11.0–54.2)^{b}
Oregon	*Alnus*^{A}	4.3 (3.2–5.9)^{a}	48.8 (36.1–66.0)^{b}	50.8 (37.6–68.7)^{b}	62.4 (45.2–86.1)^{b}
Oregon	*Pseudotsuga*	1.3 (1.0–1.8)^{a}	34.1 (25.2–46.1)^{b}	41.3 (30.6–55.8)^{b}	44.7 (33.1–60.4)^{b}
Waiakea	*Gliricidia*^{R}	0.70 (0.37–1.35)^{a}	1.1 (0.6–1.9)^{a}	1.2 (0.7–2.0)^{a}	2.2 (1.2–4.3)^{a}
Waiakea	*Casuarina*^{A}	0.48 (0.30–0.76)^{a}	0.52 (0.34–0.81)^{a}	0.71 (0.45–1.12)^{a}	1.6 (1.0–2.5)^{a}
Waiakea	*Psidium*	0.25 (0.15–0.42)^{a}	1.0 (0.5–1.8)^{a}	0.48 (0.25–0.92)^{a}	1.8 (0.7–4.5)^{a}
Volcano	*Acacia*^{R}	0.21 (0.13–0.34)^{a}	3.8 (2.3–6.2)^{b}	3.6 (2.2–5.9)^{b}	2.8 (1.7–4.6)^{b}
Volcano	*Morella*^{A}	2.0 (1.4–2.8)^{b}	1.8 (1.1–2.7)^{ab}	8.9 (5.5–14.5)^{b}	3.0 (2.1–4.4)^{b}
Volcano	*Dodonaea*	1.0 (0.6–1.7)^{ab}	1.7 (1.1–2.7)^{ab}	2.4 (1.6–3.5)^{b}	2.8 (1.6–4.9)^{b}

Note: Mean parameter values ± SE, back-transformed from analysis of log-transformed data, are shown. The parameters are estimates across all years for which we had data. Comparing within sites, treatments with the same letter are not significantly different. Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively. Data are from the top 10 cm of soil at each site in 2019 (for New York and Hawaii) or 2018 and 2019 together (for Oregon). Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively.

(Table 2). In *Betula*, foliar N content rose 36% from the control to the +15 g N m^{-2} $year^{-1}$ treatment, and in *Pseudotsuga* and *Psidium* it tended to rise from the control to both the +10 g N m^{-2} $year^{-1}$ (10% and 19%, respectively) and +15 g N m^{-2} $year^{-1}$ (14% and 23%, respectively) treatments. The exception was *Dodonaea*, where there was no discernible increase in foliar N content with N fertilization. In *Pseudotsuga* and *Psidium*, adding P along with N increased foliar N content compared to the control treatment (by 26% and 43% for *Pseudotsuga* and *Psidium*, respectively) but only tended to increase it (by 11% and 16% for *Pseudotsuga* and *Psidium*, respectively, but not significantly) compared to the +15 g N m^{-2} $year^{-1}$ treatment (Table 2).

No N-fixing tree species significantly increased its foliar N content as we added N, and only one treatment in one species tended to increase (*Acacia* in the +15 g N m^{-2} $year^{-1}$ treatment compared to the control; a 14% but nonsignificant increase; Table 2). For *Gliricidia*, adding P increased foliar N content above the control and the +10 g N m^{-2} $year^{-1}$ treatment and tended to increase it above the +15 g N m^{-2} $year^{-1}$ treatment. For the other N-fixing species, adding P had no significant effect on foliar N content, though it tended to increase foliar N content compared to the control in *Robinia* and *Morella*.

In New York, Oregon, and Waiakea, N-fixing trees had higher foliar N content than their nonfixing reference

TABLE 4 Extractable soil ammonium (μg NH_4-N g $soil^{-1}$) across treatments.

Site	Species	Control	+10 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$	+15 g N m^{-2} $year^{-1}$ +15 g P m^{-2} $year^{-1}$
New York	*Robinia*^{R}	23.3 (17.1–31.6)^{ab}	52.9 (41.2–68.1)^{abc}	64.5 (50.2–82.9)^{bc}	116.2 (85.4–158.0)^{c}
New York	*Betula*	21.4 (17.9–25.5)^{a}	46.0 (33.8–62.6)^{abc}	71.2 (55.4–91.6)^{bc}	82.3 (60.5–112.0)^{bc}
Oregon	*Alnus*^{A}	5.2 (4.1–6.6)^{a}	43.6 (34.4–55.3)^{b}	44.4 (35.0–56.3)^{b}	98.8 (76.6–127.4)^{b}
Oregon	*Pseudotsuga*	4.8 (3.8–6.1)^{a}	43.4 (34.2–55.0)^{b}	52.9 (41.7–67.0)^{b}	57.4 (45.3–72.9)^{b}
Waiakea	*Gliricidia*^{R}	3.5 (2.9–4.2)^{a}	3.3 (2.8–3.8)^{a}	3.3 (2.8–3.9)^{a}	3.8 (3.1–4.6)^{a}
Waiakea	*Casuarina*^{A}	3.7 (3.2–4.2)^{a}	3.4 (3.0–3.9)^{a}	3.1 (2.7–3.5)^{a}	4.3 (3.7–4.8)^{a}
Waiakea	*Psidium*	2.3 (2.0–2.7)^{a}	2.9 (2.4–3.5)^{a}	2.8 (2.3–3.5)^{a}	4.4 (3.3–5.7)^{a}
Volcano	*Acacia*^{R}	4.1 (3.3–5.0)^{a}	7.1 (5.8–8.7)^{a}	9.5 (7.8–11.7)^{a}	5.7 (4.7–7.0)^{a}
Volcano	*Morella*^{A}	6.6 (5.7–7.7)^{a}	5.6 (4.6–6.7)^{a}	4.1 (3.4–5.1)^{a}	5.7 (4.9–6.6)^{a}
Volcano	*Dodonaea*	9.0 (7.1–11.4)^{a}	9.3 (7.8–11.2)^{a}	6.0 (5.0–7.0)^{a}	6.5 (5.1–8.2)^{a}

Note: Mean parameter values ± SE, back-transformed from analysis of log-transformed data, are shown. The parameters are estimates across all years for which we had data. Comparing within sites, treatments with the same letter are not significantly different. Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively. Data are from the top 10 cm of soil at each site in 2019 (for New York and Hawaii) or 2018 and 2019 together (for Oregon). Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively.

trees, although the effect was only marginal for some of the treatments comparing *Casuarina* to *Psidium* (Table 2). Some of these differences were large. In the control treatments, for example, *Robinia*'s foliar N content was 32 mg N g dry $leaf^{-1}$, compared to 18 for *Betula*. We observed similarly strong differences in Oregon and Waiakea. In Volcano, however, *Acacia* and *Morella* had foliar N contents similar to that of *Dodonaea* in the control treatment.

Soil chemistry

At harvest time, which was approximately 3 months after the final fertilization, we measured KCl-extractable soil ($NO_2 + NO_3$)-N (hereafter, nitrate), NH_4-N (ammonium), total inorganic N (nitrate and ammonium), and, in Hawaii, soil pH. In New York, adding N led to dramatic increases in soil inorganic N (Tables 3 and 4, Appendix S1: Table S2). Extractable nitrate in the N-fertilized treatments, for instance, was 100- to 260-fold what it was in the control (Table 3). Ammonium in the N-fertilized treatments was 2.1- to 3.3-fold what it was in the control (Table 4), and total extractable inorganic N was 2.8- to 5.5-fold greater in the N-fertilized treatments compared to the control (Appendix S1: Table S2). Although it was not significant, extractable nitrate (Table 3) and total inorganic N (Appendix S1: Table S2) tended to be higher under *Robinia* than under *Betula* (twofold to 4.6-fold mean differences within treatments for nitrate, 10%–46% for total inorganic N).

Oregon showed the same qualitative trends as New York, though the effect of adding N was more dramatic in Oregon than New York for ammonium (eight- to 11-fold higher than controls; Table 4) and less dramatic for nitrate (11- to 32-fold higher than controls; Table 3). Total extractable inorganic N, therefore, was 9- to 14-fold higher in the N-fertilized treatments (Appendix S1: Table S2). Also similar to New York, extractable inorganic N tended to be higher under *Alnus* than under *Pseudotsuga* (Tables 3 and 4, Appendix S1: Table S2) in the control treatments (by 51%), though it was not significant.

In both Hawaii sites the effects of fertilization on extractable inorganic N were much more muted, and, especially in the fertilized treatments, soil extractable N concentrations were low compared to the temperate sites. Fertilizing with N increased soil extractable nitrate in *Acacia* (Table 3), but not in the other cases in Hawaii. In *Acacia* the effect of fertilization on nitrate concentrations was less dramatic than in the temperate sites, though it was still substantial (18-fold; Table 3). Adding ammonium nitrate tended to decrease soil pH in most cases, although the only significant comparison was a 0.52-pH unit drop from the control to the +15 g N m^{-2} $year^{-1}$ treatment in *Gliricidia* (Appendix S1: Table S3).

Foliar ^{15}N

The purpose of adding ^{15}N to the soil was to create distinct isotopic signatures of soil-derived N compared to atmospheric N, the latter of which is effectively constant on an enriched scale (0.3663 atom $\%^{15}N$). To determine how effective our ^{15}N application was, we present the raw ^{15}N data from nonfixer foliage, which we use as reference trees to estimate the soil N pool that the

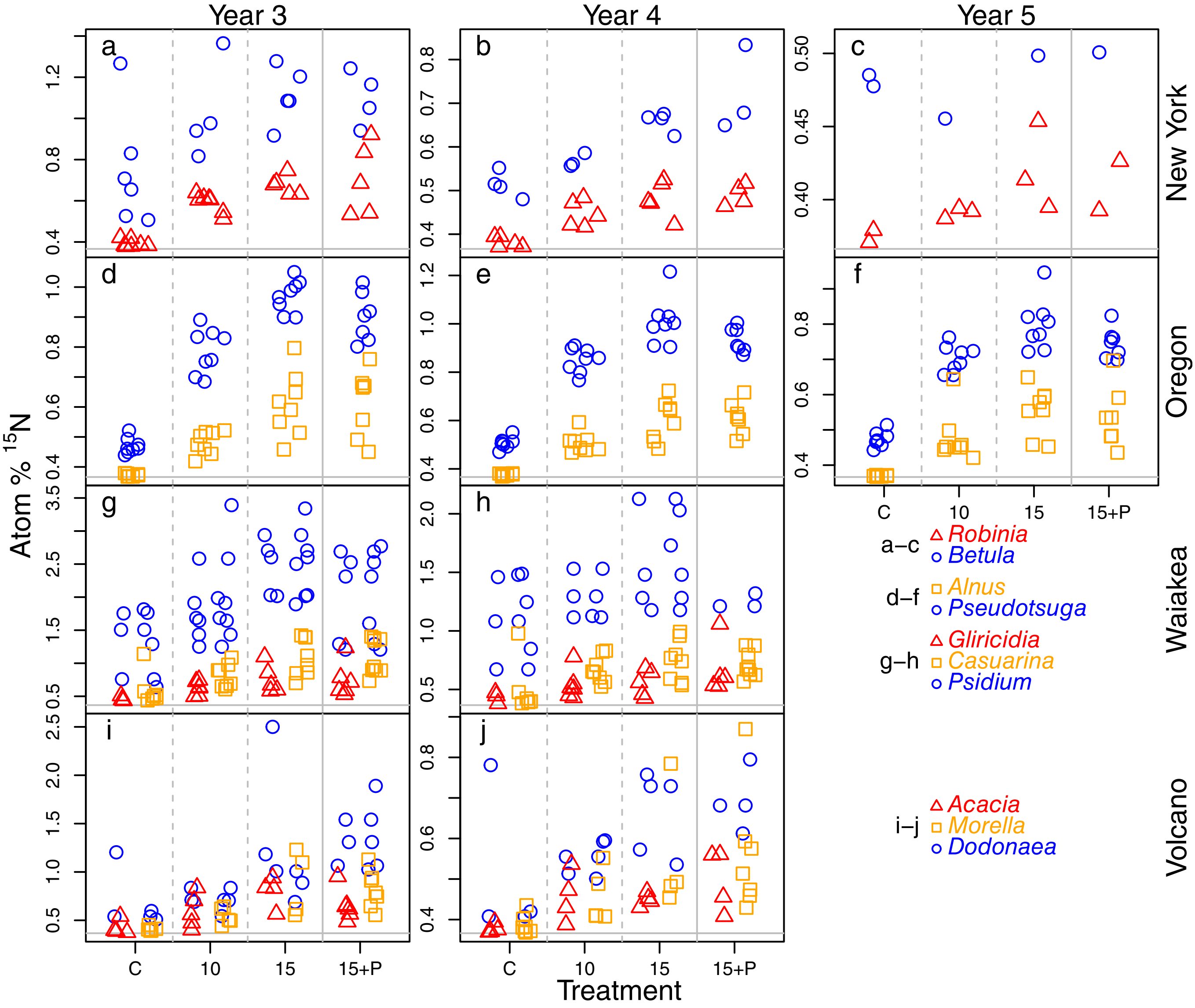


FIGURE 4 Foliar ^{15}N. Treatments, colors, symbols, jittering, and gray vertical lines are as in Figure 3. Each point is the atom percentage of ^{15}N in a single tree's foliage taken in Years 3 (a, d, g, i), 4 (b, e, h, j), and 5 (c, f) from species grown in New York (a–c), Oregon (d–f), Waiakea (g and h), and Volcano (i and j). These are the data used to calculate $\%N_{dfa}$ (Figure 5). The end members for each fixer's foliar ^{15}N are the value of pure fixation (0.3663% ^{15}N, indicated by the horizontal gray line) and the paired nonfixer's foliar ^{15}N, the latter of which is vertically aligned with the fixer. In cases where the nonfixer was dead or unhealthy, the average of all the healthy nonfixers paired to a healthy fixer in a treatment was used as the end member. Nonfixer trees are shown for each time they are paired to a fixer, which means that some of the nonfixers in Waiakea and Volcano are displayed twice. Note the different vertical axis scales in the different panels.

N fixers access. At the end of Year 2 (the first year of isotopic labeling), the signal was present but not sufficiently consistent (Appendix S1: Figure S5) in New York. Therefore, we focused on Years 3 (the second and final year of isotopic labeling) through 5, where the isotopic labeling was sufficiently consistent (Figure 4). The atom %^{15}N of the nonfixer foliage declined over time, as expected, but the value within each treatment was sufficiently distinct from 0.3663 to give reliable estimates of $\%N_{dfa}$. Figure 4 also shows the isotopic values of foliage from the N-fixing trees, which shows a visual representation of the mixing model used to calculate $\%N_{dfa}$.

Symbiotic N fixation: $\%N_{dfa}$

All species in all treatments fixed significant fractions of their N except for *Morella* in the +15 g N m^{-2} year^{-1} treatment (Figure 5, Appendix S1: Table S4). Most showed a trend toward lower $\%N_{dfa}$ with added fertilizer, which was

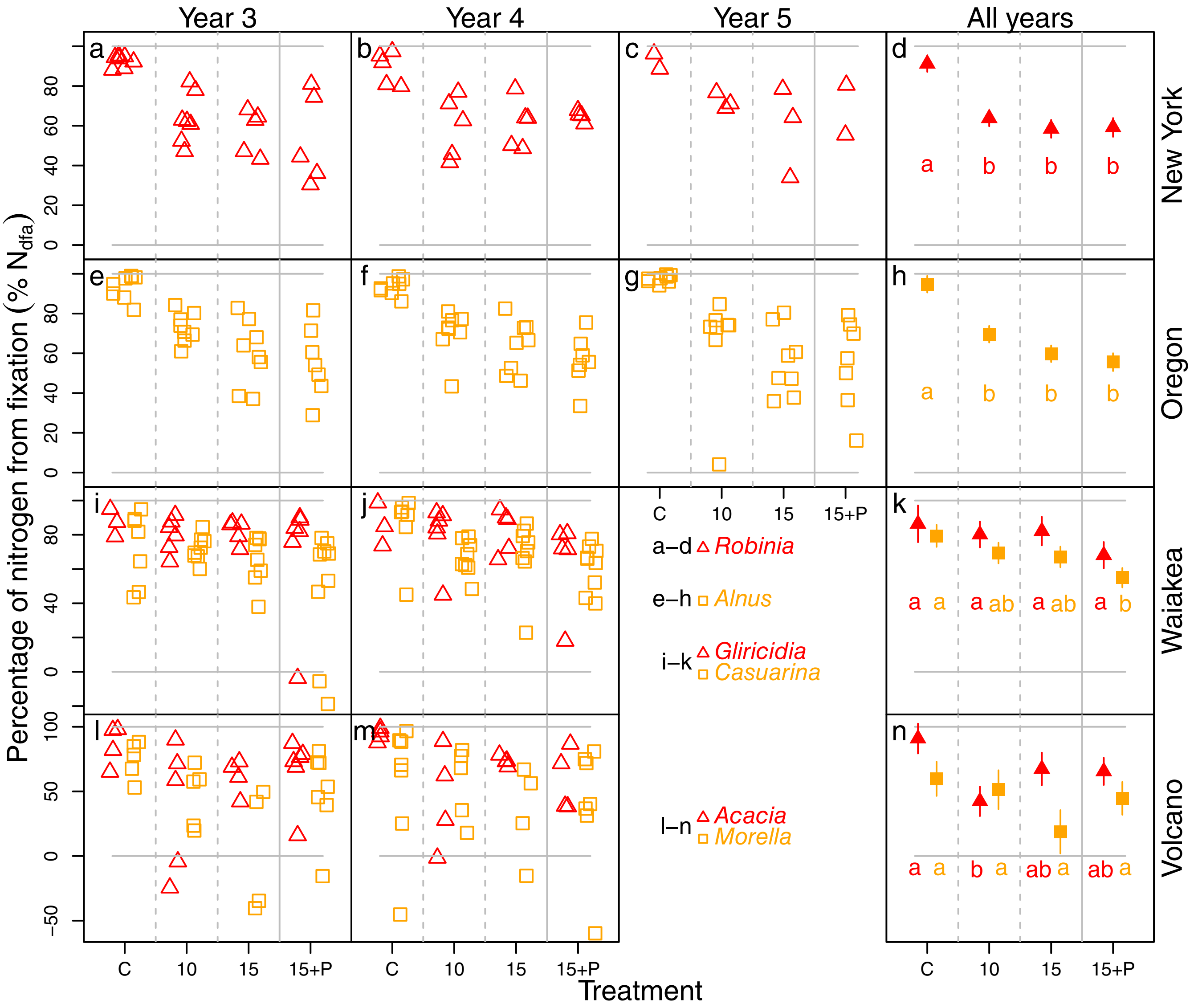


FIGURE 5 %N_{dfa} response to fertilization treatments. Treatments, colors, symbols, jittering, and gray vertical lines are as in Figure 3. Each point in (a)–(c), (e)–(g), (i), (j), (l), and (m) is the percentage of nitrogen from fixation (%N_{dfa}) (as opposed to the soil) from a single tree in Years 3 (a, e, i, l), 4 (b, f, j, m), and 5 (c, g) from species grown in New York (a–c), Oregon (e–g), Waiakea (i, j), and Volcano (l, m). These are calculated from the data in Figure 4 using the paired nonfixers as end members (or the average within a treatment where the paired nonfixers are dead or unhealthy); see Appendix S1: Table S4 for alternative ways to calculate %N_{dfa}. Gray horizontal lines indicate 0% and 100% N_{dfa}. Note the different vertical axis scales in Waiakea (i–k) and Volcano (l–n), which had some individual tree values below 0%. Points and error bars in (d), (h), (k), and (n) represent parameter estimates ± SE from a mixed-effects model fit to the data for each species, with a fixed effect for treatment and a random effect for individual trees. Parameter estimates with the same letter are not significantly different from each other. All parameter estimates are significantly different from 0 (based on 95% confidence intervals) except for *Morella* in the +15 g N m^{-2} $year^{-1}$ treatment.

significant in some species. *Robinia* fixed about 91% of its N in the control treatment, which was higher than in the +10 g N m^{-2} $year^{-1}$ and +15 g N m^{-2} $year^{-1}$ treatments (64% and 59%, respectively, which were not significantly different from each other; Figure 5a–d, Appendix S1: Table S4). *Alnus* was similar to *Robinia*, declining from 95% in the control to 70% and 60% in the two N-fertilized treatments (Figure 5e–h, Appendix S1: Table S4). *Gliricidia* and *Casuarina* fixed similar fractions of their N across the N fertilization treatments, with treatment means of 80%–86% for *Gliricidia* and 67%–79% for *Casuarina* (Figure 5i–k, Appendix S1: Table S4). *Acacia*'s %N_{dfa} declined substantially from the control (91%) to the +10 g N m^{-2} $year^{-1}$ treatment (42%), although it was somewhat higher (67%) and statistically indistinguishable from the control in the +15 g N m^{-2} $year^{-1}$ treatment

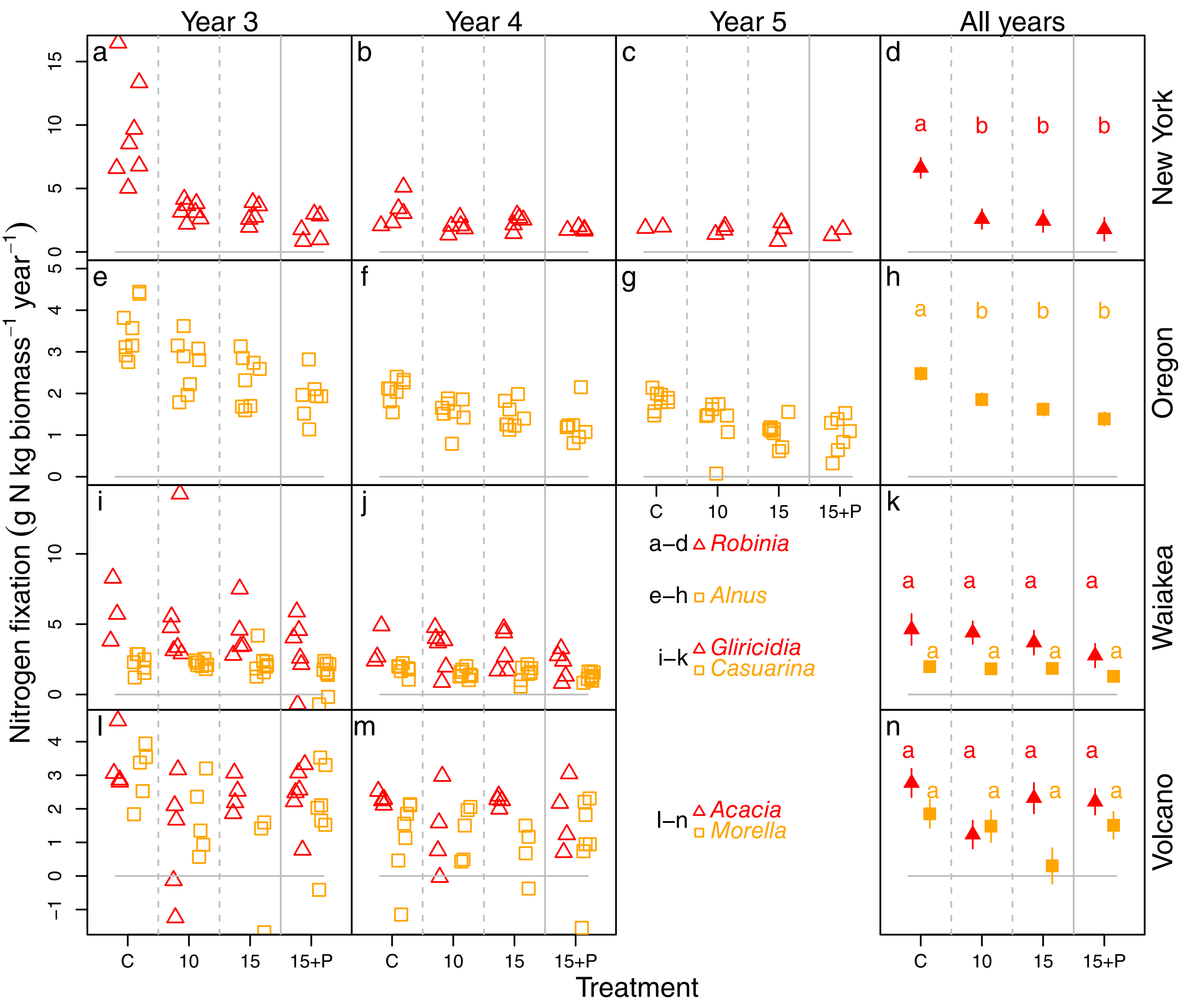


FIGURE 6 Nitrogen fixation (g N kg biomass^{-1} year^{-1}) response to fertilization treatments. All details are as in Figure 5, except that the vertical axis shows total N fixation per biomass instead of %N$_{\mathrm{dfa}}$, and there is no horizontal gray line at 100%. Nitrogen fixation per biomass is calculated using %N$_{\mathrm{dfa}}$, total plant N, and plant biomass, as described in the *Methods* section.

(Figure 5l–n, Appendix S1: Table S4). *Morella* had similar %N$_{\mathrm{dfa}}$ in the control (60%) and the +10 g N m^{-2} year^{-1} (51%) treatments, with a significant drop in the +15 g N m^{-2} year^{-1} treatment (19%), where its 95% confidence interval overlapped with 0 (Figure 5l–n, Appendix S1: Table S4).

Adding P did not stimulate %N$_{\mathrm{dfa}}$ in any species (Figure 5, Appendix S1: Table S4). Where there was a trend (none of which were significant), adding P suppressed %N$_{\mathrm{dfa}}$ relative to the +15 g N m^{-2} year^{-1} treatment, with the exception of *Morella*. For *Morella*, %N$_{\mathrm{dfa}}$ in the P addition treatment trended higher than the +15 g N m^{-2} year^{-1} treatment but lower than the +10 g N m^{-2} year^{-1} treatment.

The sensitivity checks on our %N$_{\mathrm{dfa}}$ calculation revealed that our calculations were robust. Compared to the base case (reported earlier in the text, in Figure 5, and in the base case scenario of Appendix S1: Table S4), the "mean of reference trees in treatment" case gave similar results. For *Robinia*, *Alnus*, *Gliricidia*, *Casuarina*, *Acacia*, and *Morella*, respectively, the "mean of reference trees in treatment" method changed treatment-level %N$_{\mathrm{dfa}}$ estimates by no more than 1.1%, 0.5%, 4.1%, 4.3%, 1.8%, and 5.2% (Appendix S1: Table S4).

For the sensitivity check where we assumed that N-fixing trees used half as much stored N as nonfixing trees ("modified retranslocation"), estimates of %N$_{\mathrm{dfa}}$ were mostly similar to the base case, though a couple treatments diverged more. For *Robinia*, *Alnus*, *Gliricidia*, *Casuarina*, *Acacia*, and *Morella*, respectively, the "modified retranslocation" method changed treatment-level $\%N_{dfa}$ estimates by no more than 7.3%, 2.8%, 1.5%, 4.3%, 17.2%, and 29.0% (Appendix S1: Table S4).

Even with these changes, both of the first two sensitivity calculations gave similar significance results (Appendix S1: Table S4). There were three exceptions. First, in the "mean of reference trees" sensitivity check, *Casuarina* showed no statistical difference across treatments, as opposed to a difference between the control and the $+15\ g\ N\ m^{-2}\ year^{-1}$+$15\ g\ P\ m^{-2}\ year^{-1}$ treatment in the base case. Second, in the "modified retranslocation" sensitivity check, *Acacia* showed no statistical differences across treatments as opposed to being lower in the $+10\ g\ N\ m^{-2}\ year^{-1}$ treatment in the base case. Third, in the "modified retranslocation" sensitivity check, the 95% confidence intervals of $\%N_{dfa}$ for *Morella* overlapped zero in the $+10\ g\ N\ m^{-2}\ year^{-1}$ treatment (despite a slightly higher mean estimate of $\%N_{dfa}$ than in the base case—53% vs. 51%) and in the $+15\ g\ N\ m^{-2}\ year^{-1}$ $+15\ g\ P\ m^{-2}\ year^{-1}$ treatment.

The third and final sensitivity check was an alternate interpretation of our base case, addressing the question: What fraction of N-fixing tree soil N uptake would have to occur outside the isotopically labeled zone (2-m radius), assuming none for the nonfixing reference tree, to explain the isotopic results if the N-fixing trees were not fixing any N? For *Robinia*, these numbers were 91%, 64%, and 59% for the control, $+10\ g\ N\ m^{-2}\ year^{-1}$ treatment, and $+15\ g\ N\ m^{-2}\ year^{-1}$ treatment (Figure 5, Appendix S1: Table S4). Well over half of a plant's N from outside a 2-m radius seems much larger than would be reasonable in our plants. When we harvested *Robinia* roots, we found some roots outside 2 m for the largest trees (Akana, 2022), but even so, we feel that our interpretation is robust, for two reasons. First, whereas most *Robinia* were substantially larger than most *Betula* when we harvested, the same was not true for the N fixer–nonfixer pairs in the other sites, although there were a couple of large individual *Gliricidia* and *Acacia* trees (Appendix S1: Figure S1). Therefore, a consistently larger rooting system for N fixers than nonfixers was mostly of concern in New York. Second, based on our allometric equations for *Robinia* biomass as a function of diameter, height, and canopy dimensions (Carreras Pereira et al., 2023; Appendix S3) and our allometric equations for *Robinia* lateral rooting extent as a function of biomass (Akana, 2022), we can estimate rooting extent in the past. From this estimate, trees with roots that extended beyond 2 m in Year 5 would have had many fewer roots beyond 2 m in Years 3 and 4. For example, the largest tree at harvest had about 80% of its root length outside 2 m in Year 5, compared with 5% in Year 3 and 40% in Year 4. Large quantities of N uptake from beyond 2 m would result in an increase in apparent $\%N_{dfa}$ in later years, yet the $\%N_{dfa}$ patterns were similar across all years (Figure 5). For the largest tree, the Year-3-to-Year-4-to-Year-5 trend in $\%N_{dfa}$ was 68%–79%–78%. Thus, even if more of its N was acquired from beyond 2 m in Year 5, its $\%N_{dfa}$ was still quite high in Year 3, when it had few roots outside 2 m. For the rest of the trees with final biomasses over 20 kg, the Year-3-to-Year 4-to-Year 5 trends in $\%N_{dfa}$ were 78%–63%–71%, 94%–63%–96%, 43%–64%–37%, 65%–64%–64%, 81%–66%–81%, and 61%–77%–69%. The lack of a consistent large rise in these $\%N_{dfa}$ numbers indicates that N uptake from beyond 2 m was not a major issue.

Overall, our $\%N_{dfa}$ results show strong evidence that all species we measured, regardless of their location of origin (temperate vs. tropical) or symbiotic type (rhizobial vs. actinorhizal), were either obligate or incomplete downregulators; none were perfectly facultative.

Symbiotic N fixation: Total N_{dfa}

Our two metrics of total N_{dfa}—N fixation per tree biomass per time and N fixation per tree per time—give different types of information than $\%N_{dfa}$. Both total N_{dfa} metrics are rates, whereas $\%N_{dfa}$ is a percentage. Of the two total N_{dfa} metrics, N fixation per biomass per time is more relevant for determining strategies, as it would be constant for obligate SNF. On the other hand, N fixation per tree per time is more relevant for ecosystem-level rates, as it retains the information about tree size, although we note that it only includes N in aboveground tissues. In our data, N fixation per biomass per time (Figure 6, Appendix S1: Table S5) gave nearly identical qualitative results to $\%N_{dfa}$: significant downregulation but definitively positive SNF in *Robinia* and in *Alnus*, suggesting incomplete downregulation in both (Table 5); no evidence for downregulation in *Gliricidia* or *Casuarina*, indicating either obligate SNF or incomplete downregulation (Table 5); and mixed evidence for downregulation in *Acacia* and *Morella*, indicating either obligate SNF or incomplete downregulation (Table 5). Similar to $\%N_{dfa}$, the only treatment where total N_{dfa} was not clearly positive was *Morella* in the $+15\ g\ N\ m^{-2}\ year^{-1}$ treatment.

N fixation per tree per year (Appendix S1: Figure S6 and Table S6), which is not diagnostic of SNF strategies but is relevant for ecosystem-level estimates, showed no decline in SNF with N fertilization. Even though many of the species downregulated SNF per biomass, the larger biomass in those individuals meant that tree-scale SNF was not statistically different across fertilization

TABLE 5 Symbiotic N fixation strategies observed in our experiment.

Site	Species	Strategy	Corresponding Figure 1 region(s)
New York	*Robinia*^{R}	Incomplete downregulation	Moderate for control, high for +10 and +15
Oregon	*Alnus*^{A}	Incomplete downregulation	High for all treatments
Waiakea	*Gliricidia*^{R}	Obligate or incomplete downregulation	High for all treatments
Waiakea	*Casuarina*^{A}	Obligate or incomplete downregulation	High for all treatments
Volcano	*Acacia*^{R}	Obligate or incomplete downregulation	High for all treatments
Volcano	*Morella*^{A}	Obligate or incomplete downregulation	High for all treatments

Note: Interpretations of our data in light of the theoretical N fixation strategies presented in Figure 1. Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively.

treatments. The large variation within each treatment (driven by variation in tree size) makes this an insensitive measure of responses to treatments and an insensitive measure of whether N fixation is greater than 0 (Appendix S1: Table S6).

Relationships between symbiotic N fixation and other variables

We also tested whether SNF, within individual species, enhanced growth or foliar N and whether nodule counts reflected isotope-based estimates of SNF. $\%N_{dfa}$ did not predict absolute aboveground biomass growth rates (Appendix S1: Figure S7) or foliar N content (Appendix S1: Figure S8) in any species. Nodule biomass in *Robinia* was not related to N fixation (total N_{dfa} per tree per time) in the nine trees from which we cored for nodules (Appendix S1: Figure S9).

DISCUSSION

Contrary to our hypotheses, we found that the N-fixing symbioses we examined continued to fix N at high rates even when they were not N limited. Also counter to expectations, in the symbioses we studied, we did not find that the tropical symbioses were more facultative than the temperate symbioses or that the rhizobial symbioses were more facultative than the actinorhizal symbioses. Statistically, all of our tropical symbioses fixed at similar rates regardless of N fertilization (indicating obligate N fixation), though there were tendencies toward some downregulation (indicating potentially incomplete downregulation). Both of our temperate species downregulated SNF somewhat but continued fixing a large amount of N even when not N limited (incomplete downregulation). These findings are contrary to the differential regulation hypothesis (Lu & Hedin, 2019; Menge et al., 2014; Menge, Batterman, Hedin, et al., 2017; Sheffer et al., 2015). If these findings are indicative of the average temperate and tropical SNF strategies, they suggest that different SNF strategies across latitude do not explain the latitudinal abundance distribution of N-fixing trees. This study is the most robust field-based experimental test to date of SNF strategies in these or any tree species, so, although it covers only six species, these results change our priors for understanding SNF in symbiotic trees. In the following sections, we discuss why obligate SNF or incomplete downregulation might occur, the current state of evidence for SNF strategies generally, and the implications of our findings for understanding the abundance distribution of symbiotic N-fixing trees, for ecosystem-level and climate effects of symbiotic N-fixing trees, and for modeling symbiotic N fixation.

Why obligate SNF or incomplete downregulation, and evidence for different SNF strategies

Given that N fixation is thought to be more energetically expensive than acquiring N from the soil, our findings bring to mind a basic question that Binkley et al. (1994) asked about *Alnus rubra*: Why would these species continue to fix N when they are not N limited? Theoretical work has explored reasons that plasticity in general (not just in the context of N fixation) might not be adaptive. Two main classes of explanation are that plasticity itself might be costly or that plasticity, which is imperfect, leads to a poor ability to match the environment (Auld et al., 2010; DeWitt et al., 1998; Pigliucci, 2005; van Kleunen & Fischer 2005). An inability to match the environment means that sticking to the "best" constant strategy, a form of bet hedging, can be the most adaptive (Klausmeier et al., 2007; Menge et al., 2011; Moran, 1992; Padilla & Adolph, 1996). In plants, the cost of plasticity is generally not large (Murren et al., 2015), so we do not find it to be the most compelling argument for why obligate SNF occurs. Furthermore, there is evidence that plants can turn SNF off entirely (e.g., Menge et al., 2015; Taylor & Menge, 2018), even if they did not in our study, suggesting that a cost of plasticity is not the driver of obligate SNF.

If the energetic cost of SNF itself is sufficiently close to the cost of soil N uptake, at least in some conditions, then the best strategy may be to maintain SNF at a constant rate, even when current soil N availability exceeds demand, due to uncertainty in future access to soil N. What environmental conditions could lead to a constant and high rate of SNF? A prime candidate is high light availability. Mechanistically, high light means a high capacity for photosynthesis, leading to abundant fixed carbon to fuel SNF, as hypothesized by Bormann and Gordon (1984) for alder. Abundant fixed carbon could lead to high SNF in two separate ways: source control, where excess carbon is used to fuel SNF, and sink control, where carbon drives growth, which increases N demand. The explanation that light could drive SNF, through either of these mechanisms, would help tie together our results with previous studies. The trees we studied here were spaced at least 5 m apart from each other in open fields in an attempt to separate root systems and minimize belowground competition, which also meant that they had plentiful light. Greenhouse and field work has consistently found strong effects of light on SNF, often stronger than the effects of soil N (Chou et al., 2018; McCulloch & Porder, 2021; McHargue, 1999; Myster, 2006; Sprent, 1973; Taylor & Menge, 2018, 2021). The field studies cited in the introduction (Barron et al., 2011; Batterman, Hedin, et al., 2013; Bauters et al., 2016; Binkley et al., 1992, 1994; Brookshire et al., 2019; Gei & Powers, 2015; Mead & Preston, 1992; Menge & Hedin, 2009; Ruess et al., 2009; Sullivan et al., 2014; Taylor et al., 2019; Winbourne, Feng, et al., 2018; Wong et al., 2020; Wurzburger & Hedin, 2016) did not manipulate N or light, but many of the findings are consistent with this interpretation. For example, N fixers in canopy gaps and shoreline sites in Barron et al. (2011) likely had higher light availability (light from multiple directions) than in mature forest sites, and N fixers in younger successional sites in some studies (e.g., Batterman, Hedin, et al., 2013; Sullivan et al., 2014, Wurzburger et al., 2022) likely had higher light than in older sites. The light interpretation is also consistent with another study in our experimental plots in Waiakea. *Mimosa pudica* and *Desmodium triflorum*, two ground-layer legume species that recruited naturally under and near our *Gliricidia*, *Casuarina*, and *Psidium* trees, had %N_{dfa} values similar to those of our trees (averages of 58%–82%), but their %N_{dfa} in the fertilized plots decreased with shade (Schmidt et al., 2023).

To be clear, our data do not suggest that all species in all conditions are obligate or incomplete downregulators. Instead, rather than "all rhizobial/tropical species are facultative, all actinorhizal/temperate species are obligate" (as previously hypothesized; Menge et al., 2009, 2014; Sheffer et al., 2015), our data are congruent with a different hypothesis, namely, that N fixation strategies vary with environmental conditions rather than being tied to particular taxonomic groups. For example, Taylor and Menge (2018) found that N-fixing trees are more obligate in high light and more facultative at low light, which is consistent with our field data, which are from the high light end of the spectrum. Other environmental conditions, such as temperature and water, are likely to be important as well, and other factors like ontogeny could also play roles. Studies that show declines in N fixation through succession, for example, could be explained by declines in N fixation with tree age rather than changes in soil properties or light (Wurzburger et al., 2022). Our trees were younger than most trees in successional studies, so their sustained N fixation could be a consequence of their youth.

We did manipulate one environmental condition often cited as a control on SNF: the availability of P. Some other studies have found that P can stimulate SNF (e.g., Batterman, Wurzburger, & Hedin, 2013; Crews, 1993), which is often thought to have high P requirements (Vitousek & Howarth, 1991). In our experiment, however, we found no P limitation to the growth or SNF of any of the species we studied, consistent with the lack of P limitation on other young soils of the Big Island of Hawaii (Vitousek & Farrington, 1997) and consistent with the lack of P limitation to SNF in the Pacific Northwest (Yelenik et al., 2013).

Latitudinal abundance distribution of symbiotic N-fixing trees

A main motivation for this study was to test the differential regulation hypothesis, which posits that a difference in SNF strategies (obligate at higher latitudes, facultative at lower latitudes) explains why N-fixing trees are 10 times more abundant at lower latitudes than higher latitudes in the Americas (Lu & Hedin, 2019; Menge et al. 2014; Menge, Batterman, Liao, et al., 2017; Menge, Batterman, Hedin, et al., 2017; Sheffer et al., 2015). Our results do not support the differential regulation hypothesis. As predicted by the hypothesis, both temperate species fixed plenty of N even when they had excess soil N, but, contrary to the hypothesis, all the tropical species we studied also fixed plenty of N when they had excess soil N. If the species in this study are indicative of other species in their biomes, then different strategies do not explain the latitudinal abundance distribution of N-fixing trees in the Americas.

If differential regulation does not explain the latitudinal abundance distribution of N-fixing trees in the Americas, what could? Of the four hypotheses proposed in Menge, Batterman, Hedin, et al. (2017), two remain plausible. One, the N limitation severity hypothesis, says that although N limitation of any degree is more common at higher latitudes, severe N limitation is more common at lower latitudes because of the greater growth potential at lower latitudes. If SNF is only cost-effective when N limitation is severe, then a greater prevalence of severe N limitation in the tropics could select for N fixers, who, once established, could persist for a while. The second hypothesis, the N fixation benefit–cost hypothesis, says that even if N limitation (including severe N limitation) is more common at higher latitudes, SNF is cost-effective at moderate N limitation at lower latitudes but only at severe N limitation at higher latitudes (Menge, Batterman, Hedin, et al., 2017). The high temperature optimum of SNF compared to photosynthesis (Bytnerowicz et al., 2022) suggests greater efficiency of SNF at lower latitudes, consistent with the N fixation benefit–cost hypothesis.

Ecosystem-level and climate effects of symbiotic N-fixing trees

From an ecosystem perspective, our results help explain the persistence of N-rich conditions in forests with N-fixing trees, including the paradox of N richness in tropical forests (Hedin et al., 2009), with implications for the broader climate system. According to theory, perfectly facultative SNF cannot lead to N-rich conditions, but obligate and incomplete SNF can (Hedin et al., 2009; Kou-Giesbrecht & Menge, 2019; Menge et al., 2009, 2015). Many forests where N-fixing trees are common build up large quantities of N in both biomass and soil (Binkley et al., 1992; Mayer et al., 2020; Vitousek et al., 1987; Vitousek & Walker, 1989). Obligate and incomplete SNF should increase soil N more than facultative SNF, providing additional N to increase growth and C uptake by co-occurring nonfixers. Both meta-analyses and large-scale surveys of mixed-species plots show that N fixers increase the basal area growth of nonfixers, though effects on total stand growth may be marginal or nonexistent (Lai et al., 2018; Piotto, 2008; Staccone et al., 2021; Taylor et al., 2017; Xu et al., 2020), especially in high N sites with weak N limitation (Binkley, 2003).

Obligate and incomplete SNF can also increase ecosystem N richness via legacies of fixed N in soil. The temperate N fixers we studied leave strong soil N legacies that persist after fixers are replaced (von Holle et al., 2013). This is especially true for *Alnus* in Oregon, where multiple disturbance–recovery cycles that promote SNF cause high N inputs and disequilibrium in N balances, leading to unusually high soil N accumulation and persistent N saturation (Perakis et al., 2011; Perakis & Sinkhorn, 2011). Legacy N in soil can stimulate the growth of subsequent nonfixing trees (Perakis & Sinkhorn, 2011; Schuster & Hutnik, 1987), though when in excess, it can eliminate N limitation altogether (Mainwaring et al., 2014). Most legacy N in soil is bound to soil C, and SNF frequently increases soil C storage as well (Dynarski et al., 2020; Mayer et al., 2020). Though multiple mechanisms may explain high soil C under N fixers, the presence of excess N due to obligate and incomplete SNF in particular may stabilize old organic C (i.e., slow or prevent decomposition) via inhibition of oxidative enzymes (Chen et al., 2018).

Obligate and incomplete downregulation of SNF can also explain high rates of N leaching and gaseous N loss under N-fixing trees (Devotta et al., 2021; Williard et al., 2005), including the tree species we studied (Binkley et al., 1992; Compton et al., 2003; Erickson & Perakis, 2014; Jackson et al., 2018). Losses as N_2O are of particular interest as a greenhouse gas, and direct measurements at our New York site revealed larger N_2O emissions under *Robinia* than *Betula*, as expected from incomplete downregulation of SNF (Kou-Giesbrecht, Funk, et al., 2021). According to a recent meta-analysis, N-fixing trees tend to stimulate (approximately double) N_2O emissions in general (Kou-Giesbrecht & Menge, 2021), consistent with widespread obligate SNF or incomplete downregulation of SNF.

Implications for modeling symbiotic N fixation

The regulation of SNF is included in many models (Stocker et al., 2016), ranging from simple theoretical models (e.g., Koffel et al., 2018; Kylafis & Loreau, 2008; Marleau et al., 2011) to ecosystem-scale models (e.g., Fisher et al., 2010; Levy-Varon et al., 2019; Rastetter et al., 2001; Vitousek & Field, 1999) to regional or global terrestrial biosphere models (e.g., Davies-Barnard et al., 2022; Gerber et al., 2010; Kou-Giesbrecht & Arora, 2022; Kou-Giesbrecht, Malyshev, et al., 2021; Lawrence et al., 2019; Meyerholt et al., 2016; Peng et al., 2020; Shi et al., 2016; Sulman et al., 2019; Wang et al., 2007; Wieder et al., 2015; Zhu et al., 2019). Some models include separate plant populations of nonfixers and of obligate N fixers (e.g., Sheffer et al., 2015; Vitousek & Field, 1999), which allows nitrostat dynamics to play out at the community level. Others models separate plant populations of nonfixers and of facultative N fixers (e.g., Sheffer et al., 2015), which allows for a more rapid feedback. Still others model the entire plant community as a single entity that is capable of fixing N if needed (e.g., Rastetter et al., 2001 and many of the previously listed terrestrial biosphere models), sometimes imposing different time scales to capture the essence of community-level versus individual-level nitrostats (e.g., Gerber et al., 2010).

The plethora of ways that SNF is modeled stems in part from the different goals and constraints of the different models, but different ways of modeling SNF have starkly different consequences for communities, ecosystems, and climate. Our field results suggest that modeling populations of N fixers that always fix (obligate or incomplete downregulators) is an appropriate approach, but that a strict taxonomy-based determination of strategies is not. However, our results are also consistent with a broader view that incorporates light, temperature, water, and potentially other environmental controls on within-individual regulation of SNF.

Implications for measuring symbiotic N fixation

Although it was not the main focus of our study, our results have implications for how to measure SNF. Whereas the cellulose disk method gave an accurate estimate of soil ^{15}N in a pot study (Hendricks et al., 2004), it did not in our field study, presumably due to more spatial variation in the ^{15}N signal of soil N across the rooting distribution of our trees than our disks could capture. Based on this finding, we do not recommend using cellulose disks for assessing the ^{15}N content of available N for trees in the field. Instead, we recommend the continued use of nonfixing reference trees for isotopic methods of assessing SNF in trees in the field.

Despite the known challenges of using nodule biomass to estimate SNF (Soper et al., 2021; Winbourne, Harrison, et al., 2018), we found it surprising that nodule biomass in *Robinia* (the only species we measured) showed no relationship whatsoever to our isotopic estimate of SNF. These measurements target different quantities: Our isotopic method estimates N fixed over a time span of a year or more, whereas nodule biomass gives a snapshot of current investment in SNF. Nodule biomass might have matched our isotopic estimate better if we had measured it in the middle of the growing season rather than near the end, but this further highlights the importance of choosing the right method for the question. Overall, for integrated measures of SNF, we recommend the isotopic dilution technique used here.

Conclusion

Multiple years of fertilization of field-grown trees in New York, Oregon, and Hawaii demonstrated that six N-fixing tree species maintained high rates of symbiotic N fixation even when they were demonstrably not N limited. We found no difference in SNF strategy across latitudinal provenance or bacterial association of the tree species. These findings do not support the differential regulation hypothesis: According to our findings, a transition in SNF strategy does not explain the 10-fold change in relative abundance of N-fixing trees across latitude in the Americas. Our results also help explain the tropical N paradox: Continued SNF in tropical forests even when trees are not N limited would bring excess N, leading to large N export. Finally, our results help explain why N-fixing trees double N_2O emissions.

AUTHOR CONTRIBUTIONS

Duncan N. L. Menge, Amelia A. Wolf, Jennifer L. Funk, and Steven S. Perakis designed the study. All authors conducted the experiment and collected data. Duncan N. L. Menge and Amelia A. Wolf analyzed data and wrote the manuscript. All authors edited the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data and code (Menge et al., 2022) are available in Dryad at https://doi.org/10.5061/dryad.fbg79cnxk.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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