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HIGHLIGHTED STUDENT RESEARCH

Isotopic constraints on plant nitrogen acquisition strategies during ecosystem retrogression

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Abstract

Plant root associations with microbes such as mycorrhizal fungi or N-fixing bacteria enable ecosystems to tap pools of nitrogen (N) that might otherwise be inaccessible, including atmospheric N or N in large soil organic molecules. Such microbially assisted N-foraging strategies may be particularly important in late-successional retrogressive ecosystems where productivity is low and soil nutrients are scarce. Here, we use natural N-stable isotopic composition to constrain pathways of N supplies to different plant functional groups across a well-studied natural soil fertility gradient that includes a highly retrogressive stage. We demonstrate that ectomycorrhizal fungi, ericoid mycorrhizal fungi, and N-fixing bacteria support forest N supplies at all stages of ecosystem succession, from relatively young, N-rich/phosphorus (P)-rich sites, to ancient sites (ca. 500 ky) where both N supplies and P supplies are exceedingly low. Microbially mediated N sources are most important in older ecosystems with very low soil nutrient availability, accounting for 75–96% of foliar N at the oldest, least fertile sites. These isotopically ground findings point to the key role of plant–microbe associations in shaping ecosystem processes and functioning, particularly in retrogressive-phase forest ecosystems.

Keywords Stable isotopes · Mycorrhizal fungi · N fixation · Nutrient limitation · Ecosystem retrogression

Introduction

Plant–microbe interactions are critical drivers of ecosystem functioning and nutrient cycling (Phillips et al. 2013; Averill et al. 2014; van der Putten 2017). These interactions can shape ecosystem processes and properties throughout primary succession, as nutrient capital builds during early pedogenic phases and then declines over the course of millennia (Wardle et al. 2004). In ecosystems that have developed beyond peak biomass and into a retrogressive stage, characterized by declining standing biomass, net primary productivity, and soil nutrient availability (Peltzer et al. 2010), plant–microbe relationships may be key in maintaining nutrient cycling (Dickie et al. 2013). However, changes in plant–microbe symbioses and nutrient dynamics throughout long-term ecosystem development are not well understood, with a number of possible outcomes which likely differ between plant functional types.

In retrogressive-stage ecosystems, soil nitrogen (N) supplies are typically depleted relative to earlier, more fertile stages, and what little N remains is often found in complex, organic forms such as protein–tannin complexes (Qualls et al. 1991; Kraus et al. 2003; Wardle et al. 2004). Under these circumstances, root-associated microbes such as N-fixing bacteria or mycorrhizal fungi can assist in plant N acquisition (van der Heijden et al. 2008). These ecological partnerships incur a carbon (C) cost to the plant hosts but provide access to N pools that would otherwise be unavailable (Chapman et al. 2005; Fisher et al. 2010).

Ectomycorrhizae, a symbiotic relationship between fungi from a phylogenetically diverse group of approximately

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Here, we use stable N isotopes to quantify the contribution of plant-microbe partnerships to forest N supplies throughout succession. We demonstrate that symbioses are critical to plant nutrition in the retrogressive ecosystems of late-stage succession, where soil nutrient availability is especially low.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00442-020-04606-y) contains supplementary material, which is available to authorized users.

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25,000 fungal species (Tedersoo et al. 2010) and a broad group of plants spanning both gymnosperms and angiosperms (Brundrett 2009), are one such partnership. Ectomycorrhizal fungi can acquire soil N in both mineral and organic forms (Rains and Bledsoe 2007), as well as degrade complex organic molecules to liberate N (Read and Perez-Moreno 2003), making them valuable for plant nutrition. Ericoid mycorrhizae are another important symbiosis, occurring between fungi spanning many phyla (Leopold 2016) and plants mainly in the Ericaceae family. Similar to ectomycorrhizal fungi, ericoid mycorrhizal fungi can produce extracellular enzymes and organic acids to mineralize dissolved organic N (DON) compounds and facilitate plant N uptake (Bending and Read 1997a, b; Read and Perez-Moreno 2003; Schimel and Bennett 2004). However, both lab and field experiments suggest that ericoid mycorrhizal fungi have a particular advantage in depolymerizing and accessing N found in protein–tannin complexes due to greater enzymatic potential as well as greater specific root length compared to ectomycorrhizal fungi (Read 1991; Bending and Read 1997a; Read and Perez-Moreno 2003; Wurzburger and Hendrick 2009).

Both ecto- and ericoid mycorrhizal fungi can be an important source of plant N nutrition, and have been found to supply up to 80% of plant N in N-limited ecosystems (Read and Perez-Moreno 2003; Hobbie and Hobbie 2006). Indirect evidence suggests that plant reliance on ecto- and ericoid mycorrhizal fungi partners for N foraging may increase as plant-available soil N supplies decline during ecosystem retrogression. For example, ectomycorrhizal fungi have been found to contribute more to plant nutrition under conditions of environmental stress (Pena and Polle 2014), and declining nutrient availability has been linked with greater abundance of ectomycorrhizal fungi (Courty et al. 2018) as well as more intensive fungal foraging traits (Moeller et al. 2014). Root colonization by ericoid mycorrhizal fungi has also been observed to increase in response to declines in soil nutrient availability (Wurzburger and Bledsoe 2001). However, there remains little direct characterization of changes in plant–mycorrhizal nutrient dynamics throughout ecosystem development, especially with respect to ericoid mycorrhizal fungi (Dickie et al. 2013).

Symbiotic N fixation is another strategy through which plants can meet their N demands. High rates of symbiotic N fixation are most often observed in early successional environments in the temperate zone, where soil N is still accumulating (Vitousek et al. 2002; Menge et al. 2012); in late-successional temperate forests, symbiotic N-fixing plants are often absent (Vitousek and Howarth 1991). This may be due to the high C cost of N fixation compared to soil N acquisition (Rastetter et al. 2001), climate constraints on nitrogenase activity (Houlton et al. 2008), evolutionary factors (Crews 1999; Menge et al. 2009; Sheffer et al. 2015), limitation of symbiotic N fixers by nutrients other than N (Uliassi and Ruess 2002), or all of these. In contrast, work in the boreal suggests that at least in some cases, low soil N availability in late succession can drive higher rates of N fixation (Zackrisson et al. 2004). Further work is, therefore, needed to resolve the role of symbiotic N fixation in plant N nutrition throughout succession, particularly in retrogressive ecosystems where N is depleted.

The natural abundance of stable N isotopes ($^{15}N/^{14}N$) in plant foliage can be a useful tool for examining plant N-foraging strategies and their response to changes in nutrient availability (Robinson 2001; Hobbie and Hobbie 2006; Houlton et al. 2007). Foliar $^{15}N/^{14}N$ can be used to estimate the proportion of foliar N derived from mycorrhizal transfer, as mycorrhizal fungi preferentially retain ^{15}N in their biomass and transfer low $^{15}N/^{14}N$ to their plant hosts, resulting in lower foliar $^{15}N/^{14}N$ in mycorrhizal vs. non-mycorrhizal plants (Hobbie et al. 2005). Foliar $^{15}N/^{14}N$ can also be used to calculate the proportion of N derived from symbiotic N fixation, because N derived from atmospheric N_2 is often distinguishable from soil N sources. In addition, foliar $^{15}N/^{14}N$ can be compared to that of specific soil N pools (DON, NH_4^+, and NO_3^-) to further constrain plant N sources using probabilistic mixing models (Houlton et al. 2007).

Here, we investigate plant reliance on microbial symbioses to acquire N throughout ecosystem retrogression across a well-studied chronosequence, the Ecological Staircase in Mendocino County, California (Jenny 1973), using natural stable N isotopic techniques. This chronosequence is a model for understanding plant response to nutrient deficiency throughout succession due to an extreme range in ecosystem development and soil fertility across the sequence. At the youngest terraces (100,000–200,000 years old), soils are fertile and have relatively greater concentrations of extractable mineral N forms, and support productive coniferous forests. At the older terraces (300,000–500,000 years old), soils are highly weathered and infertile, and the soil N pool is dominated by organic compounds such as protein–tannin complexes (Northup et al. 1995). Three distinct plant–microbe symbioses that can contribute to plant N acquisition are present at all sites: ectomycorrhizae, ericoid mycorrhizae, and an actinorhizal N-fixation symbiosis between the *Frankia* actinobacteria and shrub *Myrica californica*. Although *M. californica* is also taxonomically capable of forming ectomycorrhizae, it has not been observed to do so at these sites (Wurzburger and Bledsoe 2001).

We test the specific hypothesis that plants increasingly rely on root-associated microbes (ectomycorrhizal fungi, ericoid mycorrhizal fungi, or N-fixing bacteria) to obtain N as soil fertility declines throughout succession and into retrogression. If this is the case, we expect to observe a systematic decline in foliar $^{15}N/^{14}N$ in ectomycorrhizal and ericoid mycorrhizal plant species across the chronosequence, reflecting

greater reliance on N derived from mycorrhizal associates as soil N becomes increasingly held in complex organic forms. We also expect a positive correlation between foliar $^{15}N/^{14}N$ and foliar N concentration, with increased divergence of plant and soil $^{15}N/^{14}N$ reflecting isotopic discrimination during N transfer from mycorrhizal fungi to plants. In the N-fixing shrub *M. californica*, we expect that foliar $^{15}N/^{14}N$ to approach that of atmospheric N_2 when N fixation is an important source of plant N, using the standard mean $\delta^{15}N$ of 0‰, where $\delta^{15}N = \left(\frac{\frac{^{15}N}{^{14}N}\text{sample}}{\frac{^{15}N}{^{14}N}\text{atmospheric N}} - 1 \right) \times 1000$, consistent with N-fixing plant shoot sampling (Unkovich 2013). Further, we hypothesize that ericoid mycorrhizal plants will demonstrate a greater ability to take up soil N via mycorrhizal transfer than ectomycorrhizal plants, especially at the oldest terraces, reflecting the enhanced ability of ericoid mycorrhizal fungi to access N within protein–tannin complexes via enzyme production (Bending and Read 1997a). If this is the case, we expect to observe lower foliar $^{15}N/^{14}N$ in ericoid mycorrhizal plant species than in ectomycorrhizal plant species, reflecting greater reliance on N transferred from mycorrhizal fungi.

Materials and methods

Study sites

Study sites were located along a well-characterized soil chronosequence known as the Ecological Staircase. The Ecological Staircase is located in the Jug Handle State Reserve near Fort Bragg, CA, which experiences a Mediterranean climate with warm, dry summers and cool, wet winters. The chronosequence consists of five marine-cut terraces formed through a combination of ocean erosion and tectonic uplift, ranging from approximately 100,000–500,000 years in age (Jenny 1973). Soils are formed from beach and eolian deposits overlying greywacke sandstone and are classified as Typic Hapludults at the youngest terraces (T1 and T2) and Aquic Hapludults and Typic Duraquods at the older terraces (T3–T5) (Table S1). One 20 m × 20 m sampling plot was established at each of the five terraces.

Terraces 1 and 2 support productive Bishop pine (*Pinus muricata)* forests, while terraces 3–5 are characterized by pygmy forests of stunted (< 4 m at 100 years) Bishop pine and Bolander pine (*Pinus contorta var. bolanderi*), Mendocino cypress (*Cupressus pigmea*) and a thick shrub understory (dominantly *Vaccinium ovatum*, *Rhododendron macrophyllum*, *Arctostaphylos nummularia,* and *Gaultheria shallon*). Individual *M. californica* shrubs are scattered infrequently across all terraces.

Previous research at the Ecological Staircase has shown that the speciation of N in soil changes dramatically when examining subsequently older terraces. The size of the mineral N pool decreases sharply with soil age, from ~ 300 mg mineral N m^{-2} at the T1 to close to 0 mg mineral N m^{-2} at T3–T5. At all stages of development, the mineral *N* pool is dominated by NH_4^+, with < 10% of the mineral N pool present as NO_3^-, (Yu et al. 2003). On the other hand, soil-extractable DON concentrations increase from ~ 100 mg N m^{-2} at the T1 to ~ 3000 mg N m^{-2} at T3–T5 (Yu et al. 2003). Protein–tannin complexes are a particularly important part of the organic N pool at the pygmy forest terraces and contain 60% of the dissolved soil organic N (Northup et al. 1995), while free amino acids account for only 1.5–10.6% of DON (Yu et al 2002).

Foliage sampling

Current-year, fully expanded foliage was collected using a pole-saw from replicate individual plants of common species that composed the majority of aboveground biomass within the established sampling plots in August 2008. Foliage was collected from ectomycorrhizal plant species: *P. muricata, P. contorta ssp. bolanderi*; arbutoid mycorrhizal plant species: *A. columbiana, A. nummularia*; ericoid mycorrhizal plant species: *R. macrophyllum, V. ovatum, G. shallon;* and the N-fixing shrub *M. californica.* Symbiotic type was assigned based on plant taxonomy and known mycorrhizal capabilities based on the literature. Because the fungi that form arbutoid mycorrhizae are frequently the same fungi that form ectomycorrhizae in other plants, arbutoid mycorrhizae can be considered a subset of ectomycorrhizae (Brundrett 2004); thus, we assigned *A. columbiana* and *A. nummularia* to the ectomycorrhizal plant species category, though they are not technically ectomycorrhizal. Although *M. californica* is capable of hosting arbuscular mycorrhizal fungi, this association has not been observed at this set of sites (Wurzburger and Bledsoe 2001). Thus, *M. californica* was not considered to be mycorrhizal in this study.

Only cosmopolitan species found in both fertile (i.e., T1–T2) and retrogressive (i.e., T3–T5) terraces were sampled; hence, *Cupressus pigmaea* was excluded because it is only found at retrogressive terraces. When possible, foliage samples were collected from at least five individual plants of each species at each plot. When five individuals of a species were not present within a plot, foliage was sampled from as many individuals as were present. The number of individual plants sampled of each species at each terrace is shown in Table S2. While all species sampled in this study were present in fertile and retrogressive terraces, only *P. muricata* and *M. californica* were observed in all five sites across the chronosequence.

All foliage samples were placed in paper bags and air-dried upon collection. Upon returning to the lab, undamaged leaves were selected by hand, rinsed three times with Nanopure water (Thermo Scientific, Waltham, MA, USA)

to remove dust and leaf debris, and dried for three days at 50 °C. Replicate leaves collected from individual plants were then ground to a fine powder and homogenized using a Wiley mill followed by ball mill pulverization. Powdered samples were analyzed for C concentration, $^{13}C/^{12}C$, N concentration, and $^{15}N/^{14}N$ using an PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility. Sample isotope ratios were corrected for instrument variation between runs using laboratory standards that were previously calibrated against NIST Standard Reference Materials. Significant differences in foliar chemistry between plant species and across sites were determined via a two-way ANOVA with species and site as factors, followed by Tukey's honestly significant difference post hoc test. Our choice of ANOVA as a statistical test is based on a wealth of biogeochemical studies that have also used ANOVA to compare single sites spanning a wide biogeochemical gradient arrayed along a time axis (e.g., Vitousek 2004), including numerous such studies at this same chronosequence (e.g., Yu et al. 2003; Izquierdo et al. 2013; Winbourne and Houlton 2018). Linear regression was used to analyze the relationship between foliar $\delta^{15}N$ and N concentration.

Soil collection and analysis

In April 2015, ten soil samples were collected from each sampling plot from the top 0–10 cm mineral horizon (the depth of greatest soil N based on previous sampling of soil pits at these sites) using a hand coring device and placed in a plastic bag. At each sampling plot, five of the soil samples were collected from underneath known mycorrhizal plant species, and five of the soil samples were collected from underneath *M. californica* individuals. At each terrace, concentrations and isotopic composition of N in bulk soil and individual pools did not significantly differ between the soil samples collected underneath mycorrhizal plant species and the soil samples collected underneath *M. californica* individuals, so soil data at each terrace were pooled prior to statistical analyses. Soil samples were collected from no closer than 3 m and sampling locations were strategically chosen to be representative of the sampling plot. All soil samples were immediately sieved to 2 mm and 10 g was subsampled for soil solution extraction. Soil solution was extracted with 50 mL of 2 M potassium chloride (KCl) and frozen immediately.

Samples were returned to the lab at UC Davis and a subsample of each field-moist soil sample was oven-dried and field moisture content was determined by mass difference. The remaining soil samples were air-dried and ground to a fine powder using a ball mill. Ground soil samples were weighed into tin capsules on a microbalance before being submitted to the UC Davis Stable Isotope Facility. C concentration, $^{13}C/^{12}C$, N concentration, and $^{15}N/^{14}N$ were measured using an Elementar Vario EL Cube (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Significant differences in soil chemistry between sites were determined via one-way ANOVA followed by Tukey's honestly significant difference post hoc test.

Comparing $\delta^{15}N$ between foliar samples and soil samples that were collected 7 years apart required the assumption that each site was near steady state on this timeframe, with little foliar $\delta^{15}N$ variation from year to year. To validate this assumption, we collected additional foliage from selected species at each terrace in 2016 (data not shown). Within individual plant species, foliar $\delta^{15}N$ did not vary significantly between 2008 and 2016, supporting this assumption.

Analysis of soil solution extracts

NH_4^+ concentrations of soil solution extracts were measured using the indophenol formation colorimetric method described in Verdouw et al. (1978). NO_3^- concentrations of soil solution extracts were quantified using the vanadium reduction colorimetric method described in Doane and Horwath (2003). Total dissolved N (TDN) in soil solution extracts was determined by a modified persulfate oxidation protocol, which oxidizes all dissolved N species to NO_3^- (Knapp et al. 2005), followed by vanadium reduction colorimetry. All concentrations were adjusted for soil moisture and converted to μg N g^{-1} dry soil.

Because no quantifiable NO_3^- could be detected in the extracts, N isotopes were only determined for the NH_4^+ and TDN pools. To determine the $\delta^{15}N$ of TDN, the persulfate-digested TDN samples were submitted to the UC Davis Stable Isotope Facility for ^{15}N analysis of NO_3^- in water using the denitrifier method.

To determine the $\delta^{15}N$ of NH_4^+, the NH_4^+ in each extract was converted to NO_3^- using the acid trap diffusion method followed by persulfate oxidation (Sigman et al. 1997; Houlton et al. 2007). The NO_3^- concentration in each sample was measured colorimetrically using the vanadium reduction method. $\delta^{15}N$ of NO_3^- was determined using the denitrifier method (Sigman et al. 2001) at the UC Davis Stable Isotope Facility. Significant differences in concentration and $\delta^{15}N$ between extractable soil N pools and across sites were determined via two-way ANOVA with pool and site as factors, followed by Tukey's honestly significant difference post hoc test.

Isotopic blanks and standards

Nitrogen isotope composition of dissolved N forms (TDN, NH_4^+) was converted to NO_3^- via persulfate oxidation (see above). Hence, method blanks were included for every step of the extraction, oxidation, and/or acid trap diffusion followed by oxidation processes and analyzed for NO_3^- concentration and isotopic ratio as with the rest of the samples: 2.0 M KCl blanks, persulfate blanks, and diffusion blanks. Sample TDN concentrations were adjusted by subtracting the average measured NO_3^- concentration of KCl blanks that were oxidized along with the rest of the samples. Sample NH_4^+ concentrations were adjusted by subtracting the average measured NO_3^- concentration of KCl blanks that were subjected to acid trap diffusion and then oxidized along with the rest of the samples. In all cases, the background concentration of TDN and NH_4^+ in the KCl blanks constituted < 20% of the actual sample concentration. The $\delta^{15}N$ of TDN and NH_4^+ samples were corrected for the $\delta^{15}N$ of KCl blanks using the following equation:

$$\delta^{15}N_{sample} = \frac{\delta^{15}N_{total} - (\delta^{15}N_{blank} \times f_{blank})}{f_{sample}}.$$

To check the accuracy of isotopic results, USGS-26 ammonium sulfate standard ($\delta^{15}N = 53.75‰$) was treated as a sample for both the persulfate oxidation and the acid trap diffusion followed by persulfate oxidation procedures. The measured $\delta^{15}N$ of the standard was always < 4% different from the expected $\delta^{15}N$.

Soil solution extract $\delta^{15}N$ calculations

The concentration of DON was calculated using the following equation:

$$[DON] = [TDN] - \left[NH_4^+\right].$$

The $\delta^{15}N$ of DON was then calculated using the following equation:

$$\delta^{15}N_{DON} = \frac{\left(\delta^{15}N_{TDN} \times [TDN]\right) - \left(\delta^{15}N_{NH_4^+ +} \times \left[NH_4^+\right]\right)}{[DON]}.$$

Isotopic mixing model

Foliar $\delta^{15}N$ was corrected for internal N transfer. Assuming an isotope effect of internal fractionation of 4‰, plant steady state of N uptake and loss, and an equal proportion of belowground and aboveground losses, foliar $\delta^{15}N$ should be 2‰ higher than source N (Houlton et al. 2007):

$$\delta^{15}N_{foliar\ (corrected)} = \delta^{15}N_{foliar} + 2‰.$$

We considered four possible sources of foliar N:soil NH_4^+, soil DON, NH_4^+-N transferred from mycorrhizae and DON-N transferred from mycorrhizal fungi. To calculate the $\delta^{15}N$ of mycorrhizal transferred N, the isotope effect of mycorrhizal transfer (Δ) was assumed to be 9‰ (Hobbie and Hobbie 2006; Hobbie et al. 2008), and the $\delta^{15}N$ of N from mycorrhizal transfer was estimated using the following equation:

$$\delta^{15}N_{mycorrhizal} = \delta^{15}N_{DON\ or NH_4^+} - \Delta.$$

Fractional contributions of direct soil uptake and mycorrhizal transfer to foliar N were calculated using the Stable Isotope Analysis in R package (SIAR). SIAR uses a Markov Chain Monte Carlo framework to calculate the fractional contribution (*f*, expressed as a decimal fraction) of each isotopic source (direct soil DON uptake, direct soil NH_4^+ uptake, mycorrhizal transfer of DON-N, mycorrhizal transfer of NH_4^+-N) to the mixture (total N of plant foliage). This model accounts for uncertainty in the isotopic values of mixtures and sources. The model input was the recycling-corrected foliar $\delta^{15}N$ of each sample, the mean and standard deviation $\delta^{15}N$ of each N source at each terrace (Tables S3–S4). Foliar samples were grouped by terrace and mycorrhizal type (ectomycorrhizal and ericoid mycorrhizal), and the model was run for 500,000 iterations, with the initial 50,000 iterations discarded. The model output was an estimated mean *f* for each N source at each terrace for both mycorrhizal types, as well as an estimated 95% confidence interval for each *f*, which takes into account uncertainty of isotopic sources as well as variability between plants in each group (Table S5). Non-overlapping confidence intervals were interpreted to indicate significantly different *f* values.

The full mixing model included four potential N sources (i.e., unknowns) and one isotopic system (known), resulting in a mathematically undetermined system of equations (Houlton et al. 2007). This resulted in model runs with significant uncertainty, in which the different potential sources were not readily distinguished (Table S5, Fig. S1). To facilitate analysis, a simplified model was run using the same parameters and input mixtures, but with the N sources simplified to soil TDN (without differentiating between DON and NH_4^+) and mycorrhizal transfer TDN (Tables S6–7). This reduced the uncertainty of estimated *f* values, and is likely more representative of biological processes, as fungal mixing and processing of soil N occurs prior to transfer to plant hosts (Hobbie and Hobbie 2006). Our analysis thus focused on this simplified model.

The same modeling techniques were used to calculate fractional contributions of direct soil uptake and symbiotic

N fixation in *M. californica*, except the N sources were either soil TDN or atmospheric N (Tables S8–10).

All mixing models were also run without applying the 2‰ correction factor to foliar $\delta^{15}N$ values to test the validity of applying the correction factor (Tables S11–S13, Figs. S2–S4). Because similar results were obtained using the corrected vs. the uncorrected data, the uncorrected foliar $\delta^{15}N$ values were outside the range of the source $\delta^{15}N$ values causing model errors, and the 2‰ correction factor has been validated in previous studies (Houlton et al. 2007), we chose to use corrected foliar $\delta^{15}N$ values in our analysis.

Results

Foliar $\delta^{15}N$ across terraces

Ericoid mycorrhizal plant foliar $\delta^{15}N$ significantly declined between T2 and T3–T5 (Fig. 1, $F_{3,93} = 23.897$, $p < 0.001$), ectomycorrhizal plant foliar $\delta^{15}N$ significantly declined between T1 and T2–T5 (Fig. 1, $F_{4,71} = 22.769$, $p < 0.001$), and N-fixing foliar $\delta^{15}N$ significantly declined between T1–T2 and T3–T5 (Fig. 1, $F_{4,25} = 13.016$, $p < 0.001$). There was a significant correlation between $\delta^{15}N$ and N concentration in ectomycorrhizal plant foliage (Fig. 2), ($R^2 = 0.18$, $F_{1,77} = 16.65$, $p < 0.001$) and N-fixing plant foliage (Fig. 2, $R^2 = 0.18$, $F_{1,28} = 6.35$, $p = 0.018$), but not in ericoid mycorrhizal plant foliage (Fig. 2, $R^2 = 0.001$, $F_{1,92} = 0.10$, $p = 0.75$). Additional foliar chemistry data are shown in Table S14.

Soil chemistry across terraces

DON was the most abundant form of soil-extractable N, with NH_4^+ concentrations consistently low, and no detectable NO_3^- at any of the terraces (Table 1). There was significantly more DON than NH_4^+ at all terraces ($F_{1,90} = 124.827$, $p < 0.001$), although there was no significant change in the size of either N pool across the chronosequence ($F_{4,90} = 1.898$, $p = 0.118$). The $\delta^{15}N$ of DON was significantly higher than the $\delta^{15}N$ of NH_4^+ by ~ 2–5 per mil across sites ($F_{1,90} = 65.169$, $p < 0.001$); neither $\delta^{15}N$ of DON or NH_4^+ changed significantly across the chronosequence

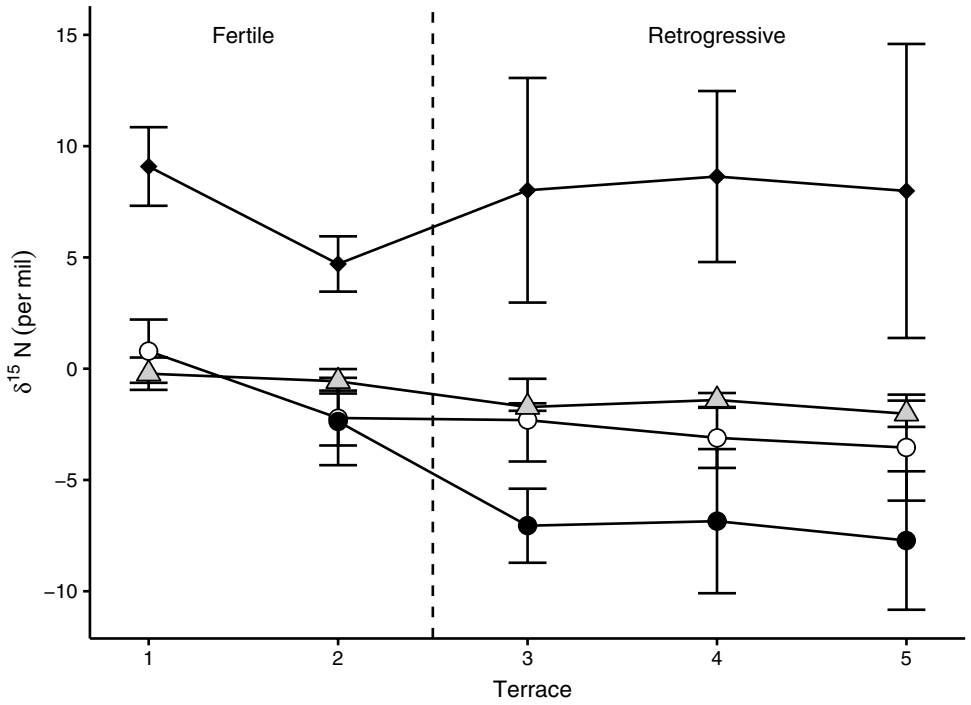

Fig. 1 Mean ± SD $\delta^{15}N$ (‰) in soil total dissolved nitrogen (black diamonds), ectomycorrhizal plant foliage (open circles), ericoid mycorrhizal plant foliage (black circles), N-fixing plant foliage (gray triangles) across the Ecological Staircase chronosequence. The terraces shown on the x-axis increase in age from ca. 100 ky at terrace 1 to ca. 500 ky at terrace 5. The vertical dashed line divides the younger, fertile terraces from the retrogressive-phase pygmy forest terraces. Ericoid mycorrhizal plant foliar $\delta^{15}N$ significantly declined between terrace 2 and terraces 3–5 ($F_{3,93} = 23.897$, $p < 0.001$), ectomycorrhizal plant foliar $\delta^{15}N$ significantly declined between terrace 1 and terrace 2–5 ($F_{4,71} = 22.769$, $p < 0.001$), and N-fixing plant foliar $\delta^{15}N$ significantly declined between terraces 1–2 and terraces 3–5 ($F_{4,25} = 13.016$, $p < 0.001$). No ericoid mycorrhizal plants were present at terrace 1

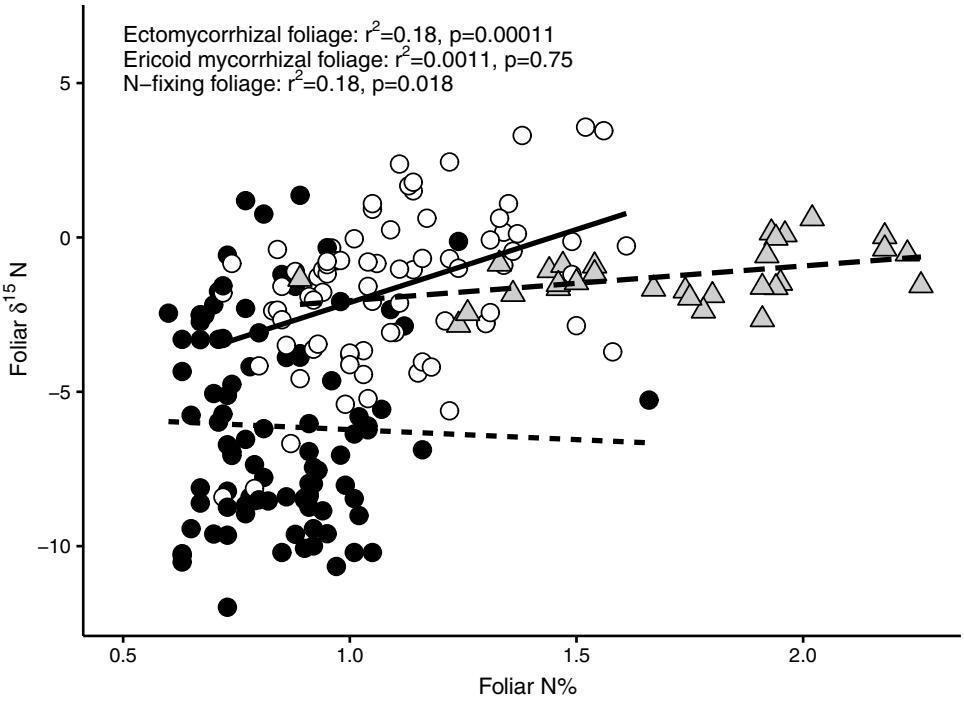

Fig. 2 Foliar $\delta^{15}N$ (‰) plotted against foliar N concentration (%) in ectomycorrhizal plant foliage (open circles and solid regression line), ericoid mycorrhizal plant foliage (black circles and short-dash regression line), and N-fixing plant foliage (gray triangles and long-dash regression line). Linear regression was used to analyze the relationship between foliar $\delta^{15}N$ and foliar N concentration within each symbiotic type individually. There is a significant correlation between $\delta^{15}N$ and N concentration in ectomycorrhizal plant foliage ($R^2 = 0.18$, $F_{1,77} = 16.65$, $p < 0.001$) and N-fixing plant foliage ($R^2 = 0.18$, $F_{1,28} = 6.35$, $p = 0.018$), but not in ericoid mycorrhizal plant foliage ($R^2 = 0.001$, $F_{1,92} = 0.10$, $p = 0.75$)

Table 1 Concentration (μg N g^{-1} dry soil) and $\delta^{15}N$ (‰) of total dissolved N (TDN), dissolved organic N (DON), and ammonium (NH_4^+) across terraces

Terrace	Total dissolved nitrogen (TDN) (μg TDN-N g^{-1} dry soil)	Dissolved organic nitrogen (DON) (μg DON-N g^{-1} dry soil)	Extractable ammonium (NH_4^+) (μg NH_4^+-N g^{-1} dry soil)	TDN $\delta^{15}N$ (‰)	DON $\delta^{15}N$ (‰)	NH_4^+ $\delta^{15}N$ (‰)
1	14.94 ± 5.59^{ab}	13.77 ± 5.36^{ab}	1.39 ± 0.71^{b}	9.09 ± 1.77^{AB}	9.65 ± 1.91^{A}	2.26 ± 1.39^{DE}
2	27.39 ± 7.70^{a}	25.75 ± 7.02^{a}	1.86 ± 0.78^{b}	4.70 ± 1.24^{ABCDE}	4.82 ± 1.26^{ABCDE}	2.44 ± 1.45^{CDE}
3	16.63 ± 12.81^{a}	15.32 ± 12.69^{ab}	1.53 ± 1.35^{b}	8.02 ± 5.05^{ABCD}	8.24 ± 5.45^{ABC}	2.30 ± 4.06^{DE}
4	22.38 ± 14.83^{a}	21.37 ± 14.11^{a}	1.24 ± 0.73^{b}	8.63 ± 3.84^{AB}	8.83 ± 4.01^{AB}	3.65 ± 2.34^{BCDE}
5	21.20 ± 14.60^{a}	20.35 ± 14.13^{a}	1.07 ± 0.51^{b}	7.99 ± 6.61^{ABCD}	8.44 ± 6.97^{AB}	0.23 ± 2.04^{E}

All data are shown as mean ± SD. Different lowercase letters following each value represent significant differences in concentrations between different pools at a single terrace based on a two-way ANOVA with pool and terrace as factors, followed by Tukey's HSD post hoc test. Different uppercase letters following each value represent significant differences in $\delta^{15}N$ values between different pools at a single terrace based on a two-way ANOVA with pool and terrace as factors, followed by Tukey's HSD post hoc test

(Table 1, $F_{4,90} = 1.848$, $p = 0.127$). Additional soil chemistry data are shown in Table S15.

Foliar $\delta^{15}N$ isotopic mixing models

Foliar $\delta^{15}N$ was consistently lower than bulk soil $\delta^{15}N$ and the $\delta^{15}N$ of all soil-extractable N pools across sites (Fig. 1). In ectomycorrhizal plant species, the estimated percentage of foliar N derived from mycorrhizal transfer ($f_{mycorrhizal}$) ranged from 54% ± 0.10% at the T1 to 78% ± 15% at T5, and exhibited significantly higher $f_{mycorrhizal}$ at T3–5 vs. at T1 (Fig. 3). In ericoid mycorrhizal plant species, $f_{mycorrhizal}$ ranged from 68% ± 11% at the T1 to 97% ± 5% at T5, and increased significantly between T2 (68% ± 11%) and T3 (97% ± 5%) (Fig. 3). At T2, both ericoid mycorrhizal plant foliage and ectomycorrhizal plant foliage displayed similar $f_{mycorrhizal}$ values (68% ± 11% in ericoid mycorrhizal species and 66% ± 22% in ectomycorrhizal species). At T3–T5, $f_{mycorrhizal}$ in ericoid mycorrhizal plant foliage was higher than $f_{mycorrhizal}$ in ectomycorrhizal plant foliage (~ 96% vs. ~ 75%), though this difference was only statistically significant at T3.

The fraction of *M. californica* foliar N derived from N fixation ($f_{fixation}$) ranged from 76% ± 17% at T1 to 98% ± 4% at T5 (Fig. 4). At T3, $f_{fixation}$ was significantly higher than at T1–T2. At the retrogressive terraces (T3-T5), $f_{fixation}$ was uniformly high (> 85%) and not significantly different between terraces.

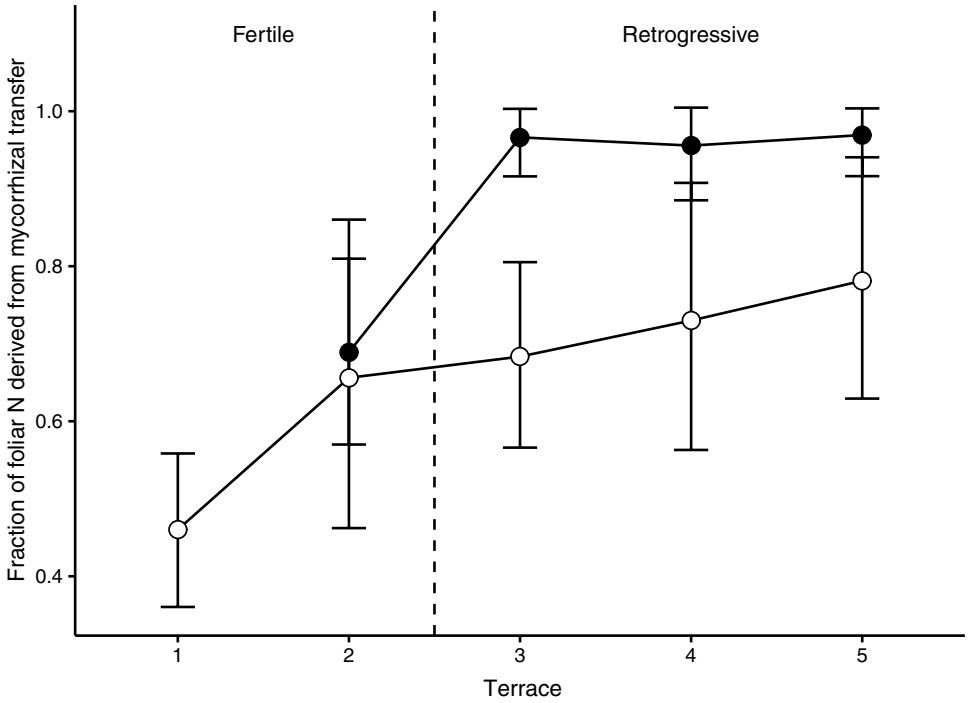

Fig. 3 Proportion of foliar N derived from mycorrhizal transfer ($f_{mycorrhizal}$) in ectomycorrhizal (open circles) and ericoid mycorrhizal (filled circles) plant foliage. Values are shown as the mean calculated fractional contribution of each N source calculated using a Markov Chain Monte Carlo framework in SIAR. Error bars represent the 95% confidence interval for *f* estimates, which take into account uncertainty of isotopic sources as well as variability between plants in each group. Non-overlapping confidence intervals indicate significantly different *f* estimates. The vertical dashed line divides the younger, fertile terraces from the retrogressive-phase pygmy forest terraces

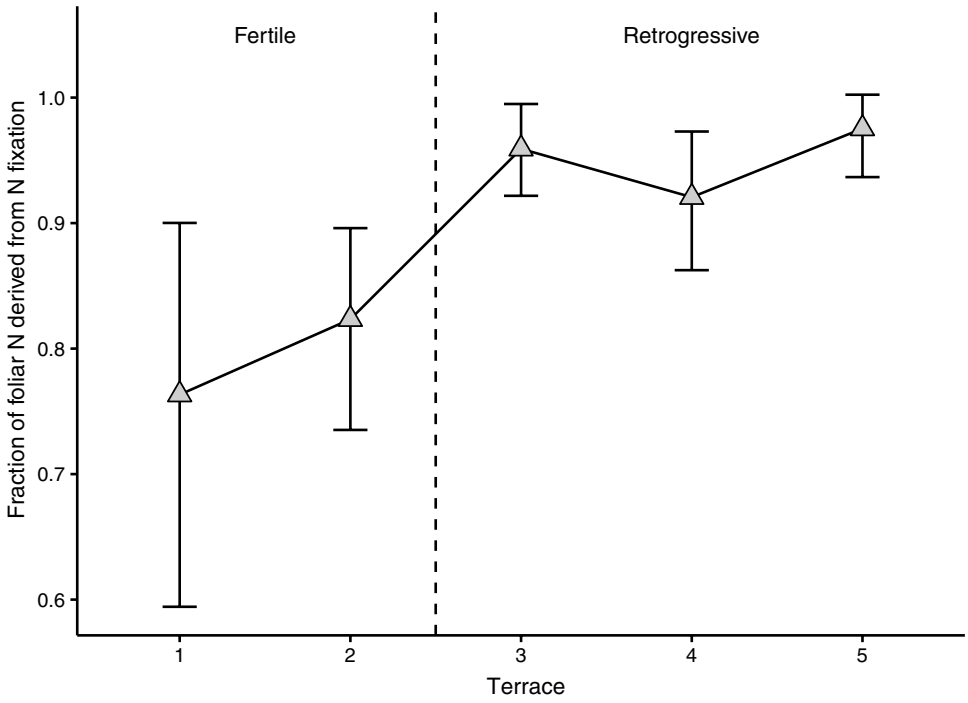

Fig. 4 Proportion of foliar N derived from atmospheric N fixation ($f_{fixation}$) in N-fixing plant foliage (gray triangles). Values are shown as the mean calculated fractional contribution of each N source calculated using a Markov Chain Monte Carlo framework in SIAR. Error bars represent the 95% confidence interval for *f* estimates, which take into account uncertainty of isotopic sources as well as variability between plants in each group. Non-overlapping confidence intervals indicate significantly different *f* estimates. The vertical dashed line divides the younger, fertile terraces from the retrogressive-phase pygmy forest terraces

Discussion

Plant reliance on microbial partnerships across the chronosequence

Our findings support the hypothesis that plants of various symbiotic types rely heavily on microbial partners to access diverse N forms throughout ecosystem succession and retrogression. Across all sites, plants with all types of microbial associates (ectomycorrhizal, ericoid mycorrhizal, and N-fixing) obtain a significant fraction of their foliar N via symbiotic pathways, with increasing reliance on microbial partners in the retrogressive pygmy forest ecosystems (i.e., T3–T5). In ectomycorrhizal plant species, foliar N concentrations

are significantly correlated with foliar $\delta^{15}N$, indicating that when N is more limiting, plant foliage expresses a greater mycorrhizal transfer isotopic signature (Fig. 2). However, this relationship is not observed in ericoid mycorrhizal plant species, suggesting that N obtained from mycorrhizal fungi may prevent their plant hosts from experiencing N limitation.

These results support past work hypothesizing that declines in nutrient availability may drive plants at retrogressive terraces to rely more heavily on mycorrhizae for N acquisition. Past studies have utilized fungal DNA sequencing to examine changes in ectomycorrhizal fungi traits across the Ecological Staircase chronosequence, and found that ectomycorrhizal fungi are more abundant at the retrogressive pygmy forest terraces and exhibit more C-intensive nutrient foraging traits than ectomycorrhizal fungi at the younger terraces (Moeller et al. 2014; Courty et al. 2018). This may be reflective of greater tree nutrient limitation, and thus greater investment in ectomycorrhizae, at the pygmy forest sites. Our findings provide a non-disruptive test of such previous hypotheses and show that mycorrhizae indeed supply a significantly greater fraction of plant N at the highly retrograded and stunted forest pygmy forest sites.

Ericoid mycorrhizal fungi are predicted to dominate over ectomycorrhizal fungi in late-successional and retrogressive ecosystems (Read 1991; Lambers et al. 2008). Several factors contribute to this expectation, including the capacity for such root-associates to degrade protein–tannin complexes (Bending and Read 1997a), thereby outcompeting ectomycorrhizal fungi for soil N when protein–tannin complexes are prevalent (Yu et al. 1999). This could result in a plant–soil feedback wherein ericaceous plants produce more foliar tannins and polyphenols when growing in acidic and infertile environments (Kraus et al. 2004); upon litterfall, these compounds can then be mineralized and taken back up by ericoid mycorrhizal fungi, contributing to a conservative soil N cycle (Chapman et al. 2005; Wurzburger and Hendrick 2009; Phillips et al. 2013). This alteration of the N cycle has been hypothesized as allowing ericaceous plants to monopolize the soil N economy in acidic and infertile soils (Northup et al. 1998; Yu et al. 2002). Indeed, past research at this chronosequence has demonstrated greater ericoid mycorrhizal colonization and decreased ectomycorrhizal colonization at the oldest terraces (Wurzburger and Bledsoe 2001). In accordance, our results reveal that ericoid mycorrhizal plants at the oldest terraces obtain significantly more of their N from mycorrhizal transfer than do ectomycorrhizal plants. Although our approach cannot address if this shift translates to a significantly greater gross amount of N, the fact that ericoid mycorrhizal plants derived nearly all of their foliar N from fungal transfer at the retrogressive-stage sites is suggestive of a closed, conservative N economy.

Symbiotic N fixation

A body of research maintains that symbiotic N fixation is not likely to be favorable in late-successional temperate and boreal forest environments (Rastetter et al. 2001). Although the N-fixing shrub *M. californica* is not a dominant species at the Ecological Staircase, we found that those individuals present are heavily reliant on symbiotic N fixation, particularly in late succession. As hypothesized, *M. californica* foliar $\delta^{15}N$ approaches 0‰ as site age increased, indicating an increased contribution of atmospheric N to foliar N. The isotopic mixing model reveals similar results, estimating that 98% of foliar N is derived from N fixation at the oldest site. Symbiotic N fixers are known to have a high demand for soil resources other than N, especially P, mostly to support the higher rates of photosynthesis needed to supply N-fixing bacteria with C (Augusto et al. 2013). The soils of the Ecological Staircase chronosequence are extremely low in available forms of P (Izquierdo et al. 2013), which may limit photosynthesis by *M. californica*. P limitation has been shown to limit free-living N fixation at the Ecological Staircase (Winbourne and Houlton 2018) and symbiotic N fixation during primary succession in Alaska (Uliassi and Ruess 2002); this could explain why *M. californica* is not a dominant species at the oldest terraces, despite its ability to access atmospheric N. However, our study clearly demonstrates that those *M. californica* individuals that do succeed in this nutrient-poor environment rely heavily on atmospheric N access made possible by their root symbionts. This supports past research in boreal forests showing increased N fixation in later successional stages (Zackrisson et al. 2004), and calls into question the assumption that all symbiotic N fixation ceases in older ecosystems, despite low overall abundance of symbiotic N-fixing trees at high latitudes (Vitousek and Howarth 1991).

Assumptions, limitations, and future research

A number of assumptions were applied to our isotopic modeling approach. We assumed that our study sites are at steady state, and that foliar and soil $\delta^{15}N$ values, therefore, reflect quasi-equilibrium conditions across each site. We assumed the isotope effect of mycorrhizal transfer was 9‰, based on an 8–10‰ range estimated by Hobbie and Hobbie (2006). However, as we were not able to directly measure the isotope effect of mycorrhizal transfer, the actual isotope effect may differ from our estimate. Implicit in our simplified mixing model for mycorrhizal plant functional types are the assumptions that there is a uniform $\delta^{15}N$ value for the entire DON pool at each site, and that all forms of DON (e.g., amino acids, peptides, and protein–tannin complexes) are equally available for direct plant root and mycorrhizal uptake. Previous research has suggested that the $\delta^{15}N$ accessible to

ectomycorrhizal fungi does not differ from that of bulk DON (Hobbie et al. 2000). However, given the large differences in DON between T2 and T3 (from ~ 25 µg DON-N g^{-1} dry soil at T2 to ~ 15 µg DON-N g^{-1} dry soil at T3, Table 1), it is likely that not all DON is available for plant uptake, resulting in hydrologic losses throughout soil development. Although the methods used here cannot distinguish between available and unavailable DON, isotopic depletion of foliar $\delta^{15}N$ values to that of soil N pools points to the likelihood of mycorrhizal transfer as a foliar N source, as observed in numerous studies of tundra ecosystems (Hobbie and Hobbie 2006; Hobbie and Högberg 2012). Given the importance of protein–tannin complexes in N cycling at the ecological staircase, future research into the availability and isotopic signature of different organic N compounds, particularly protein–tannin complexes, is worth pursuing.

Our calculations assume that changes in foliar $\delta^{15}N$ not explained by variation in soil available $\delta^{15}N$ were due to isotopic discrimination against ^{15}N via mycorrhizal transfer of N to plant hosts (Δ). However, when significant plant N is derived from mycorrhizal transfer, foliar $\delta^{15}N$ can be affected by discrimination during transfer as well as by the amount of N that mycorrhizae transfer to their plant hosts (Hobbie et al. 2005). Nutrient limitation can prompt mycorrhizal fungi to sequester a greater proportion of their N in their biomass, rather than transferring it to their plant hosts (Hobbie and Agerer 2010), thus causing foliar $\delta^{15}N$ to be lower. Previous studies of mycorrhizal nutrient transfer have similarly found that mycorrhizal fungi can regulate how much N they transfer to their plant hosts (Albarracín et al. 2013). At the old, retrogressive-stage sites, mycorrhizal fungi may, therefore, decrease the amount of N that they allocate to plants, which could also be responsible for the observed declines in foliar $\delta^{15}N$ at those sites.

Conclusions

Our study clearly demonstrates the importance of plant–microbe interactions in maintaining aboveground productivity throughout ecosystem succession, particularly in retrogressive ecosystems. At retrogressive-phase sites, plant–microbe associations involving mycorrhizal fungi and N-fixing bacteria provided the majority of plant N. This work supports illustrates the critical role that plant–microbe symbioses play in maintaining aboveground ecosystem function and productivity in response to environmental stressors (Phillips et al. 2013; Averill et al. 2014; Pena and Polle 2014), and serves as a model for exploring the biogeochemical limits to which plant–soil–microbe interactions can maintain ecosystem functioning.

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Author contribution statement KAD and BZH designed the research and the methodology. KAD conducted the fieldwork and laboratory work, analyzed the data, and wrote the original manuscript draft. KAD and BZH revised the manuscript.

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