Ecosystem-level consequences of symbiont partnerships in an N-fixing shrub from interior Alaskan floodplains

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Abstract. In long-lived N-fixing plants, environmental conditions affecting plant growth and N demand vary at multiple temporal and spatial scales, and symbiont assemblages on a given host and patterns of allocation to nodule activities have been shown to vary according to environmental factors, suggesting that hosts may alter partner choice and manipulate symbiont assemblages based on shifting plant needs. This study assessed economic trade-offs among N-fixing symbionts of thin-leaf alder (*Alnus tenuifolia*) by examining whether alder–*Frankia* associations change in response to the up-regulation (P fertilization) and down-regulation (N-fertilization) of N-fixation activities, and whether these changes are associated with differences among *Frankia* partners in their relative C cost and/or N benefit to *A. tenuifolia*.

Relative to control plots, alder in +P plots had significantly higher nodule biomass and N-fixation rates; these parameters were significantly lower in +N plots, translating to stand-level N inputs that were more than an order of magnitude greater in +P than +N plots. Nodule respiration and N-fixation rates were positively correlated, and analyses revealed that alder employs mechanisms to increase the efficiency of C use when N fixation is up-regulated. Of the eight *Frankia* OTUs (operational taxonomic units) identified, two were dominant, with significant differences in *Frankia* OTU composition across samples being explained by fertilization treatment. Dominant OTUs had similar up- and down-regulatory responses to treatments but differed in C costs of N fixation, while the most abundant sub-dominant failed to up-regulate N-fixation rates in +P plots. Differences among *Frankia* OTUs in traits relevant to plant performance may form the basis for host choice and explain successional shifts in alder–*Frankia* assemblages. We suggest that manipulation of *Frankia* assemblages is an adaptation for a species such as *A. tenuifolia* that maintains a high dependence on atmospheric N across radically different growth environments. Because of the strong effects of thin-leaf alder on soil N contents, it appears that host changes in C partitioning to nodule activities, including the physiological consequences of host specificity for *Frankia* assemblages, results in alder both driving and responding to environmental heterogeneity at small to large spatial scales.

Key words: alder; ecosystem N inputs; Frankia; *N-fixation cost; nitrogen fixation; nodule biomass; partner choice.*

Introduction

Relationships between nitrogen-fixing plants and their mutualistic symbionts serve as an ideal model system for studying the evolution of cooperation (Killingback and Doebeli 2002, Douglas 2008, Kiers and Denison 2008, Sachs et al. 2010). The supply of carbohydrates to symbionts in exchange for atmospheric nitrogen (N) fixed by bacterial nitrogenase provides the opportunity for cheating symbionts that benefit from host carbon (C) supply but fail to reciprocate (Denison et al. 2003). Although non-N-fixing "cheaters" coexist with beneficial mutualists in both legume (Friesen and Mathias

2010) and actinorhizal systems (Douglas 1998), it is believed that the evolutionary stability of this mutualistic dialogue relies on the capacity of host plants to either choose the most beneficial partners ("partner choice") and/or discriminate ("sanction") against less cooperative partners (Denison 2000, Simms and Taylor 2002, West et al. 2002, Oono et al. 2009). Putative evidence for both partner choice and sanctioning exists; however, mechanisms for and frequency of these interactions are far from resolved (Oono et al. 2009). The capacity for plants to choose partners may involve host recognition of beneficial bacterial signals during symbiosis development, coupled with the persistence of honest bacterial signaling (Heath and Tiffin 2009), or direct assessment by the plant of mutualistic benefit provided by the bacteria, and allocation of resources

accordingly (Simms and Taylor 2002, Kiers et al. 2006). Mechanisms for sanctioning include host-imposed limitation on resource transfer to symbionts, such as nutrients (Lodwig et al. 2003) or oxygen (Kiers et al. 2003), and or simply the preferential allocation of C to more beneficial partners (Oono et al. 2009).

Given the variation in benefits to hosts among symbiont strains (Parker 1995, Burdon et al. 1999), the widespread persistence of symbiont communities among nodules on an individual plant remains somewhat of an evolutionary puzzle (Oono et al. 2009). However, there may be host advantages in partnering with multiple symbionts if the performance and or cost : benefit ratio of individual symbiont strains is influenced by environmental conditions. In long-lived perennial plants, environmental conditions affecting plant growth rate and demand for N vary at multiple temporal and spatial scales. Assemblages of symbionts on a given host (Igual et al. 2006, Khan et al. 2007, Anderson et al. 2009, Kennedy et al. 2010), and patterns of allocation to nodules and nitrogenase activity (Uliassi et al. 2000, Gentili and Huss-Danell 2003) have been shown to vary systematically according to environmental factors. Moreover, greenhouse studies show that the apparent choice (allocation to nodule number and size) and performance of N-fixing partners are affected by growth conditions (Heath and Tiffin 2007, Heath et al. 2010), suggesting that in natural ecosystems, long-lived hosts may be altering partner choice and manipulating symbiont assemblages based on shifting plant needs.

Considerable research effort has been directed toward understanding the molecular and biochemical mechanisms for the up- and down-regulation of nodule production and N fixation in leguminous and actinorhizal plants (Wall 2000, Pawlowski and Sprent 2008, Valverde and Huss-Danell 2008, Wall and Berry 2008, Andrews et al. 2009, Den Herder and Parniske 2009, Gourion et al. 2009), but to our knowledge, no study has determined whether in natural ecosystems, long-lived hosts modify allocation among multiple N-fixing partners in response to changing environmental conditions. Reduced whole-plant N : phosphorus (P) ratios, resulting from decreases in soil N availability and/or increases in soil P availability, are known to increase plant C investment for the up-regulation of nodule production and nitrogenase activity (Uliassi and Ruess 2002, Binkley et al. 2003, Gentili and Huss-Danell 2003, Valverde and Wall 2003a). Increases in C allocation to nodules in response to elevated light (Lundquist et al. 2003) or CO_2 (Arnone and Gordon 1990, Temperton et al. 2003) also trigger up-regulation of N-fixation activities. It seems likely that these responses are mediated by the preferential allocation of C to partners with high nitrogenase capacity or low N-fixation costs. In contrast, when plant growth or plant N demand declines, both nodule production and nitrogenase are actively down-regulated (Wall et al. 2000, Gentili and Huss-Danell 2003, Laws and Graves 2005, Ruess et al.

2006, 2009). At least at the onset of these conditions, one might predict that C allocation to high-nitrogenase or high-cost partners also declines.

The purpose of this study was to examine the economic trade-offs among N-fixing symbionts in response to manipulated soil nutrient regimes in a long-lived shrub growing in a natural ecosystem. Thin-leaf alder (Alnus incana ssp. tenuifolia, hereafter A. tenuifolia) is a pioneering keystone species throughout interior Alaska, where it dominates early-successional floodplains and the understory of mid- and late-successional floodplain forests (Hollingsworth et al. 2010, Nossov et al. 2011). Nitrogen-fixation inputs by A. tenuifolia to early-successional stands along the Tanana River can exceed 140 kg $N·ha^{-1}·yr^{-1}$ (Van Cleve et al. 1971, Ruess et al. 2009), with inputs declining during forest development due to declines in alder density, nodule biomass, and nitrogenase activity (Uliassi and Ruess 2002, Anderson et al. 2009). Frankia assemblages within A. tenuifolia nodules differ substantially between early- and late-successional forests; where A. tenuifolia and Siberian alder (A. viridis ssp. fruticosa) co-occur as understory co-dominants in floodplain white spruce stands, they form very different Frankia partnerships (Anderson et al. 2009). This suggests that the close relationships between alder–Frankia associations and changes in patterns of soil physical/chemical characteristics observed across stand types (Anderson et al. 2009) may have more to do with host-mediated selection for specific partners than with successional patterns in soil Frankia populations.

This study examines whether alder–Frankia associations change in response to the up-regulation (P fertilization) and down-regulation (N-fertilization) of N-fixation activities, and whether these changes are associated with individual differences among Frankia partners in their relative C cost and/or N benefit to A. tenuifolia. We measured leaf chemistry, nodule biomass, N fixation (benefits), and nodule respiration rates (C cost) on plants within fertilized (N or P) and control plots, and evaluated partner performance by pairing physiological responses of nodules with the identity of individual Frankia strains within those nodules. We hypothesized that strong shifts in N-fixation activities would be associated with changes in individual Frankia partners in accordance with plant N demands. In plots where N fixation is up-regulated by P fertilization, we predicted that A. tenuifolia would up-regulate nodule activities (nodule biomass and N-fixation rates), and favor individual Frankia strains with a high but costly nitrogenase capacity. In contrast, where N-fixation activities are down-regulated by N fertilization, A. tenuifolia would reduce associations with costly, high-nitrogenase Frankia and reduce allocation to nodule production. Combined changes in Frankia partners and their associated nitrogenase activities, coupled with changes in nodule biomass, were predicted to translate to major shifts in ecosystem-level inputs of N.

Study sites

This study was conducted in mid-successional forests dominated by balsam poplar (*Populus balsamifera* L.) within the Bonanza Creek Experimental Forest, situated along the Tanana River in interior Alaska (64°51′ N, 147°43′ W; elevation, ~120 m). Fluvial processes form sand bars from glacial loess, which are colonized initially by horsetails (*Equisetum* spp.) and willows (*Salix* spp.). Within five years, *A. tenuifolia* invades and spreads to form a continuous canopy that persists for up to 30 years (Nossov et al. 2011). It is during this period that atmospheric N fixation by alder is greatest, often exceeding 140 kg N·ha^{-1}·yr^{-1} (Van Cleve et al. 1971, Uliassi and Ruess 2002, Ruess et al. 2009). Later in succession, thin-leaf alder persists as the principal subcanopy shrub when these stands are dominated by balsam poplar (45–100 years of age), and eventually by white spruce, *Picea glauca* (Moench) Voss (100–150 years of age) (Hollingsworth et al. 2010).

Our study plots were located within replicated sites (*n* = 3) established by the Bonanza Creek Long-Term Ecological Research program (BNZ LTER; information *available online*)[5] situated along a 10-km stretch of the Tanana River. Beneath the overstory of balsam poplar in these forests (~100 years of age) grows a relatively dense understory of thin-leaf alder, with rose (*Rosa acicularis*) and high-bush cranberry (*Viburnum edule*) present within the shrub layer, and a scattered ground cover of nonvascular plants, graminoids, and forbs (Hollingsworth et al. 2010) (Table 1). Soils are classified as typic cryofluvents, consisting of an alluvium of fine to medium loess grains overlain by an organic profile extending to a depth of 10 cm. Silt deposition during infrequent major flood events has produced buried organic layers in these soils; however, nodules of thin-leaf alder are found exclusively within the surface organic layer.

The climate of interior Alaska is characterized by an intensely cold snow period averaging over 200 days, with annual temperature extremes ranging from −50° to +35°C. Average daily air temperatures range from −24.9°C in January to 16.4°C in July, with an average annual temperature of −3.3°C. Potential evapotranspiration (466 mm) exceeds annual precipitation (269 mm), 65% of which falls during the growing season, which typically extends from mid-May to early September (Viereck et al. 1993b).

At each of these mid-successional sites, the BNZ LTER maintains unmanipulated (control) plots (30 × 30 m), and plots (20 × 20 m) fertilized with N (100 kg N·ha^{-1}·yr^{-1} as NH$_4$NO$_3$) or P (80 kg P·ha^{-1}·yr^{-1} as P$_2$O$_5$). N and P fertilizer is applied in three monthly doses beginning just before leaf-out in late May each year. N and P fertilizer applications have continued

since initiation in 1997 and 2004, respectively. In each treatment plot (hereafter referred to as CTL, +N, +P) at each site, we measured alder leaf and nodule chemistry, assessed nodule biomass, and obtained paired measurements of N-fixation costs and *Frankia* identity on nodule clusters from multiple alder genets.

Nitrogen fixation and nodule respiration

During July 2008, we measured ^{15}N$_2$ fixation and nodule respiration simultaneously on individual nodule lobe samples from plants from all nine plots. Five plants within each plot were randomly selected and measurements were made separately on 10 nodule clusters sampled from random locations within a 1 m diameter plot centered on each plant. Nodule samples (~60 mg dry mass) were excised from roots, brushed free of soil particles, and placed on a small tray inside a 53-mL aluminum cuvette coupled to a LI-6200 photosynthesis system (LICOR, Lincoln, Nebraska, USA). The cuvette contained a solid aluminum plug that was cooled on ice prior to measurements to facilitate equilibration to ambient soil temperatures. Cuvette temperatures were monitored and adjusted to ambient soil temperature by burying the cuvette closer to or farther from deeper, colder soil layers. We modified the LI-6200 by plumbing an injection loop, whereby once the system was closed and respiration rates had stabilized (usually within 30 s), 20 mL of 99 atom% ^{15}N$_2$ (Isotec Incorporated, Miamisburg, Ohio, USA) was introduced to the system (160 mL total volume) to produce an incubation atmosphere of 15.5% ^{15}N$_2$, enabling the simultaneous measurements of ^{15}N$_2$ uptake and nodule respiration. At the end of each measurement period (10 min), the nodule cluster was surface sterilized for 30 s in 10% bleach, rinsed thoroughly with ultrapure water, and placed in a 2-mL cryotube, which was frozen and stored in liquid N$_2$ in the field.

In the laboratory, frozen nodules were lyophilized and weighed, then homogenized and subsampled for molecular characterization of *Frankia* (see *Nodule* Frankia) and IRMS analysis of ^{15}N abundance. Subsamples were weighed out in duplicate and analyzed using a dual-inlet isotope ratio mass spectrometer (PDZ Europa Scientific Instruments, Crewe, UK). All isotope values were normalized using standards derived from IAEA ammonium sulfate. N-fixation rates (micromoles of N per gram of nodule per hour) were calculated as described previously (Ruess et al. 2006, 2009). Because these calculations require a determination of ^{15}N content of nonincubated nodules, a subsample of each nodule cluster was obtained in the field prior to incubation and frozen as a paired "control" for each sample. Nodule respiration rates (micromoles of CO$_2$ per gram of nodule per hour) were calculated, and the C cost of N fixation was expressed as the slope of the relationship between nodule respiration and N fixation (see *Statistics*).

TABLE 1. Stand structure, microclimate, and thin-leaf alder leaf chemistry from replicate ($n = 3$) stands of three successional stages along the Tanana River, south of Fairbanks, Alaska, USA.

Parameter	Successional stage		
	Early	Mid	Late
Stand structure†			
Stand age (yr)	~35	75–100	100–150
Alder density (live stems/ha)	6481 ± 2177	4815 ± 396	6883 ± 1066
Poplar density (live stems/ha)	817 ± 243	607 ± 83	42 ± 42
Spruce density (live stems/ha)	0	33 ± 17	392 ± 51
Microclimate‡			
Soil temperature at 5 cm (°C)	9.94 ± 0.82	9.74 ± 0.83	6.40 ± 0.39
Soil temperature at 10 cm (°C)	7.80 ± 1.03	6.20 ± 1.05	2.78 ± 0.24
Soil water content (v:v)	0.177 ± 0.049	0.103 ± 0.020	0.091 ± 0.044
PPFD at 2 m (μmol of photons·m^{-2}·s^{-1})			
Average	986 ± 34	206 ± 4	147 ± 83
Maximum	1609 ± 62	1241 ± 39	1037 ± 410
Alder leaf chemistry§			
Specific leaf mass (mg/cm^2)	6.25 ± 0.30 a	6.09 ± 0.17 a	5.40 ± 0.16 b
N effects on SLM (%)	−12	NS	−11
P effects on SLM (%)	−18	NS	NS
Leaf N content (%)	2.74 ± 0.17 a	2.63 ± 0.16 a	2.75 ± 0.15 a
N effects on N content (% change)	NS	NS	+10
P effects on N content (% change)	NS	+7	+14
Leaf P content (%)	0.149 ± 0.006 a	0.140 ± 0.004 a	0.139 ± 0.005 a
N effects on P content (% change)	NS	NS	NS
P effects on P content (% change)	+69	+141	+107
N resorption (%)	23.7 ± 3.1 a	26.0 ± 1.0 a	17.7 ± 6.9 a
N effects on N resorption (%)	NS	NS	NS
P effects on N resorption (%)	NS	NS	+68
P resorption (%)	55.9 ± 3.9 a	53.9 ± 3.9 a	57.6 ± 1.8 a
N effects on P resorption (%)	NS	−21	NS
P effects on P resorption (%)	−77	−100	−98

Notes: Mid-successional stands, where alder grows in the understory of balsam poplar, were a primary focus, but data from parallel N and P fertilization plots established in early- and late-successional stands (see Discussion) are also included. Data with error terms are mean ± SE. NS indicates that effects were not significant.

† Stand structure data were recorded in 2010. Alder stem canker has reduced the number of live alder stems, particularly in early succession over the past five years (Ruess et al. 2009), where densities of 13 220 ± 3124 live stems/ha were recorded in 2005. Viereck et al. (1993) reported densities of up to 20 000 alder stems/ha for similar early-successional stands along the Tanana River.

‡ Microclimate data are hourly values averaged for a six-hour period (10:00–15:00 AST) from 15 June to 15 July for 2008, 2009, and 2010 for each of three replicate stands within each successional stage. Values are stand-level averages ($n = 3$ per successional stage) for the above time periods averaged across years. PPFD (photosynthetic photon flux density) data are from one sensor recording hourly at 2 m above the ground surface in each of three stands for each successional stage. PPFD values for early-successional stands are from an LTER sensor (LTER2) recording in an open area free from trees (10:00–15:00 AST, from 15 June to 15 July for 2009, 2010, and 2011) to represent the light available to alder, which dominates the canopy in these stands.

§ Leaf chemistry data were collected in 2009 following protocols outlined in Methods. Significant effects of fertilization treatments (N or P) on leaf chemistry characteristics are listed as percentage change from control stands ($P < 0.05$), and significant differences among stand types are indicated by different letters ($P < 0.05$).

Nodule Frankia

Additional subsamples from individual ^{15}N-treated nodules were subjected to DNA amplification using alder-compatible Frankia group-specific primers. These subsamples were powdered in 2 mL Screw Cap Micro Tubes (Sarstedt, Incorporated, Newton, North Carolina, USA) using a Retsch MM-300 mixer mill (Retsch, Newtown, Pennsylvania, USA) and 3–4, 3.2-mm stainless steel beads (BioSpec Products, Bartlesville, Oklahoma, USA) per sample. Total genomic DNA was extracted using Qiagen DNeasy 96 plant kit (Qiagen, Germantown, Maryland, USA) optimized for gram-positive bacteria by the addition of 4 μg/mL of

lysozyme. The resulting genomic DNAs were utilized in PCR at a 50-fold final dilution. We selectively amplified Frankia DNA in the nifD-K intergenic spacer region using primers optimized for high GC templates. The nucleotide sequence of the forward primer (nifD1310frGC) was 5′-CGCCAGATGCACTCCTGGGACTACT-3′, and that of the reverse primer (nifK331frGC) was 5′-CGGGCGAAGTGGCTGCGGAA-3′. PCRs utilized 25 μL of Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK), 0.25 μL (50 μmol/L) primers, and the following cycling conditions: 35 cycles of denaturation at 98°C for 1 min followed by 5 min of annealing and extension at 68°C. PCR products

were cleaned using Qiagen QIAquick columns and normalized to 4 ng/μL prior to cycle-sequencing using 2.0 μL ABI BigDye 3.1 Terminator Mix (Life Technologies), 3.0 μL of 5× buffer, 3.0 μL (1 μmol/L) sequencing primer, and ~80 ng of PCR product in a total volume of 20 μL per reaction. Cycle sequence products were cleaned over Sephadex and run through an ABI Prism 3100 DNA sequencer (Life Technologies, Carlsbad, California, USA). Paired chromatograms were assembled in Aligner (CodonCode, Boston, Massachusetts, USA); phred scores were exported and all bases with phred scores <20 were converted to N. Ends of assembled sequences were trimmed using TrimSeq (ambiguity = 5, window size = 40; [Rice et al. 2000]). We assembled a set of reference sequences representing the majority of restriction fragment (RF) types we have encountered in prior published and unpublished studies (Anderson et al. 2009, Anderson 2011), and added the new sequences from this study. These strict quality controls steps helped to insure that incorrect base calls would not artificially inflate diversity estimates. Operational taxonomic units (OTUs) were defined as those sharing sequence similarity at the 99% level based on clustering with the single-linkage genome assembly program Cap3 (Huang and Madan 1999), using default settings, except the following: match score = 5, max. gap length = 2, max. overhang length = 60%, minimum identity = 99% (Taylor and Houston 2011). Note that Cap3 uses pairwise sequence alignments, rather than a single multiple sequence alignment, to group sequences into clusters, and is thus robust to alignment uncertainty and error. We chose this high sequence identity threshold because it provided the maximum congruence between OTUs and RFLP types from our previous studies, which are biologically meaningful groupings based on their consistent patterns of community turnover across successional stages (Anderson et al. 2009).

Leaf chemistry

In 2009, 10 leaves were sampled from each of five randomly selected alders in each plot during the peak growing season (first week of July) and just before leaf fall (third week of September). For each plant, the first fully mature leaf from 10 branches was removed; these were stacked together and punched with a 3 cm diameter borer. Punches from each plant were dried, pooled, and weighed to the nearest 0.1 mg to calculate specific leaf mass (SLM) in milligrams per square centimeter). Total N was determined using a LECO TruSpec CN analyzer (St. Joseph, Michigan, USA), and total P was determined colorometrically following perchloric acid digests using a modified Technicon autoanalyzer (Technicon Instruments Corporation, Tarrytown, New York, USA). N and P resorption were calculated as the difference in peak and senescent leaf nutrient contents expressed as a percentage of peak values.

Nodule biomass

During July 2010, nodule biomass was measured on each of the replicate CTL, +N, and +P plots. Six plants were randomly selected in each of the nine plots, and one soil core (15.24 cm diameter × 20 cm depth) was taken at a random radial distance up to 0.75 m from the shrub base in each of the four polar quadrants. Soil cores were stored temporarily in plastic bags, brought immediately to the laboratory, and washed free of soil. Every nodule cluster within each core was removed from roots and sorted into live and dead biomass, based on the fact that dead nodules are black and quite brittle. The number of live nodule clusters per core was counted and then sorted into the following size classes: ≤0.5 cm, >0.5–1 cm, >1–2 cm, >2–3 cm, >3–4 cm, and >4 cm. Nodule clusters were then dried at 40°C and weighed. Numbers of clusters and biomass of nodules were calculated and expressed as number per square meter. For estimations of N inputs, we assumed a uniform distribution of nodules within a 0.75-m radius of each ramet.

Statistics

All data analyses were performed using SAS 9.2 for Windows (SAS 2002). Leaf chemistry data were averaged across plants within plots, and treatment effects were tested by one-way ANOVA with sites as replicates using PROC GLM. Differences in nodule respiration and N fixation among treatments, sites, and their interaction were tested using PROC MIXED, with nodules within plants, plants within sites, and treatment-by-plants within sites as random effects. Data for nodule density and biomass were averaged across cores for each plant, and then analyzed with PROC MIXED, testing for treatment, site, and treatment × site interactions, with plants as a random effect. The C cost of N fixation was calculated as the slope of the linear relationship between nodule respiration (dependent variable) and N fixation (independent variable). Differences in slopes between treatments and OTUs were assessed using contrasts in general linear models of log-transformed data. Logistic regression was used to determine whether assemblages of *Frankia* OTUs responded to treatments or sites. We then calculated the proportion that each OTU contributed to the pool of 10 nodules for each plant, and tested for main and interactive effects of treatment and site on the proportions of individual *Frankia* OTUs, using PROC MIXED with plants as a random effect. Treatment and site responses of N fixation and nodule respiration for individual *Frankia* OTUs were analyzed using PROC MIXED with the random effects listed previously by running models separately for each OTU. For the above analyses, data were tested for normality and homogeneity of variances, and where necessary, were square-root or log_{10} transformed, or ranked and reanalyzed to meet statistical assumptions. Linear regression was used to characterize relationships between N fixation and nodule respiration.
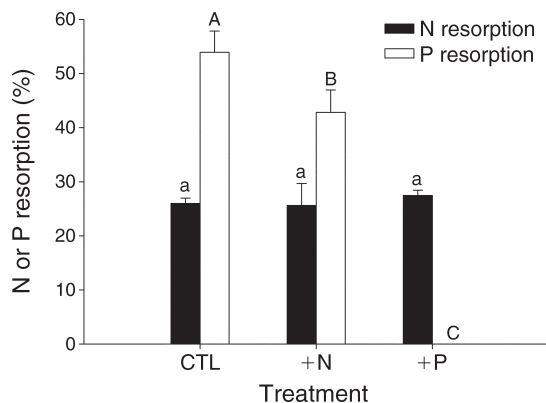
FIG. 1. Rates of autumnal N and P leaf resorption (percentage of green-leaf nutrient mass resorbed) in *Alnus tenuifolia* growing in control (CTL), +N, and +P plots during the 2009 growing season in the Bonanza Creek Experimental Forest, interior Alaska (USA). Values are means ± SE for all figures. Different letters above bars represent differences among treatments at α = 0.05 for each parameter.

Statistical significance was determined at α = 0.05 for all analyses, but marginally significant differences at α = 0.10 were also considered. Unless otherwise stated, data presented throughout are means ± SE of untransformed data.

RESULTS

Leaf nutrient contents

Plants from +P plots had significantly higher July leaf N concentrations (2.81% ± 0.06%) relative to plants from CTL plots (2.63% ± 0.05%) ($P < 0.05$), but neither differed from plants growing in +N plots (2.75% ± 0.06%). Plants from +P plots had July leaf P concentrations (0.338% ± 0.027%) that were more than double those found from plants in CTL (0.140% ± 0.004%) and +N plots (0.152% ± 0.005%) (both $P < 0.0001$) (Table 1). SLM measured in July did not vary among treatments, averaging 6.14 ± 0.09 mg/cm^2 across all plants. P resorption from leaves of plants growing in +P plots was not detectable, since P concentrations of senescing leaves (0.463% ± 0.042%) exceeded those measured in July. P resorption from plants in +N plots (42.8% ± 4.1%) was significantly less than that measured in CTL plots (53.9% ± 3.9%) ($P < 0.05$). Neither N nor P fertilization affected the magnitude of N resorption from leaves, which averaged 26.4% ± 1.3% across all plants (Fig. 1, Table 1).

Nodule biomass and N content

Relative to control plots, where nodule density averaged 911 ± 199 clusters/m^2, nodule density was higher in +P plots (4631 ± 1051 clusters/m^2) ($P < 0.001$) and lower in +N plots (346 ± 159 clusters/m^2) ($P < 0.01$). These cluster densities translated to higher live nodule biomass in +P plots (52.0 ± 6.2 g/m^2) ($P < 0.05$) and marginally lower live nodule biomass in +N plots

(21.0 ± 3.4 g/m^2) ($P = 0.13$) when compared with CTL plots (33.5 ± 5.9 g/m^2). Dead nodule biomass did not differ across treatments, averaging 4.3 ± 1.2, 6.7 ± 1.8, and 6.9 ± 1.3 g/m^2 in CTL, +N, and +P plots, respectively. Nodules in +P plots had significantly higher N concentrations (2.38% ± 0.15%) relative to CTL (1.725% ± 0.06%) and +N plots (1.97% ± 0.28%) ($P < 0.001$), which did not differ.

N fixation, nodule respiration, and fixation costs

Relative to rates measured in control plots (4.01 ± 0.32 μmol N·[g nodule]$^{-1}$·h^{-1}), N-fixation rates were increased by P fertilization (7.65 ± 0.50 μmol N·[g nodule]$^{-1}$·h^{-1}) ($P < 0.0001$) and reduced by N fertilization (2.75 ± 0.27 μmol N·[g nodule]$^{-1}$·h^{-1}) ($P < 0.05$). N fixation did not differ among replicate sites, nor were there interactive effects of treatment and site on N-fixation rates. Control and +N plots had more samples of what we consider very low N-fixation values (between 0.01 and 0.5 μmol N·[g nodule]$^{-1}$·h^{-1}) compared with +P plots ($\chi^2 = 16.5$, $P < 0.001$). Statistical differences among CTL, +P, and +N plots remain unchanged if we exclude these low values, although means are shifted upward slightly (4.67 ± 0.34, 7.88 ± 0.51, and 3.29 ± 0.31 μmol N·[g nodule]$^{-1}$·h^{-1}, respectively). We speculate on the reasons for these low samples below, and their implications for estimations of N-fixation cost.

Similar patterns were found among treatments for nodule respiration rates, which were highest in +P plots (66.30 ± 3.27 μmol CO$_2$·[g nodule]$^{-1}$·h^{-1}) and lowest in +N plots (30.02 ± 1.36 μmol CO$_2$·[g nodule]$^{-1}$·h^{-1}) (Fig. 2). Exclusion of the low N-fixation samples, described previously, led to only slightly higher average nodule respiration rates in CTL, +N, and +P plots (45.34 ± 2.23, 32.53 ± 1.68, and 66.99 ± 3.31 μmol CO$_2$·[g nodule]$^{-1}$·h^{-1}, respectively). Nodule respiration rates varied among sites ($P < 0.05$), with significantly higher rates at BP1 relative to BP3 ($P < 0.05$), and intermediate rates at BP2.
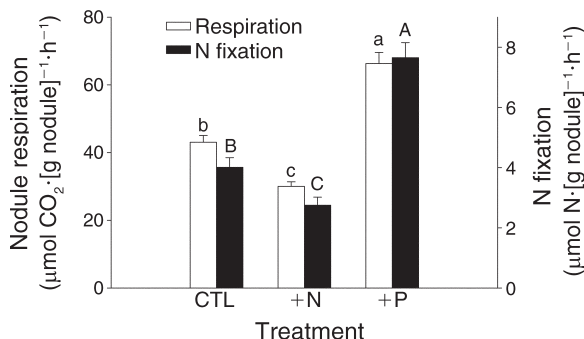

FIG. 2. Effects of N and P fertilization on rates of nodule respiration and N fixation of individual *A. tenuifolia* nodules averaged across three replicated balsam poplar stands. Different letters above bars represent differences among treatments at α = 0.05 for each parameter.
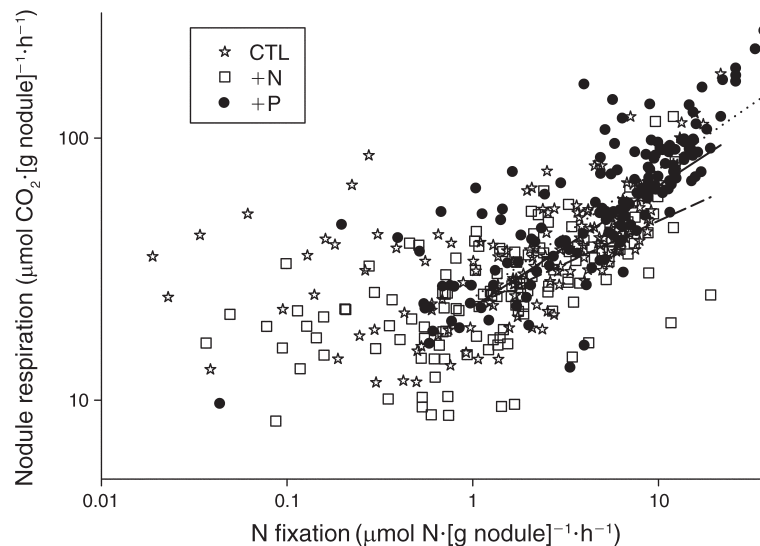
FIG. 3. The relationship between nodule respiration and N-fixation rate for individual nodules sampled in CTL, +N, and +P plots. Lines are linear regressions for values above an N-fixation rate of 1 µmol N·[g nodule]$^{-1}$·h^{-1} ($r^2 = 0.65, 0.26$, and 0.67 for CTL, +N, and +P plots, respectively; all $P < 0.0001$). Below this level, the two fluxes were not correlated, and nodule respiration rates were significantly lower in +N plots (19.66 ± 1.10 µmol CO_2·[g nodule]$^{-1}$·h^{-1} [mean ± SE]) relative to CTL (28.66 ± 2.70 µmol CO_2·[g nodule]$^{-1}$·h^{-1}; $P < 0.001$) and +P plots (27.00 ± 2.71 µmol CO_2·[g nodule]$^{-1}$·h^{-1}; $P < 0.05$), which did not differ.

Across all samples, nodule respiration and N-fixation rates were positively correlated for plants growing in control, +N, and +P plots ($r^2 = 0.61, 0.37$, and 0.71, respectively; all $P < 0.0001$). However, below an N-fixation rate of 1 µmol N·[g nodule]$^{-1}$·h^{-1}, the two fluxes were not correlated for any treatment, but rates for this "maintenance nodule respiration" were significantly lower in +N plots relative to CTL ($P < 0.001$) and +P plots ($P < 0.05$) (Fig. 3).

N-fixation cost, calculated as the linear regression slope of nodule respiration vs. N fixation across all samples, was significantly lower in +N plots (3.30 ± 0.36 µmol CO_2/µmol N) compared with CTL (4.95 ± 0.33 µmol CO_2/µmol N) and +P plots (5.06 ± 0.27 µmol CO_2/µmol N) ($P < 0.05$), which did not differ. Treatment differences in fixation costs for the lines shown in Fig. 3 (above an N-fixation rate of 1 µmol N·[g nodule]$^{-1}$·h^{-1}) differed similarly, and averaged 2.78 ± 0.49, 5.33 ± 0.37, and 5.00 ± 0.30 µmol CO_2/µmol N for +N, CTL, and +P plots, respectively. At higher fixation rates, e.g., above an N-fixation rate of 5 µmol N·[g nodule]$^{-1}$·h^{-1}, nodule respiration and N-fixation rates were not correlated for +N plots; however, costs for plants in +P plots (5.25 ± 0.38 µmol CO_2/µmol N) were significantly lower than for CTL plots (7.22 ± 0.63 µmol CO_2/µmol N) ($P < 0.05$), despite higher N-fixation rates in +P plots.

Frankia composition and ecophysiology

Grouping of *Frankia* nifD-K sequences resulted in 11 OTUs, of which 8 were recovered from nodules from the current study. OTUs 1–3 were found only in nodules outside the current study site; hence we discuss only OTUs 4–11 below. Logistic regression suggested that treatment (CTL, +N, or +P) partially explained *Frankia* OTU composition ($\chi^2 = 5.02, P = 0.08$), with significant differences between CTL and +N plots ($\chi^2 = 5.08, P < 0.05$), CTL and +P plots ($\chi^2 = 4.08, P < 0.05$), and +N and +P plots ($\chi^2 = 3.72, P < 0.05$). Only two OTUs, OTU4 and OTU8, combined to account for >95% of all nodules sampled across control and treatment plots (Fig. 4). In control plots, OTU4 and OTU8 were of similar abundance (52.7% ± 4.6% and 42.4% ± 4.4%, respectively, of 142 nodules) ($t = 1.19$, not significant), but OTU4 increased in abundance relative to OTU8 in both +N ($t = 2.93, P < 0.01$) and +P plots ($t = 2.51, P < 0.05$). Among the six subdominant OTUs, only OTU5 was found across all plots. Other subdominants were found only in CTL plots (OTU6), +N plots (OTU10), or other combinations of treatments (OTUs 7, 9, 11).

The two *Frankia* dominants (OTU4 and OTU8) had statistically similar nodule respiration and N-fixation rates in control plots, and showed similar physiological responses to treatments, where rates of both nodule respiration and N fixation were increased in +P plots and decreased in +N plots (Fig. 5). However, the C cost of N fixation was significantly greater in OTU8 compared with OTU4, particularly at higher N-fixation rates. For example, in CTL plots above an N-fixation rate of rate of 5 µmol N·[g nodule]$^{-1}$·h^{-1}, N-fixation costs for OTU8 (7.94 ± 0.38 µmol CO_2/µmol N) were significantly higher than for OTU4 (5.52 ± 0.57 µmol CO_2/µmol N) ($P < 0.10$). Similar cost differences between the two dominants were found in +P plots (66% higher cost in OTU8 relative to OTU4, $P < 0.01$). OTU5 was the only subdominant abundant enough to
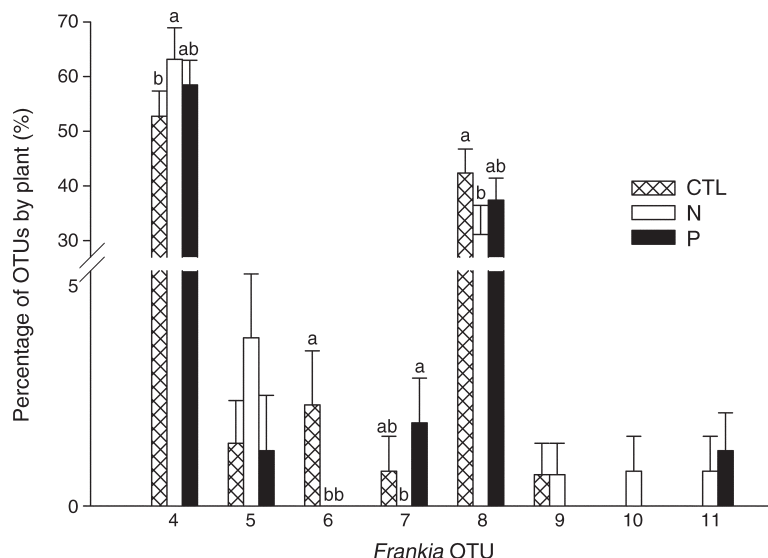
FIG. 4.   The proportion of individual *Frankia* OTUs (operational taxonomic units) in CTL, +N, and +P plots. Bars represent the proportion of each of the eight OTUs for each plant, averaged across plants and sites for each treatment. Within OTUs, bars with different letters represent treatment differences at $P < 0.10$ (OTU4) and $P < 0.05$ (OTU8).

be compared across treatments, and had similar respiration and N-fixation rates to the two dominant OTUs in CTL and +N plots (Fig. 5). However, OTU5 failed to up-regulate nodule respiration or N-fixation rates in +P plots. Moreover, none of the N-fixation data points >5 μmol N·[g nodule]$^{-1}$·h^{-1} in Fig. 3 are from OTU5 nodules.

DISCUSSION

Fertilization effects on N fixation and plant N:P balance

We found significant increases in leaf P concentration (+141%), nodule N concentration (+38%), density (+408%), and biomass (+55%), and N fixation per gram of nodule (+97%) in response to P fertilization. Leaf %N was also higher in P-fertilized relative to control plots (+7%), likely as a function of increased N-fixation rates. Up-regulation of these factors is typical of N-fixing plants, because of their inherently high P requirements relative to non-N-fixing plants. Although the biochemical mechanisms explaining this higher P requirement are not fully understood, the high P dependency of nodule C and N metabolism is believed to be the primary reason (Valverde et al. 2002, Gentili and Huss-Danell 2003, Bargaz et al. 2011). Because of this elevated P requirement, N-fixing plants are thought to invest more C and N in extracellular enzymes for soil P mobilization in P-limited environments than are non-N-fixing plants (Giardina et al. 1995, Zou et al. 1995, Wang et al. 2007, Houlton et al. 2008, Venterink 2011). Costs associated with supporting N-fixing symbionts, coupled with enzymatic costs of P acquisition, either by roots or symbiotic mycorrhizae, explain why the up-regulation of N-fixing activities is more closely linked to plant N:P

balance than to N or P availability separately (Wall et al. 2000, Huss-Danell et al. 2002, Valverde et al. 2002).

Whether increased nodule biomass in our P-fertilized plots resulted from parallel increases in plant and nodule growth or from greater partitioning to nodule production is unknown. Greenhouse studies show that actinorhizal plants often up-regulate nitrogenase activity, nodule production, and biomass partitioning to nodules as mechanisms to increase plant growth when fertilized with P (Uliassi et al. 2000, Wall et al. 2000, Valverde et al. 2002). A limited number of field studies have measured N-fixation rates following P fertilization (Vitousek 1999, Uliassi and Ruess 2002, Gokkaya et al. 2006) and along natural gradients of soil P availability (Pearson and Vitousek 2001). However, measures of nodule biomass in natural stands are rare (Pearson and Vitousek 2001, Uliassi and Ruess 2002, Ruess et al. 2009), and we know of no field study that has quantified biomass partitioning to nodules. The smaller average nodule cluster size in our +P plots in combination with greater nodule biomass suggests that nodule turnover increased relative to control plots, similar to fine-root responses to fertilization seen in some studies (Pregitzer et al. 1995, King et al. 2002). Thus C allocation to nodules in +P plots was likely higher than is implied by biomass differences alone. Nodule senescence can be triggered by disturbances such as pruning (Nygren and Ramirez 1995) or disease (Ruess et al. 2009), but the complex signaling that regulates such stress responses and those governing age-dependent senescence are poorly understood (Puppo et al. 2005, Van de Velde et al. 2006). Nodules of actinorhizal plants are characterized by indeterminate growth from an apical meristem resulting in a develop-

mental gradient that includes a zone of senescence at the nodule base containing fewer healthy *Frankia* vesicles (these are specialized *Frankia* structures where N fixation is localized) and having reduced rates of nitrogenase gene expression (Pawlowski et al. 1995, Pawlowski and Sprent 2008). As nodules age, the proportional volume occupied by this senescent zone increases, bacterial material is degraded, and nutrients are presumably resorbed by the host, explaining the decline in N-fixing capacity in older nodules. Rates of gas diffusion into and within nodules also decline with increasing nodule age (Harris and Silvester 1994, Silvester et al. 2008). Given the decline in N-fixation rates as nodules age (Huss-Danell 1997), a younger population of nodules maintained through increased nodule turnover likely contributes to higher N-fixation rates in P-fertilized plants.

Increased rates of nodule growth and N fixation in P-fertilized plots may be supported by an up-regulation of photosynthesis, as has been shown in legumes (Kaschuk et al. 2009). However, we have failed to detect significant photosynthetic responses of *A. tenuifolia* to P fertilization in either greenhouse (Uliassi et al. 2000) or field (Uliassi and Ruess 2002) experiments, suggesting that changes in whole-plant C partitioning may be more important than C acquisition in explaining the increased N fixation and aboveground growth commonly seen in P-fertilized alder. Brown (2002) and Brown and Courtin (2003) reported that positive growth responses of red alder to P fertilization were more closely tied to increased partitioning to leaves and branches than to increased photosynthetic rates. We previously found that total biomass of greenhouse-grown thin-leaf alder was nearly four times higher when grown in soil cores from P-fertilized plots compared with unfertilized plots, and biomass partitioning to nodule biomass increased by 56% while root/shoot ratio was reduced 42% (Uliassi et al. 2000). Our observations indicate that nodule density per unit root length increased in P-fertilized plots, but we don't know whether decreased partitioning to roots accompanied up-regulation of nodule growth in response to P, as seen in our previous greenhouse study.

The most likely shift in C partitioning for support of increased N-fixation activities may involve decreased investments in mycorrhizae and production of extracellular P-mobilizing enzymes (Treseder and Vitousek 2001, Lynch and Ho 2005). Alders have been shown to associate with a less diverse and more distinct community of ectomycorrhizal fungi (EMF) relative to other hosts growing in the same environment (Becerra et al. 2005, Tedersoo et al. 2009, Kennedy and Hill 2010, Bent et al. 2011, Rochet et al. 2011). Kennedy and Hill (2010) speculated that tolerance to high soil N and specialization for acquisition of some other nutrient may explain the low richness and host-specific taxa of EMF found on *Alnus rubra*. We suspect such specialization is tied to mobilization and uptake of soil P, and are completing a parallel study to test this hypothesis. Field
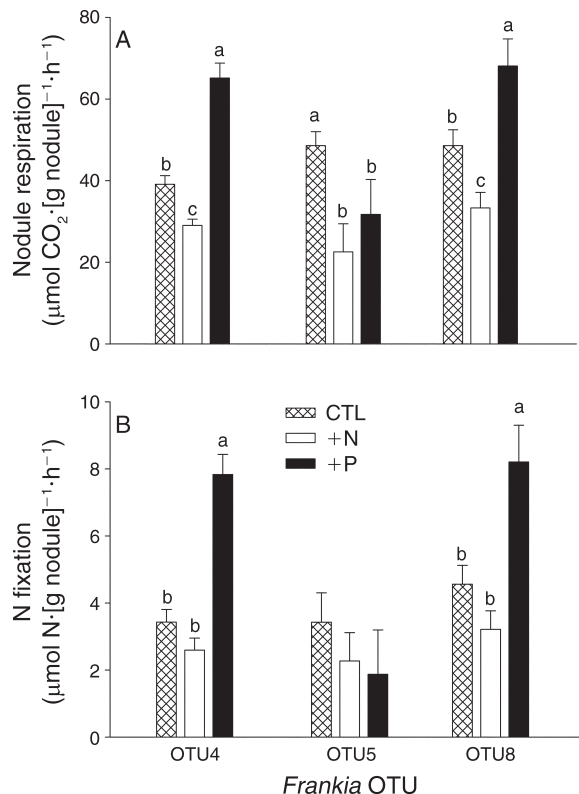

FIG. 5. Effects of N and P fertilization on (A) rates of nodule respiration and (B) N fixation averaged across all samples for the two *Frankia* dominants (OTU4 and OTU8), and the one subdominant found across all treatments (OTU5). Within OTUs, bars with different letters represent treatment differences at $P < 0.10$.

experiments conducted in the same plots as those used for the present study have found that EMF colonization is high in *A. tenuifolia*, but relative to control plots, thin-leaf alder growing in +P plots had lower levels of EMF colonization (D. L. Taylor, *unpublished data*) and lower rates of key P-mobilizing enzymes (acid phosphatase, phosphodiesterase, and phytase) (M. Swanson, *unpublished data*). In agreement with theory outlined by Houlton et al. (2008), activities of these enzymes were higher and more variable in response to soil P in alder compared with the dominant conifer in the same plots (M. Swanson, *unpublished data*). These results suggest that high costs of P acquisition may be partially diverted to support increased nodule production and specific N-fixation rates in P-fertilized plots. Reduced C partitioning to mycorrhizae has been similarly proposed as a mechanism explaining growth responses of sugar maple to fertilization, where aboveground growth but not photosynthesis increased following chronic N additions (Talhelm et al. 2011). Finally, additional growth benefits from P fertilization may derive from improved water-use efficiency, as has been suggested for red alder (Brown et al. 2011). Interannual variability in growth of thin-leaf alder growing along the Tanana River floodplain is

closely tied to river heights in the first part of the growing season, when precipitation is typically low and plants appear to be utilizing hyporheic flow (Nossov et al. 2010, Koyama and Kielland 2011). Thus, any improvement in water-use efficiency afforded by P fertilization may allow the species to divert C resources from root growth to support nodule functions.

Down-regulation of N fixation following N fertilization involves mechanisms for detection of whole-plant N:P balance similar to those for the up-regulation of these same activities in response to P additions. Relative to control plots, plants growing in N-fertilized plots had lower N-fixation rates (−31%, $P < 0.05$), lower nodule respiration rates (−28%, $P < 0.05$), and trended toward lower nodule biomass (−37%, $P = 0.13$). Relative to +P plots, these same variables were reduced by 65%, 51%, and 60%, respectively. N fertilization had no effect on leaf N resorption, but decreased P resorption by 21%, likely because of reduced P demand when N fixation was down-regulated. Reduced demand for fixed N lowers carbohydrate supply to nodules and reduces associated rates of nodule respiration. This is thought to be mediated by long-distance signals transmitted in phloem from shoots to nodules, which ultimately affect nitrogenase or other key enzymes involved with C or N metabolism of N fixation (Valverde and Huss-Danell 2008). Lower nodule respiration reduces respiratory ATP production and increases pO_2 within vesicles, both of which serve to further reduce nitrogenase activity and N-fixation rates (Silvester et al. 2008). Greenhouse experiments with *A. incana* demonstrate that nodule initiation and C partitioning to nodule growth are reduced by elevated ratios of N:P in growth solutions (Huss-Danell et al. 1982, Wall et al. 1998, 2000, Gentili and Huss-Danell 2003), but we are unaware of field experiments that have quantified N-induced down-regulation of N fixation, nodule respiration, and nodule biomass. Factors limiting growth, such as shade or defoliation, also reduce plant N demand and associated C supply to nodules, leading to nitrogenase inactivation and degradation (Lundquist et al. 2003, Valverde and Wall 2003a, b, Ruess et al. 2006, 2009).

Nutritional effects on respiratory C costs of nitrogen fixation

Values for respiratory C costs of N fixation that we measured in the field on nodules of mature *A. tenuifolia* are remarkably similar to those reported from greenhouse studies, the vast majority of which come from legumes. Our N-fixation costs averaged 5.12 ± 0.16 µmol CO_2/µmol N across all plants, and 5.55 ± 0.32 µmol CO_2/µmol N above N-fixation rates of 5 µmol N·[g nodule]$^{-1}$·h^{-1}. An extensive literature review of respiratory C costs in legumes reported a range of 3.5–6 µmol CO_2/µmol N when measured on a nodule basis (Minchin and Witty 2005). Early work measuring C costs using CO_2 evolution and acetylene reduction on *Alnus rugosa* nodules reported 3.6 µmol CO_2/µmol

C_2H_4, which translates to 5.4 µmol CO_2/µmol N, assuming a C_2H_4:N_2 reduction ratio of 3:1 (Tjepkema and Winship 1980). The similarity of our estimates to the legume values reported by Minchin and Witty (2005) and to other actinorhizal species (Tjepkema and Winship 1980, Tjepkema 1985) provides support that our field method provided reliable measures of $^{15}N_2$ uptake and nodule respiration. Although we don't fully understand what accounts for the variability in N-fixation rate among nodules within a given plant, rates of nodule respiration were orders of magnitude less variable than those for N fixation (Fig. 3). Moreover, there appeared to be a baseline respiration rate below a N-fixation rate of 2 µmol N·[g nodule]$^{-1}$·h^{-1} that was significantly lower in +N plots (22.4 ± 0.9 µmol CO_2·[g nodule]$^{-1}$·h^{-1}) relative to CTL (29.0 ± 1.9 µmol CO_2·[g nodule]$^{-1}$·h^{-1}) and +P plots (32.4 ± 2.7 µmol CO_2·[g nodule]$^{-1}$·h^{-1}). This likely represents the minimum respiratory cost necessary for maintaining sufficiently low pO_2 to prevent inhibition and inactivation of nitrogenase, coupled with maintenance and growth of nonbacterial nodule tissue. Such a mechanism allows plants to maintain nodule function during periods of reduced N demand, such as at night or during periods of brief C or P stress, without damage to nitrogenase complexes (Valverde and Huss-Danell 2008). In the low range of N fixation, e.g., between 1 and 2 µmol N·[g nodule]$^{-1}$·h^{-1}, plants in +P plots had significantly higher respiration rates (39.1 ± 4.5 µmol CO_2·[g nodule]$^{-1}$·h^{-1}) than plants in either CTL (29.6 ± 2.2 µmol CO_2·[g nodule]$^{-1}$·h^{-1}) or +N plots (26.1 ± 1.4 µmol CO_2·[g nodule]$^{-1}$·h^{-1}), which suggests a higher maintenance cost in nodules up-regulated by P fertilization. This higher nodule respiration rate in +P plots relative to +N plots persists at higher N-fixation rates (see Fig. 3 and *Results* section), and may be due to younger, more active nodules with a higher density of *Frankia* vesicles per gram of nodule in +P plots.

Although nodule respiration rates and N-fixation rates were both significantly higher in plants from +P plots relative to control plots, the C cost of N fixation did not differ between +P and CTL plots across most of the range in N fixation. In fact, at N-fixation rates >5 µmol N·[g nodule]$^{-1}$·h^{-1}, C costs of N fixation in plants from +P plots were significantly lower than in plants from CTL plots (5.25 ± 0.38 vs. 7.22 ± 0.63 µmol CO_2/ µmol N, respectively) ($P < 0.05$). Another way to characterize the effects of N and P fertilization on N-fixation economics is to examine standard major axis (SMA) slopes of log_{10} (nodule respiration) vs. log_{10} (N fixation) using Model II regression, which provide evidence for isometric vs. allometric scaling (Sokal and Rohlf 1981, Wright et al. 2004). Above an N fixation of 1 µmol N·[g nodule]$^{-1}$·h^{-1}, nodule respiration scaled allometrically with N fixation for CTL, +N, and +P plots, increasing by factors of 4.3, 4.1, and 4.6, respectively, for a 10-fold increase in N-fixation rate (Table 2). The SMA slope of 0.63 from plants in CTL

TABLE 2. Standardized major-axis slopes from Model II regressions of $\log_{10}$(N fixation) (dependent variable) on $\log_{10}$(nodule respiration) (independent variable).

N fixation (μmol N·[g nodule]$^{-1}$·h^{-1})	CTL	+N	+P
<1			
Mid	NS	NS	NS
Early	NS		
>1			
Mid	0.630 (4.27‡)	0.608 (4.05‡)	0.659 (4.56‡)
Early	0.568 (3.70‡)		
>3			
Mid	0.893 A	0.946	0.884 (7.66†)
Early	0.610 (4.07‡) B		
>5			
Mid	1.15 (14.19†) A, a	NS	0.875 (7.50†) b
Early	0.786 (6.11‡) B		

Notes: "Mid" refers to alder growing in mid-successional stands (this study), and "Early" refers to a pilot study conducted in early-successional stands during 2007 (R. W. Ruess, *unpublished data*). Slopes differing significantly from 1.0 are indicative of allometric scaling, and the value in parentheses is the factor by which nodule respiration increases for every 10-fold increase in N fixation, calculated as 10^{slope}. Slopes not followed by parentheses indicate isometric scaling and do not differ significantly from 1.0. Significance of deviations from a slope of 1.0 are based on 90% or 95% asymmetric confidence intervals (not shown; see footnotes). Models were run for a number of ranges of N fixation to determine how scaling varied with increasing N-fixation rates. Data followed by different lowercase letters within rows indicate treatment differences in standardized major axis (SMA) slopes at $P < 0.10$. Data followed by different uppercase letters within cells indicate differences between successional stages in SMA slopes at $P < 0.10$. Unless noted, all models above an N-fixation rate of 1 μmol N·[g nodule]$^{-1}$·h^{-1} are significant at $P < 0.001$. NS indicates "significant."

† Significance based on 90% asymmetric confidence interval.
‡ Significance based on 95% asymmetric confidence interval.

plots is very similar to the SMA value of 0.59 for mass-based $\log_{10}$(leaf respiration) on $\log_{10}$(photosynthetic rate) reported by Wright et al. (2004) across a global leaf-trait data set, suggesting that respiratory costs are constrained across diverse plant physiological functions. As expected from the data distributions shown in Fig. 3, this scaling factor for nodule respiration increased at higher N-fixation rates. Above an N-fixation rate of 5 μmol N·[g nodule]$^{-1}$·h^{-1}, a 10-fold increase in N fixation translated to a 14.2-fold increase in nodule respiration in CTL plants, but a significantly lower (7.5-fold) increase in +P plants ($P < 0.10$) (Table 2). Nodule respiration is the net cost of a number of highly integrated physiological processes associated with N fixation, including nitrogenase activity, H_2 production and uptake, N assimilation and transport, CO_2 recycling by PEP carboxylase within nodules, and growth and maintenance respiration of plant and bacterial tissues. We don't know whether or how cost scaling of these processes is affected during up-regulation of N fixation, but changes in morphological characteristics such as vesicle density or size may influence cost scaling through effects on gas diffusion or membrane transport functions. Another intriguing possibility is the shift toward less-expensive *Frankia* OTUs in both +N and +P plots as discussed in *Partner choice*. Whatever the mechanism, it appears that alder employs mechanisms to increase the efficiency of C use when N fixation is up-regulated.

Partner choice

A major objective of the present study was to test the hypothesis that host choice might be utilized by *A. tenuifolia* to respond to changing environmental conditions across succession. The question of whether plants are able to choose superior-performing genotypes of N-fixing bacteria is currently a very active area of experimental inquiry, and cannot be answered definitively in a single field study. Our goal in the present study was to determine whether alders in the field respond to manipulation of N-fixation physiology in a manner consistent with this hypothesis.

In two prior field studies (Anderson et al. 2009) we have observed a strong relationship between successional habitat and genetic structure of *Frankia* assemblages in *A. tenuifolia* root nodules. In early-succession sites ($n = 3$), nodules were consistently dominated by a single genotype ("RF7"), which occurred in 65% to 95% of nodules collected within a site, while late-succession nodules contained much greater richness and evenness. RF7 corresponds to OTU3 in the sequence-based analysis presented here. The present study was conducted in mid-succession sites in the same primary sere and in close proximity (1–15 km) to the early and late-succession sites. *Frankia* composition in this study appears to be transitional between early- and late-succession assemblages: >95% of the 408 nodules yielding interpretable data contained either OTU4 or

OTU8. OTU4 sequences are identical to sequences from RF1, which is uncommon in early, but common in late succession in our previous studies; OTU8 corresponds to RF3, which is uncommon in both early and late succession. Thus OTU4 is a mid–late succession genotype, while OTU8 is abundant only in mid-succession. The differences in abundance of these two OTUs compared to early and late succession, and the consistency of composition among replicate mid-succession sites, further support a close relationship between symbiotic *Frankia* composition and successional habitat in *A. tenuifolia*.

We have previously hypothesized that this relationship is primarily due to differences in host choice across succession, mostly as a result of early succession alders favoring RF7/OTU3, and late-succession alders reducing selectivity as C partitioning to nodule biomass diminishes over succession (Anderson et al. 2009, Anderson 2011). Early-succession alders maintain high leaf N concentrations (Anderson et al. 2009), probably to allow high photosynthetic rates made possible by the position of alder in the canopy and demanded by the high growth rates (Viereck et al. 1993*a*) resulting from high intraspecific competition for light in these dense stands. Moreover, early-succession soils have very low N concentrations and N mineralization rates (Kielland et al. 2006), so alders in these habitats are highly reliant on fixed N. Under these circumstances, selectivity for optimally performing *Frankia* genotypes should be very high, which is consistent with the dominance of RF7/OTU3 observed in early succession. Host choice of nutritional micro-symbionts (N-fixing bacteria and/or mycorrhizae) based on performance is predicted by a great deal of evolutionary theory (Simms and Taylor 2002, Kiers and Denison 2008), and has empirical support in several legume–rhizobial (Kiers et al. 2003, 2006, Simms et al. 2006) and mycorrhizal (Bever et al. 2009) systems, but remains untested in long-lived actinorhizal symbioses. Whether such a system could respond to changing environmental conditions as we have hypothesized is completely unknown. Data from the present study are broadly consistent with this hypothesis, but, as can be expected from a field study, are complex and far from definitive.

An important prerequisite for host choice is that bacterial genotypes differ in physiological traits relevant to host fitness. Specifically, we predicted that N-fixation rate and C cost would differ among genotypes, and that there would be a trade-off between the two traits. Cost differences between OTU4 and OTU8 were clear in CTL plots at N-fixation rates >3 μmol N·[g nodule]$^{-1}$·h^{-1}, with OTU4 being the consistently cheaper type (Table 3). Differences in N-fixation rate were less clear, but were in the direction predicted by a trade-off. ANOVA found no significant differences in either N-fixation or respiration, but unpaired *t* tests on transformed data were significant for respiration (two-tailed $P < 0.04$), and marginally significant for N fixation (one-tailed $P <$

0.07) in CTL plots, with OTU8 having the highest mean value for both. Further support for bacterial differences relevant to host choice is provided by C cost data for RF7/OTU3, the early-succession dominant genotype. In a pilot study, RF7/OTU3 was found to have a lower C cost than the two next most common OTUs present in early succession (R. W. Ruess and M. D. Anderson, *unpublished data*). This difference was statistically significant, but not robust, due to unavoidably low replication for the less common OTUs in early succession ($n = 72$ for RF7, $n \leq 13$ for others). Comparison with OTU4 and OTU8 in control plots in the present study indicates a robust difference from OTU8 and a consistent difference from OTU4 across the range of N-fixation rates, with RF7/OTU3 having the lowest slope (Table 3). Thus the dominant genotype in early succession has the lowest C cost we have observed in any genotype to date, consistent with the hypothesis that early-succession alders select superior (i.e., low-cost) symbionts.

Across fertilization treatments in mid-succession sites of the present study, support for the host choice hypothesis is less clear. We predicted that up- and down- regulation of nodulation and N fixation by fertilization with P and N, respectively, would result in parallel shifts in symbiotic *Frankia* composition. We did not observe changes in identity of the dominant genotypes in response to our manipulations: in all treatments nodules were strongly dominated by OTU4 and OTU8. Differences in the relative proportion of these genotypes were detected across treatments, but these differences were relatively modest (Fig. 4). Nevertheless, the largest observed difference supported our prediction that down-regulation of N fixation would disproportionately affect the higher-cost, higher-fixing genotype. This was in the +N plots, in which the frequency of OTU8 (31% of 125 nodules) dropped to half that of OTU4 (63% of 125 nodules) as nodulation and N fixation decreased relative to CTL plots, and N fixation and respiration became decoupled at high fixation rates (Tables 2 and 3). In +P plots, frequencies of OTU4 (59% of 141 nodules) and OTU8 (36% of 141 nodules) also differed significantly ($P < 0.05$), but in the opposite direction to our prediction; rather than favoring the high-cost/fixation genotype, up-regulation of fixation favored the low-cost one. Overall, shifts in OTU abundance in +N and +P plots, while modest, suggest that altered plant resource availability feeds back to physiologically meaningful shifts in *Frankia* community composition. In our view, the most parsimonious explanation for this observation is the occurrence of host choice in *A. tenuifolia*.

This study is the only field study we are aware of examining the possibility of host choice based on differential physiological benefits in an N-fixing symbiosis, and also the most comprehensive measurement of the cost of N fixation for any individual N-fixing system we are aware of. While several of our observations are

TABLE 3. Standardized major axis (SMA) slopes from Model II regressions of $\log_{10}$(N fixation) (dependent variable) on $\log_{10}$(nodule respiration) (independent variable) for the two dominant *Frankia* operational taxonomic units (OTU4 and OTU8) found in this study.

N fixation (μmol N·[g nodule]$^{-1}$·h^{-1})	CTL	+N	+P
<1	NS	NS	NS
>1			
OTU4	0.558 (3.61‡)	0.572 (3.73‡)	0.613 (4.10‡)
OTU8	0.684 (4.83‡)	0.715 (5.19†)	0.699 (5.00‡)
RF7a	0.567 (3.69‡)		
>3			
OTU4	0.780 (6.03†) B, b	0.670 (4.68†) b	0.946 a
OTU8	0.982 A	NS	0.895
RF7a	0.610 (4.07‡) B		
>5			
OTU4	0.888 (7.73†) B	NS	0.844
OTU8	1.326 (21.2†) A, a	NS	0.933 b
RF7a	0.786 (6.10†) B		

Notes: Data are also included for "RF7a," the dominant *Frankia* OTU encountered in early-successional stands during our 2007 pilot study (R. W. Ruess, *unpublished data*). Slopes differing significantly from 1.0 are indicative of allometric scaling, and the value in parentheses is the factor by which nodule respiration increases for every 10-fold increase in N fixation, calculated as 10^{slope}. Slopes not followed by values in parentheses indicate isometric scaling and do not differ significantly from 1.0. Significance of deviations from a slope of 1.0 are based on 90% or 95% asymmetric confidence intervals (not shown; see footnotes). Models were run for a number of ranges of N fixation to determine how scaling varied with increasing N-fixation rates. Data followed by different uppercase letters within cells indicate OTU differences in SMA slopes at $P <$ 0.10. Data followed by different lowercase letters within rows indicate treatment differences in SMA slopes at $P < 0.10$. Unless noted, all models above an N-fixation rate of 1 μmol N·[g nodule]$^{-1}$·h^{-1} are significant at $P < 0.001$.

† Significance based on 90% asymmetric confidence interval.

‡ Significance based on 95% asymmetric confidence interval.

consistent with the host-choice hypothesis, some are not, and even for the former, several caveats must be issued. One observation that is difficult to reconcile with host choice is the negative correspondence between changes in relative OTU frequency and changes in physiological differences between OTU4 and OTU8 across treatments. The largest differences in C cost, respiration, and fixation rate occurred in CTL plots (Table 3), in which the frequencies of the two OTUs were not statistically different. In other words, in plots in which the basis for choice between the two dominant OTUs was clearest, alders appeared not to choose. This may reflect a lack of selective mechanisms in alder, i.e., our host choice hypothesis may be wrong, or may be due to ambiguities arising from the fact that this study was conducted in the field, and multiple factors known to affect nodule formation and physiology were neither controlled nor measured. This is also a major caveat for our observations supporting host choice. In addition to limited control, field experiments are also limited to observation of OTUs forming appreciable numbers of nodules in situ, in our case OTU4 and OTU8. For example, OTU5 also occurred in host nodules across all three treatments, but not in sufficient numbers to obtain reliable estimates of N fixation or respiration, so the degree to which host choice may be involved in low nodulation by this genotype is unclear. Controlled-inoculation experiments utilizing *Frankia* isolates from our field sites will be crucial to characterizing this role.

A second caveat is that the present study was restricted in terms of the level of choice examined. Some proposed selective mechanisms in N-fixing plants should result in differences in frequency of nodules containing specific bacterial genotypes: e.g., selective nodule initiation in response to honest bacterial signals (Heath and Tiffin 2009, Gubry-Rangin et al. 2010), or selective senescence of nodules in response to host assessment of bacterial performance (Denison 2000). Other proposed mechanisms should result in differences in nodule size and/or bacterial density among genotypes, e.g., sanctions not involving nodule senescence (Kiers et al. 2003, 2006, Oono et al. 2011), and differential biomass allocation (Simms et al. 2006). In the present study we examined frequency of OTUs in nodules; we did not measure size of nodules containing specific OTUs or bacterial density within nodules. These will be important parameters to measure in future studies.

Finally, the present study could not distinguish between host-mediated treatment effects and direct effects on soil-dwelling *Frankia*. In other words, it is possible that fertilization affected the relative frequency of OTU4 and OTU8 in soil, and that alder simply formed nodules unselectively with these genotypes in proportion to their occurrence in soil. We are currently examining this possibility using DNA cloning to

characterize assemblages of *A. tenuifolia*-compatible *Frankia* genotypes in alder rhizospheres across our three treatments. These data indicate that *Frankia* genotypes outside the OTU4/8 group that have previously been observed in *A. tenuifolia* nodules vary in abundance by an order of magnitude in soils across fertilization treatments without any detectable variation in nodules across the same treatments (M. D. Anderson, D. L. Taylor, and R. W. Ruess, *unpublished data*). This suggests that *A. tenuifolia* do not simply associate with compatible genotypes in proportion to their occurrence in soils.

Ecosystem implications

Stand-level estimates of N inputs by *A. tenuifolia* derived from measures of N-fixation rate, nodule biomass, and plant density across treatment plots require a number of assumptions (Ruess et al. 2009). First, we assume fluxes were constant over a 24-hour period, as we have observed previously (R. W. Ruess, *unpublished data*), and has been shown elsewhere at high latitudes (Weisz and Sinclair 1988, Huss-Danell et al. 1992). Secondly, we assume fluxes follow a simple step function of plant growth and N fixation based on our previous studies (Uliassi and Ruess 2002, Anderson et al. 2004) that ramps up in May and declines in mid-August. Finally, we assume measures of within-canopy nodule biomass density are representative of nodule biomass within 0.75 m of the base of each stem (the sampling distance), which allows for stand-level nodule biomass estimates based on stem density. Of these three, scaling nodule biomass to the stand level may be the most uncertain given the large number of alder stems 1 cm diameter (dbh) now occurring in these stands due to a recent acceleration in vegetative recruitment. Whether nodule biomass of 1 cm diameter stems extends from the base as far as 0.75 m is the question, but we have previously shown that stems >1 cm in diameter have fairly evenly distributed nodule biomass within this radius (Ruess et al. 2009). While including 1 cm diameter stems may overestimate stand-level estimates of nodule biomass, we probably are underestimating nodule biomass for larger-diameter shrubs because nodules are frequently found well beyond 1 m from a stem base. Moreover, younger plants may be growing closer to their maximum relative growth rates and thus have higher N-fixation rates than the midsized plants we sampled, as greenhouse experiments have demonstrated (Ruess et al. 2006). The actual value for N-fixation inputs might thus fall between estimates that include or preclude these 1 cm diameter stems. This produces N input estimates of 26.3 ± 7.5 to 37.6 ± 11.5, 8.2 ± 2.9 to 11.5 ± 4.1, and 75.1 ± 23.1 to 106.2 ± 32.7 kg N·ha^{-1}·yr^{-1} for CTL, +N, and +P stands, respectively ($n = 3$ plots per treatment). This value for CTL stands is similar to that estimated by Uliassi and Ruess (2002) (38 ± 11 kg N·ha^{-1}·yr^{-1}) for nearby balsam poplar stands measured during the 1993–1994 growing seasons, while

the value for +P stands is approaching that estimated for early-successional alder thickets during 2005 (Ruess et al. 2009). These data indicate that while there is a substantial decline from early to mid-succession in N-fixation inputs, plant availability of P and N are important factors contributing to the reduction in N inputs as stands age.

Thin-leaf alder plays an important role in soil development across interior Alaskan floodplains, where rapid growth and N fixation by alder during the first 25 years of forest succession account for ~60% of soil N accumulated over the 200-year transition to dominance by balsam poplar and then white spruce (Van Cleve et al. 1993*a*). However, unlike many temperate forests where prominent N-fixing plants are limited to early stages of succession or open-canopy woodlands (Vitousek and Field 1999, Rastetter et al. 2001, Vitousek et al. 2002, Dawson 2008), alder often remains the dominant understory species throughout successional development of both upland and floodplain forests in interior Alaska. The persistence of N fixation by alder across such diverse environmental conditions (Anderson et al. 2004) as those seen throughout floodplain succession in interior Alaska (Marion et al. 1993, Van Cleve et al. 1993*a*) suggests a high degree of adaptation to growth under dramatically variable environmental (notably the light environment) and soil physical and chemical conditions. Alluvial soils along the Tanana River are derived from glacial weathering of metamorphic rocks from the Alaska Range, and have a high content of calcium carbonate, which results in alkaline soils and low concentrations of plant-available phosphorus. Most total phosphorus in early-successional soils is immobilized as β-tricalcium phosphate and hydroxyapatite complexes, and plant-available P is extremely low (Marion et al. 1993, Van Cleve et al. 1993*a*). Throughout this successional sequence the proportion of total P in organic forms increases due to cycling by vegetation. Alder mobilizes reduced P forms by mediating a decline in soil pH via high rates of nitrification (Van Cleve et al. 1993*b*, Kielland et al. 2006), and by producing high amounts of P-mobilizing enzymes that degrade complex inorganic and organic molecules (Giardina et al. 1995, Mitchell and Ruess 2009). Although P availability clearly remains a limiting factor in N fixation in both early (Uliassi and Ruess 2002) and mid-successional stands (see *Results*), it is likely that reduction in light, cooler soils, and negative feedbacks from N accumulation also contribute to the down-regulation of N-fixation rates measured between early- and mid-successional stages of forest succession along the Tanana River (Uliassi and Ruess 2002, Anderson et al. 2004).

Siberian alder (*Alnus viridis* subsp. *fruticosa* (Rupr.) Nym.) is more common than thin-leaf alder in late-successional white spruce stands, which occur on elevated terraces along the Tanana River floodplain; however, thin-leaf alder occurs in pockets of moister soil within white spruce stands (Viereck et al. 1993*a*). Our

previous studies found similar N-fixation rates by thin-leaf alder growing in mid- and late-successional floodplain stands, but different and more diverse nodule *Frankia* assemblages in late-successional stands (Anderson et al. 2004, 2009). M. D. Anderson (*unpublished manuscript*) found greater variation in *Frankia* assemblages within plants, among plants within sites, and among sites within late-successional forests compared with early-successional forests, and argued that environmental heterogeneity may partially explain these patterns. Factors contributing to spatial heterogeneity in soil physical and chemical parameters within late-successional stands at the scale of the rooting zone of an individual plant include interspecific competition, decaying logs, canopy gaps, and proximity to old sloughs. Variability in microclimate and soil resources are highest among late-successional stands (Table 1), due to differences such as terrace height, recruitment patterns, and disturbance history. Patches of thin-leaf alder within late-successional stands can have densities similar to those seen in mid-succession (Table 1), but these wetter patches, usually defined by relic sloughs, are relatively rare among the dominance by Siberian alder, which is more tolerant of drier soil conditions. Where they co-occur, these conspecific hosts associate with *Frankia* assemblages that differ in both composition and phylogeny (Anderson et al. 2009, Anderson 2011; M. D. Anderson, *unpublished manuscript*). Given the differences in cost and physiological performance among *Frankia* partners in response to changing soil nutritional conditions characterized here, this suggests a physiological basis underlying host specificity for these assemblages. We suspect successional shifts in *Frankia* assemblages are an important adaptation for a species such as *A. tenuifolia* that maintains a high growth rate and dependence on atmospheric N across radically different growth environments. Because of the strong effects that thin-leaf alder has on soil N contents, it appears that these complex physiological adjustments result in the species both driving and responding to environmental heterogeneity at small to large spatial scales. Preliminary data indicate that among the diverse array of *Frankia* found in floodplain soils along the Tanana River, *A. tenuifolia* partners with only a relatively small clade of closely related *Frankia* OTUs (D. L. Taylor and M. D. Anderson, *unpublished data*). Whether these *Frankia* are the most successful competitors during infection, or are evaluated and selected through a host-controlled sanction/reward process, will require greenhouse experiments where exposure of plant roots to specific *Frankia* genotypes can be controlled and plant growth and C partitioning can be precisely measured.

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SUPPLEMENTAL MATERIAL

Data Availability