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Interspecific competition enhances nitrogen fixation in an actinorhizal shrub

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Abstract In forest understory restoration, the establishment of reintroduced species may be strongly linked to their ability to compete for belowground resources. In this study, we provide isotopic and morphological evidence for competition-induced increases in nitrogen fixation by *Morella cerifera* (L.) Small (wax myrtle) when planted with *Pinus palustris* Mill (longleaf pine). Compared to a competition-free treatment, we found no significant differences in tissue N concentrations for *M. cerifera*. However, ^{15}N enrichment in leaves, stems and roots, as well as whole-plant values for nitrogen derived from fertilizer were significantly lower when the plants were subject to interspecific competition from *P. palustris*. Plants in the competition treatment also allocated a significantly greater percentage of belowground biomass to root nodules than those in the competition-free treatment (0.65 vs. 0.41%). This strongly suggests that *M. cerifera* is capable of upregulating nitrogen fixation in response to interspecific competition. This may help explain why *M. cerifera* outperformed non-nitrogen-fixing species reintroduced on the same site.

Keywords Competition · Nitrogen fixation · *Morella cerifera* · *Pinus palustris* · Stable isotopes · ^{15}N

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Introduction

The understory and ground-layer communities of many forest ecosystems are characterized by high levels of floristic diversity, species richness and endemism (Gilliam and Platt 2006; Barbier et al. 2007). Consequently, the reintroduction of multiple functional groups such as forbs, grasses and shrubs is increasingly considered an essential component of forest restoration projects (Cox et al. 2004; Young et al. 2005; Jose et al. 2006). The success of such efforts, however, is frequently limited by an inadequate understanding of how biophysical interactions and other site factors affect the establishment of the reintroduced species (Hastings et al. 2007).

The biophysical reasons that explain why certain reintroduced plant species thrive, while others do not, have been the subject of frequent speculation among restoration ecologists (Harrington and Edwards 1999; Harrington et al. 2003; Rodrigues et al. 2007; Laughlin et al. 2007). Upon reintroduction to a forest ecosystem, it can, however, be assumed that understory plants

immediately face numerous barriers to establishment. These include, but are not limited to, competition for space, water, nutrients, and light (Harrington et al. 2003; Rodrigues et al. 2007), as well as the potential absence of appropriate mycorrhizal inocula (Koide and Dickie 2002). Litterfall from the overstory can also affect reintroduced understory plant species, either by acting as a barrier to rainfall infiltration or as moisture-retaining soil mulch (Ginter et al. 1979; Harrington et al. 2003). These effects would likely be magnified in densely planted or older forests, as the frequency and strength of interspecific interactions typically decreases with increasing stand basal area (Harrington and Edwards 1999). Of particular importance in such stands would be the availability of nitrogen, a macronutrient which is frequently limiting in forest ecosystems (Vitousek and Howarth 1991).

The ability to fix atmospheric nitrogen, a characteristic shared by many leguminous (*Rhizobium* associative) and actinorhizal (*Frankia* associative) understory and ground-layer plant species, may confer a competitive advantage in certain nitrogen-limited forest ecosystems (e.g. those in which resources such as light and phosphorus are not limiting) (Vitousek and Howarth 1991). At the cellular level, for example, nitrogen-fixing bacteria have been shown to significantly upregulate nitrogenase activity in response to nitrogen deficit (Quesada et al. 1997). At the whole-plant level, legumes have been shown to derive a greater proportion of their nitrogen from fixation when forced to compete with non-leguminous species (Awonaike et al. 1996; Karpenstein-Machan and Stuelpnagel 2000). Unfortunately, however, most studies that have addressed plant to plant competition and nitrogen fixation have been agronomic in nature and focused primarily on legumes (Vitousek et al. 2002). The effects of competition on the nitrogen dynamics of actinorhizal species—an important understory component in many forest ecosystems—have not been thoroughly addressed in the literature.

In recent years, methods involving the stable nitrogen isotope ^{15}N have been used to detect and quantify biological nitrogen fixation in plants (Hogberg 1997; Busse 2000; Stahl et al. 2005). These methods work by exploiting the natural or fertilizer-enhanced differences in ^{15}N concentrations between atmospheric and soil nitrogen pools. Here, we present the results of a field study to test the hypothesis that actinorhizal species derive a greater proportion of their nitrogen from fixation when subjected to interspecific competition. We provide isotopic (^{15}N) and morphological evidence of elevated nitrogen fixation by actinorhizal *Morella cerifera* (L.) Small (wax myrtle) (Myricaceae) when subjected to intense belowground competition from *Pinus palustris* Mill (longleaf pine) (Pinaceae). For comparison purposes, we have included tissue chemistry and biomass data for two non-actinorhizal species, *Callicarpa americana* L. (Verbenaceae) and *Ilex glabra* (L.) A.Gray (Aquifoliaceae) grown under the same conditions. This trial was conducted as part of a larger study of interspecific interactions between *P. palustris* and reintroduced native woody perennials (Hagan et al. 2009).

Study site and experimental design

This study was conducted on a private 15-year-old longleaf pine plantation in Santa Rosa County, FL, USA (30°37′ N, 87°2′ W). The climate of the region is temperate, with mild winters and hot, humid summers. Mean annual precipitation is 1,645 mm and mean January and June temperatures are 8.9 and 27.2°C, respectively. The soil is classified as a Fuquay sand (loamy, kaolinitic, thermic Arenic Plinthic Kandiudult), a nutrient poor, deep, well-drained sand over loamy marine or fluviomarine deposits.

Trees in the study site were uniformly spaced, oriented east to west in rows, with approximately 3 m between rows and 1.5 m between stems within the row. Mean diameter at breast height (DBH) at the initiation of the study was 8.3 cm. Mean basal area was 12.6 m^2 ha^{-1}. In December 2005, containerized *M. cerifera*, as well as *Callicarpa americana* and *Ilex glabra*, grown from cuttings or seeds obtained locally, were incorporated into the existing between-row spacing of the site (competition treatment) and as monocultures in an adjacent open field (competition-free treatment). Shrubs were approximately 1-year old at the time of planting, having been grown in containers outside near full sun at a local nursery. Soils in the two treatments were very similar with respect to macro and micronutrient concentrations, pH, cation exchange capacity, % base saturation and % organic matter. By design, the only major difference was the complete lack of competing vegetation in the competition-free treatment.

Shrubs were given a year for establishment prior to the initiation of the study. The effect of competition on the nitrogen dynamics of this system was assessed via comparisons with the competition-free treatment. The trial was laid out as a split-plot completely randomized design with treatment as the whole plot factor and shrub species as the split-plot factor. There were four replications, each consisting of six 6×10 m subplots (one for each species by treatment combination) with eight shrubs each. These subplots were two alleys wide, oriented east to west within the alley (or equivalent distance and orientation in the competition-free treatment) with shrubs planted in two rows of four at a spacing of 3 m.

Fertilizer application and plot maintenance

Three doses of ^{15}N ammonium sulfate [$(NH_4)_2SO_4$] at 5% atom enrichment (2.26 g/per application) were applied at approximately 60-day intervals at the base of two shrubs per subplot. The six remaining shrubs in each subplot received non-enriched $(NH_4)_2SO_4$ at the same application rate. The first application was on 21 March 2007, shortly after bud swelling and new leaf development were observed. Pesticide and herbicide application, along with manual weed removal, were conducted as needed throughout the growing season.

Harvest, Sampling and Analysis

At the end of the growing season (Fall 2007), each plant that received ^{15}N fertilizer was harvested and separated into leaf, stem, root and nodule components. Plant material was dried to constant weight at 70°C, weighed, subsampled and ground with a coffee grinder to a fine (<1 mm) particle size. Samples were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) Percent ^{15}N atom enrichment (A) was determined using the following formula (Robinson 2001):

$$A = 100\big(\big(R_{\text{sample}}\big)/\big(R_{\text{sample}} + 1\big)\big),$$

where R_{sample} = the ^{15}N:^{14}N is the isotopic ratio of the sample; and R_{standard} = the atmospheric background ^{15}N:^{14}N ratio (0.3663%).

The results of these analyses were then used to calculate percent plant nitrogen derived from fertilizer (NDF), a measure of the amount of fertilizer that a plant obtains from labeled fertilizer. The following formula was used (Allen et al. 2004):

$$\text{NDF}(\%) = 100 \times (\text{a} - \text{b})/(\text{c} - \text{d}),$$

where $a = \%^{15}N$ abundance in plant tissue; b = percent abundance in control (unlabeled) plant tissue; $c = \%\ ^{15}N$ abundance of fertilizer (5%); and d = natural abundance of ^{15}N (0.3663%)

Subsample NDF values were then scaled up, based on biomass and tissue N concentrations, to obtain an estimate of whole-plant NDF. The percent of belowground biomass allocation to nodules was determined by dividing dry nodule biomass by total dry root biomass.

Data were analyzed with an analysis of variance (ANOVA), separately for each tissue type, using the PROC MIXED procedure in SAS 9.1 (SAS Institute 2004). This experimental setup resulted in 1 degree of freedom for treatments and 14 for error. The Shapiro–Wilk test, in concert with the frequency distributions, was used to check the assumption of normality.

Results and discussion

The application of labeled fertilizer raised the soil ^{15}N atom enrichment (A) to 0.476% (no significant difference between treatments) an amount that is considerably greater than the 0.3663% that is most commonly reported as the atmospheric standard (Hogberg 1997). Since nitrogen-fixing plants draw their nitrogen from both atmospheric and soil sources, the ^{15}N atom enrichment of their tissues would be expected to be lower than those of a non-nitrogen-fixing plant, or a less active N_2 fixer growing in this same soil (Busse 2000; Robinson 2001). In our study, A values were significantly lower in *M. cerifera* leaves ($F = 5.3017$, $P = 0.0372$), stems ($F = 15.0644$, $P = 0.0017$) and roots ($F = 10.6310$, $P = 0.0057$) when plants were subject to interspecific competition with *P. palustris*. No such differences were observed for *C. americana* or *I. glabra*. Subsequently, whole-plant NDF values were significantly lower ($F = 16.3943$, $P < 0.01$) for *M. cerifera* in this treatment (Table 1).

The large decreases in ^{15}N enrichment and whole-plant NDF, despite no significant treatment differences in whole-plant tissue N concentrations, suggest that interspecific competition led *M. cerifera* to derive a greater percentage of its nitrogen from atmospheric sources. A similar pattern has been observed in mixed-species forestry plantings of *Eucalyptus* and actinorhizal *Casuarina* (Baker et al. 1994). However, due to the overall reduction in biomass production, whole-plant N yield was significantly lower ($F = 10.1473$, $P < 0.01$) in this treatment (Table 1). The reduction in biomass production was attributed to the interspecific competition with *P. palustris*, as demonstrated in a companion study (Hagan et al. 2009). While shading was minimal ($\approx$35% canopy closure), belowground competition was likely a major factor. *P. palustris*, for example, had the majority of its fine roots in the uppermost 30 cm of the soil profile. Significant reductions in soil water availability (compared to the competition-free treatment) were also observed (Hagan et al. 2009).

It is not possible to state with certainty, based solely on our ^{15}N data, that an increase in nitrogen fixation occurred in this system (Chalk 1991). However, corroborating evidence is provided by the root nodule data. In leguminous and actinorhizal plants, root nodules house N_2-fixing bacteria (*Rhizobia* and *Frankia*, respectively), providing an oxygen-free environment and carbohydrates in exchange for biologically-fixed nitrogen. While time consuming and difficult to quantify, the degree of nodulation can be used as an index of nitrogen fixation (Binkley 1981), if we assume that the fixation rate per unit nodule biomass does not differ between treatments. In this study, we found that *M. cerifera*, when subjected to interspecific competition, allocated a greater percentage of its belowground biomass to nodules than it did in the absence of competition [0.65 vs. 0.41 percent, respectively ($F = 4.6442$, $P = 0.049$)] (Fig. 1). Plotting whole-plant NDF against percent belowground biomass in nodules illustrates that nodulation decreases logarithmically ($r^2 = 0.41$, $P = 0.014$) with increases in fertilizer uptake by *M. cerifera* (Fig. 2). Thus, in this study, whole-plant NDF values served as a reasonable index for comparing seasonal N_2 fixation rates for *M. cerifera* between treatments.

The increased N_2 fixation in *M. cerifera* perhaps explains its better survival and growth compared to *I. glabra* and *C. americana* in the competition treatment. In this competitive environment, *M. cerifera* performed considerably better than the other two species, having the highest survival rate (81%, compared to 75 and 53% for *I. glabra* and *C. americana*, respectively) as well as having the smallest reduction in biomass production (50.6 vs. 68.7% and 75.6% for *I. glabra* and *C. americana*, respectively) compared to the competition-free treatment (Hagan et al. 2009).

While our study does shed light on the effects of interspecific competition on nitrogen fixation, it is important to acknowledge that our experimental design was fundamentally limited by pseudoreplication. That is to say that competition, our main effect, was not randomly applied to the different plots. This, in turn, increases the possibility of committing a type 1 error (Hurlburt 1984). However, considering the inherent difficulty in establishing true replicates in field studies, and the fact that soil properties did not differ between the competition and competition-free treatments, we feel confident that our experimental design was sufficiently robust to test our hypothesis.

Table 1 Tissue chemistry (with respect to nitrogen) for *M. cerifera*, *C. americana* and *I. glabra* grown in the presence and absence of competition with *P. palustris* in the Southeastern USA

Species	Treatment	*A*			%N			Whole-plant N yield (g)	Whole-plant %NDF
		Leaves	Stems	Roots	Leaves	Stems	Roots		
M. cerifera	Competition	0.5924[a]	0.5475a	0.5909[a]	1.6185[a]	0.8257[a]	1.0181[a]	0.929[a]	4.378[a]
	No competition	0.9118[b]	0.8087[b]	0.8548[b]	1.5834[a]	0.7982[a]	0.9265[a]	2.031[b]	14.603[b]
C.americana	Competition	1.1955[a]	0.7714[a]	0.7801[a]	1.5856[a]	0.6596[a]	2.0589[a]	0.554[a]	28.820[a]
	No competition	0.8169[a]	0.8062[a]	0.7822[a]	2.0617[b]	0.8031[a]	1.0449[b]	4.314[b]	2.303[b]
I. glabra	Competition	1.1453[a]	1.0458[a]	0.8358[a]	1.0301[a]	0.3211[a]	0.7817[a]	0.433[a]	28.166[a]
	No competition	1.3337[a]	1.0395[a]	0.9559[a]	0.8840[a]	0.4403[a]	0.7097[a]	1.495[b]	13.317[a]

Species-wise means with different lower case letters are statistically different at ($\alpha < 0.05$)

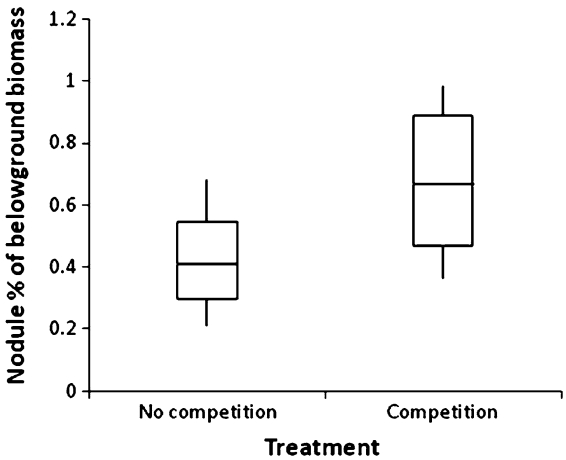

Fig. 1 Percentage of belowground biomass allocation to nodules for *M. cerifera* grown in the presence and absence of competition with *P. palustris* in the southeastern USA. Means and standard errors

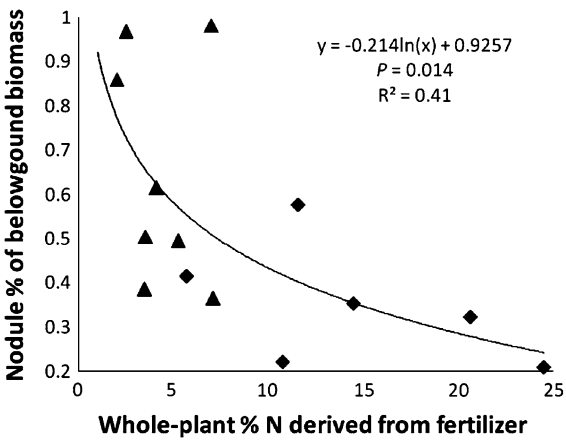

Fig. 2 Relationship between whole-plant percent nitrogen derived from fertilizer (NDF) and percentage of belowground biomass in nodules for *M. cerifera* grown in the presence and absence of competition with *P. palustris* in the southeastern USA. *Triangles* represent plants grown in the competition treatment and *diamonds* represent plants grown in the competition-free treatment

Pseudoreplication is, nonetheless, an issue that should be addressed in future studies.

Conclusion

Improving our understanding of interspecific nitrogen dynamics is a key to ensuring the success of understory restoration plantings. This is particularly true for actinorhizal nitrogen fixers, which have received comparatively less research attention in this area than have understory and agronomic legumes. In this study, decreases in ^{15}N enrichment and NDF and increases in nodule production strongly suggest that *M. cerifera* responds to interspecific competition by upregulating the fixation of atmospheric nitrogen. This, in turn, may help explain why *M. cerifera* outperformed non-nitrogen-fixing species reintroduced on the same site. If this is true, then understory plantings with native actinorhizal species could be an effective first step in the restoration of nitrogen-limited forest communities, especially if overstory trees are already in place. In the longer term, nitrogen additions from these species could enrich the soil, thereby helping to facilitate the establishment and growth of non-nitrogen fixers. Care would have to be taken, however, to ensure that the incorporation of these plants does not promote nitrophillic nonnative species.

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