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Bottom-up rather than top-down processes regulate the abundance and activity of nitrogen fixing plants in two Connecticut old-field ecosystems

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Abstract The maintenance of nitrogen limitation in terrestrial ecosystems remains a central paradox in biogeochemistry. Although plants that form a symbiotic association with nitrogen fixing bacteria should be at a competitive advantage over non-fixing plant species in N limited environments, N_2 fixing plants are uncommon in most mid- to high-latitude ecosystems. Theory and observation suggest that preferential grazing on N-rich tissues by herbivores, resource limitations to growth, reproduction and N_2 fixation, and temperature limitations to the activity of the N_2 fixing enzyme nitrogenase, explain the rarity of N_2 fixing plants. These ideas, however, have never been confronted by multifactor experiments in the field. In a 3 year field experiment, we found that the abundance, growth, reproductive output and fraction of plant-N derived from N_2 fixation in temperate, old-field ecosystems was constrained by the availability of phosphorus (P). Although the availability of light was crucial to the performance of old-field N_2 fixing plants, the largest gains in biomass and the rate of N_2 fixation were observed in the plots fertilized with P. By contrast, herbivory had no effect on the abundance, biomass and activity of N_2 fixing plants and inconsistent effects on foliar nitrogen concentrations (opposing directions, depending upon year), suggesting that herbivores do not affect the ecology of N_2 fixing plants in old field ecosystems, at least not over the course of 3 years. Together with a recent study demonstrating that C limitation explains the absence of N_2 fixing trees in temperate forests our analysis suggests that stand replacing disturbances shift the limitation on the abundance and activity of N_2 fixing plants from P early in secondary succession to light later in succession, as the forest canopy closes and incident light levels decline precipitously.

Keywords Nitrogen fixation · Oldfield · Temperate · N cycle · Biomass

Introduction

Nitrogen fixation is the main pathway by which reactive N enters into terrestrial ecosystems (Cleveland et al. 1999; Galloway et al. 2004). Many plant species form a symbiotic association with bacteria where the exchange of plant carbon for microbial N benefits both groups (Vitousek and Field 1999). In N limited ecosystems it stands to reason that N_2 fixing plant species should be at a competitive advantage over non-fixing species because of their direct access

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to an essential, limiting resource. Moreover through site occupancy and in the absence of disturbance, N_2 fixing plants should steadily increase N availability to the point where it is no longer limiting to growth (Vitousek et al. 2002).

Why then do terrestrial ecosystems remain N limited? Both conceptual and quantitative models argue that some combination of bottom up (i.e., resources) and top down (i.e., herbivores) ecological processes ultimately constrains the abundance and activity of N_2 fixing plants (Eisele et al. 1989; Ritchie and Tilman 1995; Ritchie et al. 1998; Vitousek and Field 1999; Rastetter et al. 2001; Vitousek et al. 2002). In combination with losses of N via leaching and gaseous emissions, these ecological constraints are hypothesized to maintain N limitation for tens to hundreds of thousands of years of ecosystem development (Chadwick et al. 1999; Perakis and Hedin 2002; Vitousek et al. 2002; Menge et al. 2008).

Nutrient limitation to the synthesis of nitrogenase, the enzyme responsible for fixing atmospheric N_2 into ammonia, represents an important source of bottom up control. Nitrogenase is rich in phosphorus (P), iron (Fe) and molybdenum (Mo), and limitation by one or more of these resources can limit the growth or activity of N_2 fixing organisms (Schindler 1977; Howarth and Cole 1985; Smith 1992; Barron et al. 2009). The availability of light availability is another important bottom-up constraint (Vitousek and Howarth 1991). N_2 fixation requires a large expenditure of energy (i.e. carbon, Hartwig 1998) and as a result of forgoing carbon allocation to growth, storage or defense (Bazzaz et al. 1987) in favor of fixation, N_2 fixing plants are likely to be shade intolerant, losing their competitive edge over non-fixing plants even in marginally shaded, N-limited habitats. Exacerbating shade intolerance, evolutionary constraints favor high tissue-N and P concentrations in N_2 fixing plants (McKey 1994), which tend to increase the rate of basal respiration (Reich et al. 2008). In addition, cold temperatures in northern latitudes limit the activity of the nitrogenase enzyme, effectively increasing the energetic cost of N_2 fixation with increasing latitude (i.e., more enzyme is required to fix N_2, Houlton et al. 2008).

Top down control is thought to occur when herbivores preferentially graze the N-rich tissues of N_2 fixing plants (Ritchie and Tilman 1995; Ritchie et al. 1998). Top down control may also arise indirectly when herbivores alter competitive interactions among plant species in the community (McInnes et al. 1992; Schmitz 2008), although the importance of this indirect mechanism limiting N_2 fixing plants *per se* is as yet unknown. Importantly, if top down control were to constrain to the abundance and activity of N_2 fixing plants, herbivores would play a central role in the maintenance of N limitation in terrestrial ecosystems.

To date there have been no multifactor experiments in terrestrial ecosystems testing the importance of top-down vs. bottom-up controls over the abundance, biomass and activity of N_2 fixing plants. To assess the relative importance of soil resource limitation, light limitation and herbivore pressure on the abundance, biomass and activity of N_2 fixing plants, we developed a field and laboratory based experimental framework to test the null hypothesis that the three factors equally affect the performance of N_2 fixing plant species. To test this hypothesis in the field, we designed a replicated experiment in old-field communities of northwestern Connecticut where we increased P, Fe and Mo availability together through fertilization, decreased light availability with shade cloth, and reduced herbivory by building exclosures and applying insecticide. To isolate the individual effects of soil nutrients on N_2 fixing plants we designed a greenhouse experiment where two N_2 fixing plants species were grown with factorial additions of P, Fe and Mo.

Materials and Methods

This research was conducted in the town of Norfolk, CT USA (41°59′N, 73°12′E), where we established a replicated, factorial-design field experiment in two old-field communities. The experimental treatments (fertilization, shade cloth, herbivore exclusion) were randomly assigned to four replicate 1.4 × 1.4 m treatment plots in each field where N_2 fixing plants were already present. Hence the experiment comprised 64 experimental plots (i.e., 2 replicated old fields × 8 experimental treatment combinations × 4 replicates of each experimental treatment). A distance of 100 m separated the two old fields. The experimental manipulations began in April of 2004 and continued through the end of 2006, the third growing season of treatment.

In both old-field communities, the most abundant N_2 fixing plant was *Trifolium pratense* L. (red clover), comprising >90% of stems in this functional group. *Trifolium campestre* was also found in both fields, albeit at substantially lower abundance. In the first of two replicate fields (here in "Field 1"), there were three additional N_2 fixing plants species: *T. repens*, *Medicago lupulina* and *Lotus corniculatus*. There were several dominant species common to both fields including: *Agrostis alba* (C3 Grass), *Anthoxanthum odoratum* (C3 Grass), *Calamogrostis canadensis* (C4 Grass), *Potentilla norvegica* (Forb), *Fragaria virginiana* (Forb), and *Sphagnum* spp. (non-vascular plant). Together these species accounted >70% plant cover.

The soils in the two fields are classified as loams with an average of 38 ± 0.9% sand, 47 ± 1.5% silt and 15 ± 0.6% clay. Soil pH in both field averaged 5.2 ± 0.1. Soil moisture content throughout the growing season averaged 31 ± 0.5% (w/w). None of the soil properties were significantly different between the fields.

Phosphorus, Fe and Mo were added together at rates of 10, 0.8 and 0.5 g m^{-2} $year^{-1}$, (Vitousek and Farrington 1997). Phosphorus was added as triple superphosphate, Fe was added as ferrous sulfate, and Mo was added as ammonium molybdate. Incident light availability (~1,700 μmol m^{-2} s^{-1}) was reduced to 300 μmol m^{-2} s^{-1} through the use of commercially available shade cloth. The shade cloth was draped over the plot leaving a 30 cm gap between the soil surface and the bottom of the shade cloth allowing access to all herbivores with the possible exception of some flying insects that may have been discouraged by the presence of the mesh. Herbivores were excluded from plots by 1.5 cm mesh hardware cloth extending 5 cm into the soil and 30 cm above the soil. To account for any changes in plant growth associated with "trenching" during fence installation in 2004, we used a shovel to trench around all non-exclosure plots. To exclude deer, the top of the enclosure was strung with 2.54 cm mesh deer-X protective garden fencing draped over 1.75 m tall wood stakes driven into the corner of each plot. Insecticide was applied biweekly throughout the growing season. At the end of every growing season (October) the fields were mowed to prevent woody encroachment but all aboveground biomass remains on the site (Jody Bronson, Great Mountain Forest Corporation, *personal communication*). This management practice is ongoing >20 years.

In August of 2005 and 2006 demographic aspects of the N_2 fixing plant population were measured (i.e. abundance of stems and the number of inflorescences per plant), as was the aboveground biomass of red clover. In 2005, *T. pratense* biomass was estimated from allometric relationships developed from harvests conducted outside the plots in 2004. Biomass estimation was a two step process. First, we established a relationship between the number of non-reproductive stems and their biomass ($r^2 = 0.88$, $P < 0.0001$). Second, to that quantity we added the biomass of the reproductive stems. The biomass of reproductive stems was best predicted by the product of the reproductive stem diamater and reproductive stem height ($r^2 = 0.68$, $P < 0.0001$). In 2006, the biomass of red clover was measured directly following harvest and drying of the material. The relative abundance of all species within the community was measured using the point-intersection method (Shaver et al. 2001), and for simplicity of data presentation, classified into one of four functional groups: N_2 fixing plants, non-N_2-fixing forbs, grasses and non-vascular plants.

To determine which of the three soil resources controlled the growth of the N_2 fixing plants in the field, we conducted a greenhouse experiment with *T. pratense* and *T. repens* seedlings inoculated with *Rhizobium*, the N_2 fixing symbiont. Field soil was excavated from both fields to a depth of 15 cm, brought to the Arnold Arboretum in Jamaica Plain, MA, and combined into a single composite sample. The soil was sieved to remove rocks and coarse roots and mixed with acid-washed sand (30% w/w) to facilitate root harvest. The germinated plants were potted in 1,000 cm^3 containers. The soil was kept at field-moist conditions throughout the experiment.

Triple superphosphate contains trace quantities of Mo, so there may have been a 'hidden' Mo treatment associated with the superphosphate addition. Superphosphate contains 8–17 mg Mo kg^{-1}, with a median value of 13 mg kg^{-1} (Charter et al. 1995). Assuming the median concentration of Mo in superphosphate, the application of 10 g P m^{-2} $year^{-1}$ in the +P the field and in the greenhouse amounted to an addition of 17.2 mg Mo m^{-2} $year^{-1}$, 29 times less Mo than applied in the +Mo treatment (i.e. 3.4% of the +Mo treatment). Thus, it is unlikely that the superphosphate treatment added sufficient Mo to elicit an effect on growth or N_2-fixation.

Similar to the superphosphate treatment, molybdenum was added as ammonium molybdate tetrahydrate (molecular mass 1,235.86) raising the possibility that there was a 'hidden' N addition in the Mo treatment. As a result of adding 0.5 g Mo m^{-2} however, the Mo treatment added 0.11 g N m^{-2}. If we very conservatively assume that the rate of net N mineralization in the soil is 2.0 g m^{-2} $year^{-1}$ (Burke et al. 1997), 18-fold less N was supplied to the N_2 fixing plants via the Mo treatment compared to what was supplied by mineralization (i.e., a 5.5% pool increase). This amounts to a tracer level addition of N, one that is unlikely to affect the activity of N_2 fixing plants, particularly given the positive charge on $NH4^+$ and the strong sorption of $NH4^+$ to the cation exchange capacity of soil (Gallet-Budynek et al. 2009).

The greenhouse experiment was designed as a randomized block with four replications of blocks, three separate fertilizer treatments ($\mp$ P, $\mp$ Mo, $\mp$ Fe) applied in factorial combination at the same rate applied in the field, and four replicate pots per treatment, block and species ($n = 256$ pots). The seedlings grew for 82 days at a light intensity of ~1,200 μmol m^{-2} s^{-1} and watered regularly. At the end of the end of the 82 days the entire seedling was harvested (foliage, stem, roots), dried and weighed. Because Mo was added independently from P (as triple superphosphate) the greenhouse experiment enabled us to separate the direct effects of Mo limitation from co-limitation by P and Mo.

Changes in the quantity of N derived from N_2 fixation in field-grown *T. pratense* were assessed via natural abundance $\delta^{15}N$ analysis of foliage in 2006 (Hogberg 1997). The same technique was used to assay changes in N derived from N_2 fixation in the greenhouse study with *T. pratense* and *T. repens*. The isotopic composition of ^{15}N in red clover was compared to that of the non-N_2-fixing plant species *Fragaria virginiana* that was present in every plot. Atmospheric N_2 is the standard for analysis with a $\delta^{15}N = 0$. Most plant species, including those studied here, are depleted in ^{15}N relative to the atmosphere (i.e., $\delta^{15}N < 0$). Hence, we concluded that there was an increase in the rate N_2 fixation only when there was a significant increase in foliar $\delta^{15}N$ in *T. pratense* and no corresponding change in foliar $\delta^{15}N$ in *F. virginiana* (Hogberg 1997). The assumptions behind this method and their impact on data interpretation are presented in the "Discussion".

The field data were analyzed by three-way ANOVA with light (ambient, shaded), fertilization (native fertility, fertilization with P plus Fe and Mo) and herbivore status (included, excluded) as main effects. There were significant differences in the magnitude of treatment effects between years and fields; hence the data were analyzed separately for each year of measurement in each field ($n = 4$). The greenhouse data were also analyzed by 3-way ANOVA, but in this case the three treatments were P addition (ambient, +P), Mo addition (ambient, +Mo) and Fe addition (ambient, +Fe). The block effect was not statistically significant, so the term was dropped from the model. All data were assessed for normality and homogeneity of variance and log-transformed to meet model assumptions when needed. When transformations of the data did not stabilize the variance or improve normality, we used the non-parametric Kruskall Wallace test to asses treatment effects on N_2 fixing plants.

Results

In the field, the abundance and biomass of N_2 fixing plants was significantly stimulated by fertilization with P plus Mo and Fe (herein abbreviated 'PMoFe'), and significantly reduced by shading (Figs. 1, 2). There was a significant interaction between light depletion and PMoFe fertilization in both fields. In all cases, the largest gains in $_N2$-fixer biomass were observed under ambient, high light conditions with fertilization (Fig. 2b, d). The significantly lower biomass of N_2 fixing plants in 2006 compared to 2005 was due to demographic attributes of the *T. pratense* populations. In 2005 there were several large *T. pratense* plants per plot (Fig. 1a, c) that produced a large number of inflorescences. After seed dehiscence a subset of these individuals died resulting in a 2006 growing season characterized by fewer, larger plants and an abundance of 1 year plants (Fig. 1b, d).

In the greenhouse, fertilization with superphosphate alone ($P < 0.001$) increased total seedling biomass in *T. pratense* and *T. repens* (Fig. 3). There was no main effect of Fe or Mo fertilization on biomass for either species, nor were there any

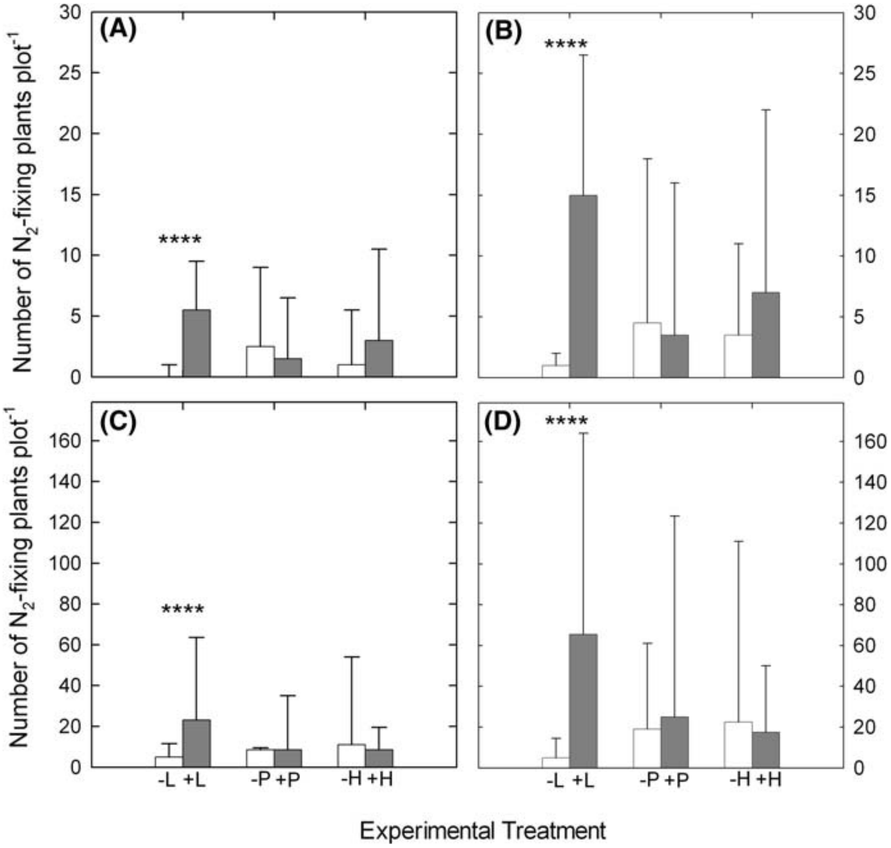

Fig. 1 The median abundance (+upper-quartile range) of all N_2 fixing plant species in response to the main effects of the experimental treatments. **a** Field 1, 2005 growing season, **b** Field 2, 2005 growing season. **c** Field 1 2006, growing season. **d** Field 2, 2006 growing season. Along the x-axis "−L" = shaded plots, "+L" = ambient, high light, "−P" = unfertilized plots, "+P" = plots fertilized with P plus Fe and Mo, "−H" = herbivores excluded, and "+H" = herbivores present. The data were analyzed using non-parametric statistical analysis (see Methods). In all figures, the level of statistical significance for main effects is indicated by * = $P < 0.05$, ** = $P < 0.01$, or *** = $P < 0.001$

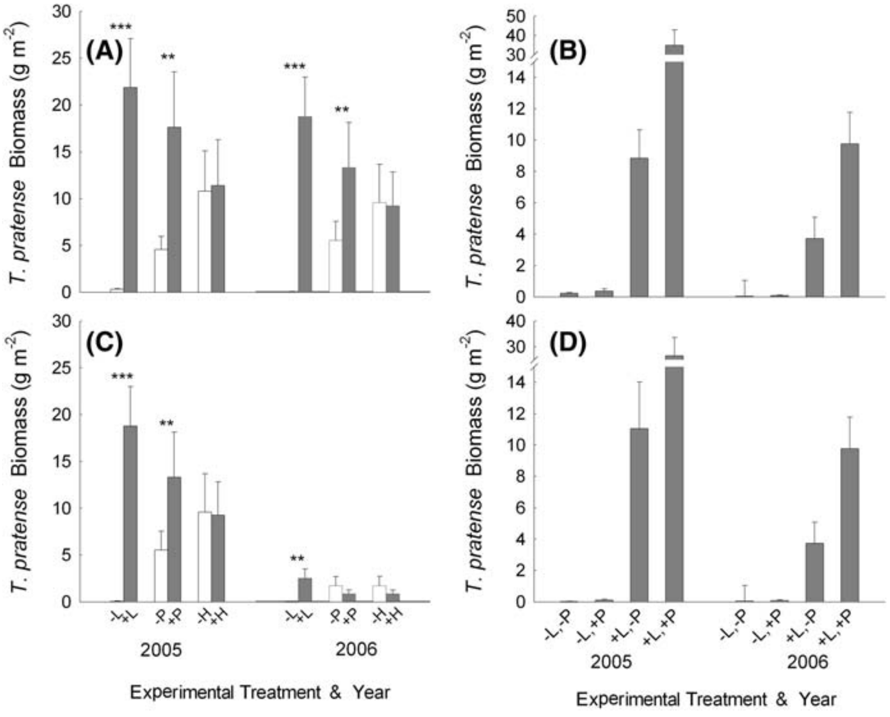

Fig. 2 The mean (± 1 SE) response of *T. pratense* to shading and fertilization with P plus Mo and Fe in 2005 and 2006. **a** Main effects on biomass in Field 1 in 2005 and 2006. **b** Interactive effects of light and P on biomass in Field 1 in 2005 ($P < 0.05$) and 2006 ($P < 0.01$). **c** Main effects on biomass in Field 2 in 2005 and 2006. **d** Interactive effects of light and P on biomass in Field 2 in 2005 ($P = 0.10$) and 2006 (NS). X-axis labels and levels of statistical significance are the same as those in Fig. 1

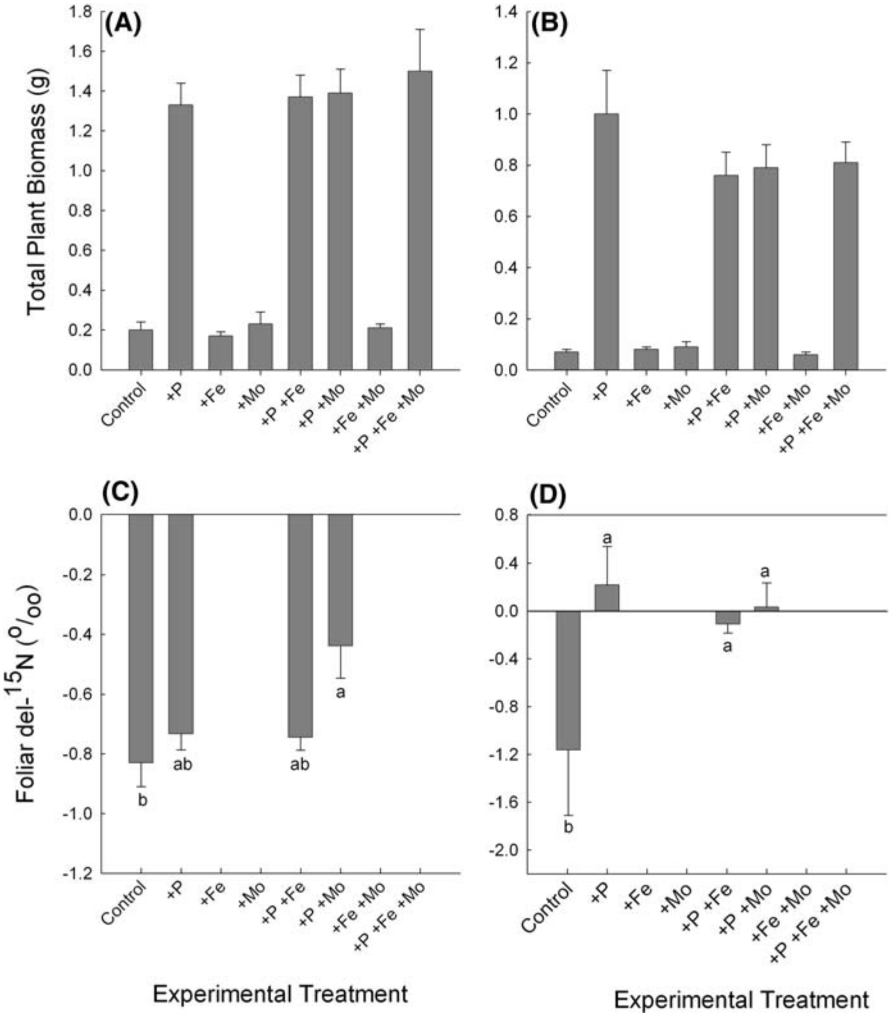

Fig. 3 Mean (± 1 SE) plant biomass of **a** *T. pratense* and **b** *T. repens* seedlings grown in a greenhouse for 82 days under factorial combinations of superphosphate, Fe and Mo fertilization at the same rate applied in the field. The seedlings were grown at a light intensity of ~1200 μmol m^{-2} s^{-1}. **c** Significant ($P < 0.05$) differences in the mean (± 1 SE) foliar $\delta^{15}N$ in *T. pratense* fertilized with P alone or in factorial combination with Mo and Fe. **d** Significant ($P < 0.05$) differences in the mean (± 1 SE) foliar $\delta^{15}N$ in *T. repens* with P alone or in factorial combination with Mo and Fe

interactive effects of these elements with the superphosphate treatment.

In the field, the most enriched foliar $\delta^{15}N$ values in the N_2-fixing plants were observed in the plots under ambient light or those fertilized with PMoFe (Fig. 4a), with a significant interaction between treatments. Under shaded conditions or under ambient light without added PMoFe, foliar $\delta^{15}N$ in *T. pratense* in both fields was significantly ($P < 0.05$) lower than under ambient light with PMoFe fertilization (Fig. 4b, d). Associated with the change in foliar $\delta^{15}N$, the concentration of N in *T. pratense* foliage was significantly greater in the plots fertilized with PMoFe than in the plots under ambient light without fertilization (Fig. 4c). The variation in foliar $\delta^{15}N$ in *T. pratense* and *T. repens* in the greenhouse mirrored that observed in the field (Fig. 3c, d). Foliar $\delta^{15}N$ in *T. pratense* was significantly more enriched in the pots fertilized with superphosphate plus Mo than in the control pots (Fig. 3c). Fertilization with superphosphate alone or in combination with Fe and Mo resulted in enriched foliar $\delta^{15}N$ in *T. repens* compared to control pots (Fig. 3d).

The variation in the isotopic composition and concentration of N in the foliage of the non-N_2-fixing plant *F. virginiana* was substantially different from that observed for *T. pratense* (Fig. 5). Shading significantly ($P < 0.07$) increased foliar $\delta^{15}N$ in *F. virginiana.* Fertilization with superphosphate alone had no effect on the isotopic composition of foliage. In Field 1, however, there was a significant light × fertilization interaction with the most depleted foliar $\delta^{15}N$ signatures found in the plots under ambient light and fertilization (Fig. 5b). Foliar-N

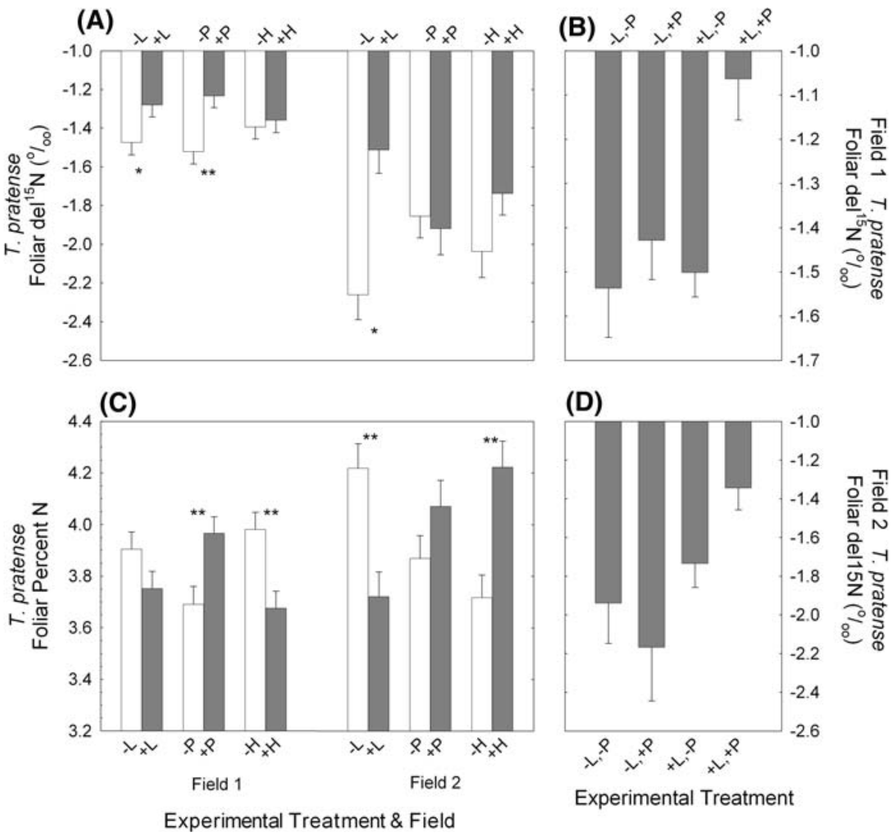

Fig. 4 **a** Foliar δ^{15}N of *T. pratense* in response to shading, fertilization and herbivore removal in Field 1 and Field 2 in 2006. **b** The interaction between shading and fertilization on foliar δ^{15}N in *T. pratense* ($P < 0.05$) in Field 1. **c** Foliar percent N in *T. pratense* in response to shading, fertilization and herbivore removal in Field 1 and Field 2 in 2006. **d** The significant ($P < 0.05$) interaction between shading and fertilization on foliar δ^{15}N of *T. pratense* in Field 2. All values are means (± 1 SE). X-axis labels and levels of statistical significance are the same as those in Fig. 1

concentrations in *F. virginiana* were significantly lower in the plots fertilized with PMoFe, the opposite of what occurred in *T. pratense*.

The exclusion of herbivores had no significant effect on the abundance or biomass of the N_2 fixing plants in either field (Figs. 1, 2), nor on foliar δ^{15}N (Fig. 4). Herbivore exclusion in Field 1 did however result in higher foliar-N concentrations in *T, pratense*. In Field 2 the opposite was observed. Foliar-N concentrations in *T. pratense* were significantly lower in the plots where herbivores were excluded (Fig. 4c).

Discussion

The results presented here provide the first multifactor analysis of top-down vs. bottom-up constraints on the ecology and activity of N_2 fixing plants. Light limitation clearly decreased the abundance, growth and activity of N_2 fixing plants (Figs. 1, 2, 4), consistent with the geographical distribution of N_2 fixing plants in the temperate zone; because of substantial shade intolerance (Fig. 2), N_2 fixing plants are relegated to high light ecosystems dominated by non-woody vegetation (Paul et al. 1971; Turkington et al. 1977; Crews 1999). Importantly however, the abundance, biomass and rate of N_2 fixation were significantly stimulated by the addition of P plus micronutrients in the old field ecosystems studied here. In the absence of nutrient addition, the largest gains in biomass and N_2 fixation were not possible. By comparison, the effects of herbivores (via exclusion) on N_2 fixing plants were weak during the course of this 3 year study (Figs. 1, 2, 4). Hence the ecology of the N_2 fixing plants in these old-field communities is largely constrained by bottom-up processes.

Inference regarding that rate of N_2 fixation in this study depends upon the analysis and interpretation of natural abundance foliar δ^{15}N. There is no question that variations in foliar δ^{15}N can arise from changes in N cycling unrelated to changes in the rate of N_2 fixation (Hogberg 1997). Fertilization in particular can affect root depth distributions, the colonization of roots by mycorrhizal fungi, and there are suggestions that P fertilization affects the rate of N mineralization from soil organic matter (SOM). In our study there

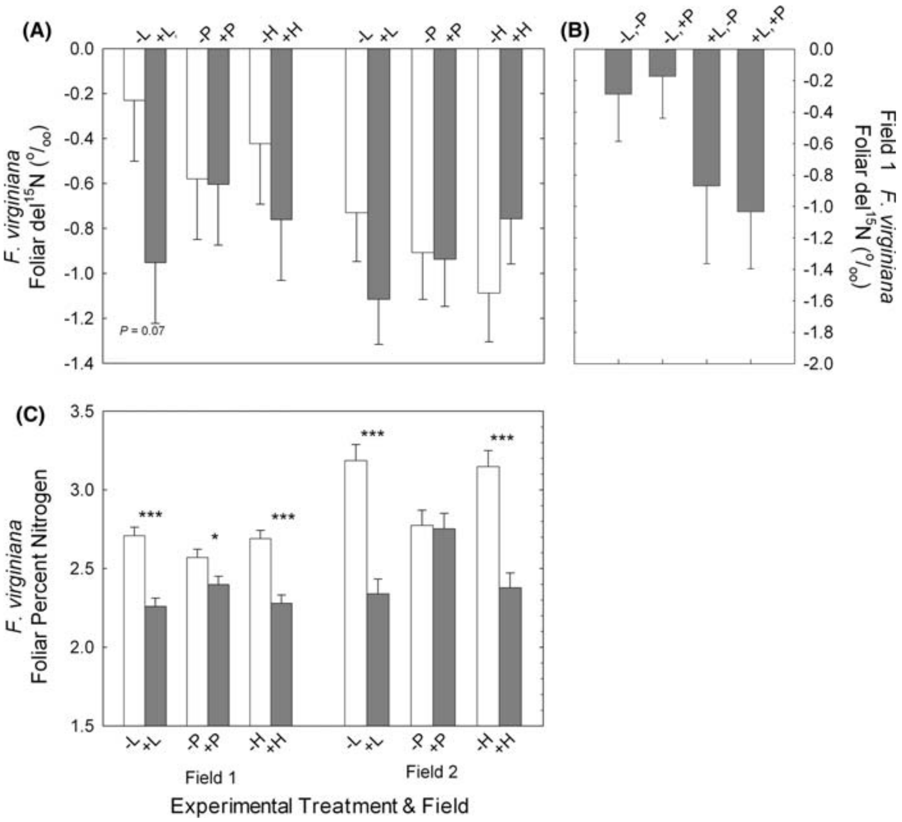

Fig. 5 **a** Foliar δ^{15}N of *F. virginiana* in response to shading, fertilization and herbivore removal in Field 1 and Field 2 in 2006. **b** The interaction between shading and fertilization on foliar δ^{15}N in *F. virginiana* ($P < 0.05$) in Field 1. **c** Foliar percent N in *F. virginiana* in response to shading, fertilization and herbivore removal in Field 1 and Field 2 in 2006. All values are means (± 1 SE). X-axis labels and levels of statistical significance are the same as those in Fig. 1

also existed the possibility of "hidden" experimental treatments (i.e., NH_4^+ applied in the Mo treatment repressing the activity of N_2 fixation, see *Methods* for additional details). Any of these effects could alter our assessment that the increase in the demand for N in the plots fertilized with PMoFe under ambient light was in part, satisfied by higher rates of N_2 fixation in *T. pratense*. Below we address major concerns regarding the effects of fertilization and 'hidden' treatments on our interpretation of the foliar δ^{15}N data. Our analysis suggests that the increase in foliar δ^{15}N is best explained by increases in the rate of N_2 fixation:

- The variation in the isotopic composition of the non-N_2 fixing plant *F. virginiana* (Fig. 5) was nearly the opposite of that observed in *T. pratense* (Fig. 4). The increase in foliar δ^{15}N in *T. pratense* in response to ambient light and P fertilization contrasts with the significant decrease in foliar δ^{15}N in *F. virginiana* in the same treatments (Fig. 5). These data suggest that under ambient light conditions an increasing fraction of N was derived from fixation in *T. pratense* but that an increasing fraction of N was derived from the decomposition of SOM in *F. virginiana*.
- Fertilization and irrigation tend to increase root proliferation in surface soil (Safford 1974; Prior et al. 1994). Surface pools of SOM are more depleted in ^{15}N relative to deeper soil horizons, thus if greater N uptake occurred in the top of the soil profile in the PMoFe treatment, we would expect the foliage of *T. pratense* to be depleted in foliar δ^{15}N relative to the control treatments, the opposite of what was observed (Fig. 4). A shift in root depth distribution would be consistent with the variation in foliar δ^{15}N in *F. virginiana* (Fig. 5).
- There is a clearly established relationship between soil P supply and the accumulation of N during ecosystem succession as a result of biological N_2 fixation (e.g., (Syers et al. 1970; Walker and Syers 1976; Cole and Heil 1981; Chadwick et al. 1999). Although less well established, there is some indication in the experimental literature that P

fertilization increases inorganic N concentrations in the soil (Ross and Bridger 1978; Tate and Salcedo 1988; Kranabetter et al. 2005). Ion exchange resin samples collected from the fields in 2005 show that the availability of N was not significantly different between the control and PMoFe-fertilized plots (control: 1.84 ± 0.57 μg N g resin^{-1} day^{-1}; +PMoFe: 1.94 ± 0.39 μg N g resin^{-1} day^{-1}, no significant difference between fields). Even if there were a positive relationship between P fertilization and N mineralization, this relationship would result in more depleted foliar δ^{15}N values in the foliage of *T. pratense* in the fertilized plots, not enriched values as we observed (Fig. 4). Mineralization results in a pool of plant-available N that is depleted in ^{15}N relative to the source pool (i.e., SOM). This mechanism could however explain variations in foliar δ^{15}N in *F. virginiana.*

- Typically fertilization decreases the activity of mycorrhizal fungi, their colonization of plant roots and the production of extracellular enzymes (Egerton-Warburton and Allen 2000; Lilleskov et al. 2002). The plants in this old-field ecosystem are colonized by AM fungi and mounting evidence suggests that AM fungi contribute to the decomposition of soil organic matter and the transfer of N to host plants (e.g., (Hodge et al. 2001; Joanne Leigh 2009). If fertilization with P reduced AM activity or their colonization of roots, it is likely that plant access to soil-N would have declined. By contrast, we found larger N_2 fixing plants with elevated concentrations of N in foliage in the P treatment in the field (Fig. 4) and greenhouse (Fig. 3) indicating gains in plant-N acquisition. Given no measurable changes in N availability among treatments (see above), the increase in foliar δ^{15}N in *T. pratense* suggests a larger effect of fertilization on N_2 fixation than on the transfer of N mediated by mycorrhizal fungi.
- The ammonium molybdate treatment contained tracer levels of N, and rates of N_2 fixation are repressed by added N (Hartwig 1998). Given that the Mo treatment applied no more, and likely less, than 1/18th the amount of N provided by the mineralization of N from SOM (Burke et al. 1997), and that the positive charge on NH_4^+ results in substantial sorption of NH_4^+ to the cation exchange capacity of the soil (Gallet-Budynek et al. 2009), it is unlikely that the tracer-level addition of NH_4^+ in the Mo treatment repressed N_2 fixation.

In addition to the points raised above, it is worth noting that (1) fertilization with P increases the growth rate and biomass of N_2 fixing organisms in terrestrial and aquatic environments (Schindler 1977; Smith 1992; Cassman et al. 1993; Tyrrell 1999; Cech et al. 2008), indicating that P limitation is common and taxonomically widespread, (2) rates of acetylene reduction in N_2 fixing plants fertilized with P are significantly higher than in plants without added P fertilizer (Smith 1992; Uliassi and Ruess 2002), and (3) compared to the control plots we observed substantially more roots with nodules at a higher density in the field and in the laboratory grown clover species fertilized with superphosphate (Finzi and Rodgers *personal observations).*

The field experiment added P, Fe, and Mo to the same plot making it difficult to separate individual vs. interactive effects of these nutrients on growth and N_2 fixation. In addition superphosphate contains trace quantities of Mo (Crews et al. 2000). We used the greenhouse study to address the individual vs. combined effects of P, Mo, and Fe on growth and N_2 fixation, and the potential for a 'hidden' effect of Mo fertilization in the P treatment. In the greenhouse, seedling biomass in both clover species was significantly higher in the pots fertilized with P but not in response to Mo or Fe addition alone (Fig. 3a, b). Thus there is no evidence that clover growth is directly limited by either micronutrient alone or in combination when added at high levels to overwhelm physicochemical sinks in the soil. Moreover, there were no interactive effects between P fertilization and Mo or Fe fertilization on clover growth. Given that the superphosphate treatment applied only 3.4% of the Mo applied in the Mo treatment, the biomass data strongly suggest that P was the element most limiting growth in the two clover species. The natural abundance foliar δ^{15}N data also support this idea. Foliar δ^{15}N in *T. pratense* was significantly enriched over control pots in the +P, +Mo treatment. Foliar δ^{15}N in *T. repens* was significantly higher in the P treatment independent of Fe and Mo additions. Thus Mo may have limited fixation in *T. pratense* but in both clover species there was clearly a dominant effect of P addition.

The increase in the rate of N_2 fixation with fertilization may reflect greater availability of P and/or Mo for the synthesis of the nitrogenase enzyme (Hartwig 1998), a greater demand for N once P and micronutrient limitation to growth was removed (Houlton et al. 2008), or most likely both. To satisfy the P demand for growth and N_2 fixation (Figs. 2, 4), N_2 fixing plants could take up more P from the soil if they increased the production of N-rich extracellular phosphatase enzymes that cleave ester-bound P from soil organic matter into a plant available form (Treseder and Vitousek 2001). Paradoxically however, the high N cost of phosphatase synthesis suggests that the growth of N_2 fixing plants in old fields is as N limited as non-N_2 fixing members of the same plant community. We tested for N limitation to growth and reproductive output in a series of eight, single-factor N addition plots (four per field), fertilized for 3 years (2004–2006) under ambient light conditions. N was added at a rate of 20 g N m^{-2} $year^{-1}$ in the form urea, split into four equal concentration applications throughout the growing season. Fertilization with N significantly reduced the biomass of the N_2 fixing plants (2005 $P < 0.05$, Control = 17.3 ± 3.6 g/plot, +N = 6.3 ± 1.6 g/plot; 2006 $P < 0.05$, Control = 5.1 ± 1.6 g/plot, +N = 0.8 ± 0.3 g/plot) and reproductive output of N_2 fixing plants, a response that has been observed elsewhere (Suding et al. 2005). On the one hand, the N-fertilization results suggest that the old field N_2 fixing plants studied here are co-limited by N and P. That is, to increase their growth rate these species need more P to fix more N_2 from the atmosphere, and at the same time they need to fix more N_2 to acquire more P because they cannot compete with non-fixing species for available soil N. On the other hand, N fertilization may have promoted widespread P limitation such that all species aggressively scavenged P from the soil. Because of their high demand for P, N_2 fixers may have not acquired sufficient P and been excluded from the community. These alternative explanations will need additional analysis.

Cost-benefit modeling suggests that N_2 fixing trees in the temperate zone must invest a large quantity of energy and nutrients to support nodulation and enzyme synthesis (Houlton et al. 2008). This is thought to occur because cold temperatures limit the activity of the nitrogenase enzyme raising the energetic cost of N_2 fixation. In N-limited, temperate and boreal forests where light availability is low, temperature limitation to the activity of nitrogenase is theoretically sufficient to explain the near absence of N_2 fixing tree species. In temperate, old-field communities C availability to support the N_2-fixing habit is comparatively plentiful. Hence N_2 fixing plants are an important component of the community where their productivity and N_2-fixing activity are primarily P limited. Together with the results presented by Houlton et al. (2008), our data suggest that stand-replacing disturbances result in P limitation of N_2 fixing plants early in secondary succession which transitions to light limitation later in succession, as the forest canopy closes and incident light levels decline precipitously (Canham et al. 1994).

Herbivores affect the structure and composition of plant communities through preferential grazing of plant species (Pastor et al. 1993), or as an indirect result of altering competitive interactions among species (McInnes et al. 1992; Schmitz 2008), both of which can feedback to ecosystem function (e.g. Frank and Groffman 1998; Knops et al. 2000). For example, herbivore exclusion in the oak savannas of the Cedar Creek Natural History Area resulted in a large increase in the abundance of the N_2 fixing plant *Lathyrus venosus* but not *Amorpha canescens* or *Lespedeza capitata* (Ritchie and Tilman 1995; Knops et al. 2000). Similarly, the exclusion of top predators (spiders) in old-field ecosystems of northeastern CT reduced the activity of grasshoppers (herbivores) thereby increasing the abundance of the dominant plant *Solidago rugosa* at the expense of forbs (Schmitz 2008, 2003, 2006). Here, we found that herbivore exclusion had negligible effects on the abundance, biomass and activity of N_2 fixing plant species (Figs. 1, 2, 4), and opposing effects on foliar %N (lower with herbivores in 2005, higher in 2006, Fig. 4). These data suggest that herbivores do not substantially affect the abundance or activity of N_2 fixing plants in old-field ecosystems, at least not over the 3 year time scale of this study.

To demonstrate that the ecological constraints studied here are specific to N_2 fixing plants, and hence that these factors uniquely account for their low abundance, the response of the N_2 fixing plants to the experimental manipulations must differ from the other plant functional groups (Vitousek et al. 2002). Relative to the >10 fold reduction in the relative abundance of N_2 fixing plants in both years (Fig. 6),

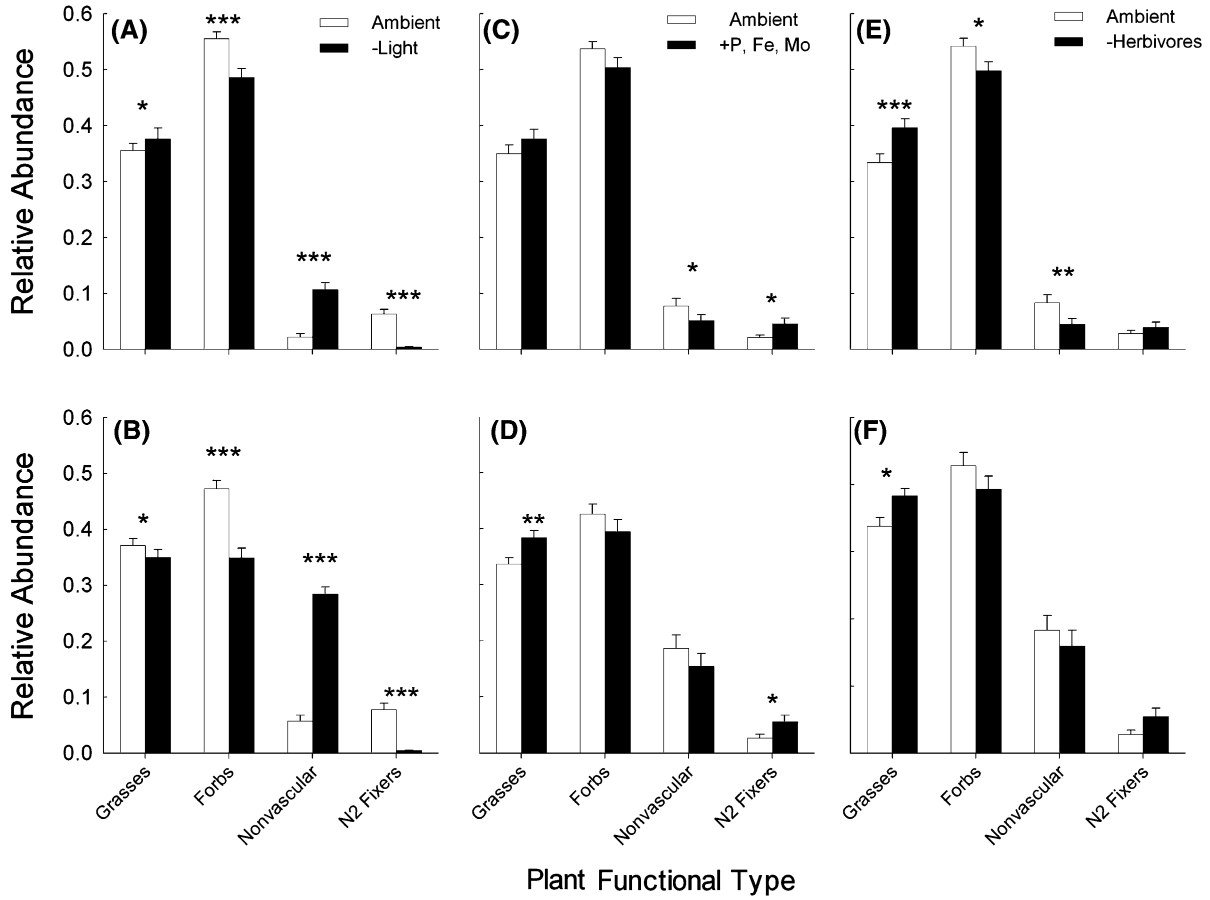

Fig. 6 The mean (± 1 SE) relative abundance of N2 fixers, grasses, forbs (excluding N_2 fixing species) and non-vascular plants in 2005 (**a**, **c**, **e**) and 2006 (**b**, **d**, **f**) in response to shading (**a**, **b**), fertilization with PMoFe (**c**, **d**), and herbivore exclusion (**e**, **f**). The level of statistical significance for main effects is as listed in Fig. 1. There were no statistically significant interactions among the three treatment variables

shading decreased the abundance of non-fixing forbs by <1.4 fold, had no effect on the abundance of grasses, and increased the abundance of nonvascular plants (Fig. 6). Differential responses of the N_2 fixing plants were also observed in response to PMoFe fertilization and the exclusion of herbivores (Fig. 6). The relative abundance of N_2 fixing plants in temperate old-field communities is thus uniquely constrained by substantial shade intolerance, strong P limitation, and susceptibility to herbivory.

Conclusion

Bottom up rather than top down processes affected the persistence of N_2 fixing plants within the old-field communities studied here. Under ambient light conditions fertilization with P significantly increased the biomass of N_2 fixing plants and the quantity of N derived from N_2 fixation. Increases in biomass and N_2 fixation are necessary conditions for N_2 fixing plants to relax N limitation. Given the significant interaction between light and P availability, it appears that as post-disturbance succession proceeds the dominance of P limitation on the abundance and activity of N_2 fixing plants is replaced by light limitation because of the rapid development of a closed-canopy, low light environment and substantial shade intolerance in N_2 fixing plants.

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