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Title page

Title:

Legumes regulate grassland soil N cycling and its response to variation in species diversity and N supply but not CO_2

Running title:

Legumes regulate grassland soil N cycling

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Abstract:

Legumes are an important component of plant diversity that modulate nitrogen (N) cycling in many terrestrial ecosystems. Limited knowledge of legume effects on soil N cycling and its response to global change factors and plant diversity hinders a general understanding of whether and how legumes broadly regulate the response of soil N availability to those factors. In a 17-year study of perennial grassland species grown under ambient and elevated (+180 ppm) CO_2 and ambient and enriched (+4 g N m^{-2} yr^{-1}) N environments, we compared pure legume plots with plots dominated by or including other herbaceous functional groups (and containing 1 or 4 species) to assess the effect of legumes on N cycling (net N mineralization rate and inorganic N pools). We also examined the effects of numbers of legume species (from 0 to 4) in 4-species mixed plots on soil N cycling. We hypothesized that legumes would increase N mineralization rates most in those treatments with the greatest diversity and the greatest relative limitation by and competition for N. Results partially supported these hypotheses. Plots with greater dominance by legumes had greater soil nitrate concentrations and mineralization rates. Higher species richness significantly increased the impact of legumes on soil N metrics, with 349% and 505% higher mineralization rates and nitrate concentrations in 4-species plots containing legumes compared to legume-free 4-species plots, in contrast to 185% and 129% greater values, respectively, in pure legume than non-legume monoculture plots. N-fertilized plots had greater legume effects on soil nitrate, but lower legume effects on net N mineralization. In contrast, neither elevated CO_2 nor its interaction with legumes affected net N mineralization. These results indicate that legumes markedly influence the response of soil N cycling to some but not all global change drivers.

Introduction

As an important source of protein and calories for humans and animals and of nitrogen (N) for plants, legumes are second only to the Poaceae in their importance to humans and terrestrial ecosystems (Graham & Vance, 2003; Rogers, Ainsworth, & Leakey, 2009; Vitousek, Menge, Reed, & Cleveland, 2013). Nitrogen fixation by legumes is a major driver of carbon (C) and N cycling and of succession in various ecosystems (Hu, Liu, Yin, & Song, 2016; Vitousek et al., 2013). The introduction of legumes can alleviate N limitation in many ecosystems due to their potential to fix N from the atmosphere, which can increase N inputs to the ecosystem. Legume litter has high concentrations of N and decomposes and releases N rapidly (Makkonen et al., 2012; Mclaren & Turkington, 2010). Legume litter can enhance the decomposition of other soil organic materials and thus increase soil N cycling and availability due to the relatively higher N concentrations and lower lignin/N ratios in legume tissues (Cong, Hoffland, Li, Janssen, & van der Werf, 2015; Makkonen et al., 2012; Mclaren & Turkington, 2010; Milcu, Partsch, Scherber, Weisser, & Scheu, 2008). Thus, legumes increase N mineralization rates, increasing the overall N availability to all members of the plant community and increasing the growth of co-existing, non-fixing plant species (Hoogmoed, Cunningham, Baker, Beringer, & Cavagnaro, 2014; Lee, Reich, & Tjoelker, 2003a; Mulder, Jumpponen, Högberg, & Huss-Danell, 2002; Pirhofer-Walzl et al., 2012; Temperton, Mwangi, Scherer-Lorenzen, Schmid, & Buchmann, 2007). Therefore, the presence of legumes has the potential to regulate N cycling and ecological processes in terrestrial ecosystems (Jensen et al., 2012).

Species richness (SR) is an important component of plant diversity that influences ecosystem processes (Hooper et al., 2012; Loreau et al., 2001; Wardle, Bardgett, Callaway, & Van der Putten, 2011). Increasing SR often decreases the size of soil inorganic N pools because of greater plant N uptake and greater microbial N immobilization (Knops, Bradley, & Wedin, 2002; Mueller, Hobbie, Tilman, & Reich, 2013). Ultimately, however, total soil N pools become larger in more species-rich communities than in species-poor communities (Fornara & Tilman, 2008; Oelmann et al., 2011). In the long-term BioCON grassland study (Biodiversity, CO_2, and N experimental platform), in Minnesota USA, net N mineralization rates were reduced in more diverse plots initially (contrasting 1-species plots with 16-species plots), but after a decade, SR had near neutral or positive effects on net N mineralization (Mueller et al., 2013; Reich et al., 2012). Such changes in N availability with increasing SR should influence the response of N cycling to legumes in mixed communities (legumes and non-legumes), as N fixation by legumes may gradually become suppressed as soil N supply rates increase (Lee et al., 2003a; Lee, Tjoelker, Reich, & Russelle, 2003b), although empirical evidence for this indirect SR effect is scarce.

Elevated CO_2 and N are two important global change factors that might modulate the effects of legumes on N cycling, but predicting the response of N cycling in ecosystems with legumes to elevated CO_2 and N supply remains uncertain (Ebersberger, Niklaus, & Kandeler, 2003; Gill et al., 2002; Larsen et al., 2011). Although elevated N supply often reduces decomposition of soil organic matter (Janssens et al., 2010; Riggs, Hobbie, Bach, Hofmockel, & Kazanski, 2015), it increases inorganic N pools and net N mineralization rates, likely by increasing soil N pools and reducing soil C:N ratios (Gruber & Galloway, 2008; Lu et al., 2011; Mueller et al., 2013). Larger N pools and net N mineralization rates in turn suppress N fixation and the amount of N derived from the atmosphere by legume plants (Lu et al., 2011; Salvagiotti et al., 2008; West, HilleRisLambers, Lee, Hobbie, & Reich, 2005). Elevated CO_2 has been hypothesized to decrease N availability by suppressing soil organic matter decomposition and immobilizing N into organic materials (De Graaff, Six, & Van Kessel, 2007; Hu, Chapin III, Firestone, Field, & Chiariello, 2001), or to increase N availability by priming organic matter decomposition and N release (Blagodatskaya, Blagodatsky, Dorodnikov, & Kuzyakov, 2010; Hoosbeek et al., 2004; Zak et al., 1993). Moreover, elevated CO_2 either increases N fixation by increasing the availability of C for legumes to exchange with the bacterial symbiont (Rogers et al., 2006; Soussana & Hartwig, 1995; Zanetti et al., 1996), or decreases N fixation after several years' CO_2 enrichment by reducing the availability of other nutrients, particularly the micro-nutrient molybdenum, to legumes (Hungate et al., 2004). Changes in N availability with elevated CO_2 or N supply will thus influence N fixation by legumes, but such effects have not been examined previously.

Given that legume effects on N cycling derive from changes in N fixation, which declines as soil N availability increases (Lee et al., 2003b; Lu et al., 2011; Salvagiotti et al., 2008; Vitousek et al., 2013; West et al., 2005), and that legume N fixation response to soil N availability likely influences legume responses to CO_2 and SR, and thus their effects on soil N cycling, we generated our hypotheses (which are illustrated in **Fig. 1**). For each of the following hypotheses, we consider first the effects of greater SR, CO_2 elevation, or N enrichment in the absence of legumes, and then consider (in italics) how legumes might alter those effects, given our *overall hypothesis that legumes promote N fixation, which should increase soil inorganic N pools and promote net N mineralization and decrease N immobilization by microbes, because of lower root C:N ratios*.

H1: (*Species Richness Effects*) By increasing soil organic matter inputs to soils and demand for N by plants and soil microbes, increasing SR will reduce inorganic N pools. *In communities that include legumes, their N fixation will offset the negative effect of SR on inorganic N pools such that higher SR will reduce inorganic N pools less in communities with legumes than in communities without legumes*.

H2: (*CO_2 Effects*) By increasing labile C inputs to soils, elevated CO_2 will enhance soil organic matter decomposition through priming and increase net N mineralization rates. Alternatively, greater labile C inputs to soils under elevated CO_2 could promote plant inorganic N uptake and soil microbes immobilization by increasing plant and microbial demand for N. *In communities with legumes, increases in C availability with elevated CO_2*

should promote N fixation and favor increased inorganic N pools and net N mineralization compared to communities without legumes.

H3: (*N Enrichment Effects*) Increased N supply will increase the inorganic N pools and promote soil N mineralization by reducing soil C:N ratios. *N enrichment will reduce positive effects of legume presence or abundance on soil N pools and net N mineralization by suppressing N fixation, minimizing the differences in N pools and mineralization between communities with and without legumes.*

To test these hypotheses, we present 17 years of results of soil net N mineralization and solution inorganic N pools measurements from subsets of plots from the BioCON experiment, in temperate grassland in central Minnesota, USA. In BioCON, plant diversity and composition were experimentally controlled in various planted communities drawing from a species pool that comprised four legume, four non-fixing forb, four C_3 grass and four C_4 grass (all perennials) species. In this study, we restricted our analyses (1) to pure legume and non-legume plots with 1-species monocultures and 4-species mixtures made up of single functional groups, and (2) to 4-species communities with species from across the four functional groups and variable numbers of legume species (from 0 to 4) (as illustrated in Fig. S1). All levels of species richness and legume treatments were exposed to all combinations of ambient and elevated CO_2 and N treatments.

Materials and Methods

Experimental design

This study was conducted within the BioCON experiment (see Reich et al., 2001a, 2004 for detail) at the Cedar Creek Ecosystem Science Reserve in east-central Minnesota, USA. The experiment was initiated in 1997 on sandy glacial outwash soils (>90% sand) and was designed to address the interactive effects of biodiversity, elevated CO_2 and enriched N on grassland ecosystem function. It consists of six 20-m diameter rings, three with an ambient CO_2 environment and three with an elevated CO_2 environment maintained at an average CO_2 concentration of 560 μmol mol^{-1} by a free-air CO_2 enrichment (FACE) system. Each ring contains 61 individual 2×2-m plots, half of which were randomly selected to receive 4 g N (NH_4NO_3) m^{-2} y^{-1}, which was divided into three applications, in May, June, and July. Plant diversity was experimentally controlled using four C_3 grasses (*Agropyron repens, Bromus inermis, Koeleria cristata* and *Poa pratensis*), four C_4 grasses (*Andropogon gerardii, Bouteloua gracilis, Schizachyrium scoparium* and *Sorghastrum nutans*), four legume forbs (*Amorpha canescens, Lespedeza capitata, Lupinus perennis* and *Petalostemum villosum*), and four other forbs (*Achillea millefolium, Anemone cylindrica, Asclepias tuberosa* and *Solidago rigida*), and the plots were planted at various levels of species and functional diversity. The 16 species are all perennials and native or naturalized to the Cedar Creek Ecosystem Science Reserve, and all levels of plant diversity were exposed to all combinations of the ambient and elevated CO_2 and N treatments.

Here we focused on a subset of the plots that allowed us to test our hypotheses. Plots were either monocultures (n=128), four-species plots planted with a single functional group ("monogroups") (n=48), or four-species plots planted with mixtures of species from across the four functional groups and with varying numbers of legume species (from 0 to 4) (n=123) (Fig. S1). Each diversity treatment was distributed among the four CO_2 × N levels. To compare the effects of legumes with those of other functional groups as well as the interaction between functional group identity and species richness (1 or 4), we compared monocultures and 4-species monogroups containing only legumes with plots containing only one of each of the other three functional groups. To determine the effects of different numbers of legume species in mixtures, we also compared 4-species mixed communities that contained varying numbers of legume species, from 0 to 4. We conducted these comparisons across the combinations of the ambient and elevated CO_2 and N treatments. These comparisons enabled tests of the differences of soil N metrics between legume plots and non-legume plots and variations among the numbers of legume species, and of whether these differences varied with species richness (H1), elevated CO_2 (H2) or enriched N (H3). Other plots in BioCON that were planted with 9 and 16 species were not used in the present study, as these all had similar proportional abundances of legumes in the initial plantings.

Measurement of soil N and root traits

In situ soil net N mineralization was measured in each plot with one replicate of four composite cores using the undisturbed buried core method (Reich, Peterson, Wedin, & Wrage, 2001b; Robertson et al., 1999), which consisted of incubating soil cores (20 cm long by 2 cm in diameter) in the field for one mid-summer month each year from 1998 to 2014 (except 2008). Measurements from 2001 showed that rates in one mid-summer month represented one-third of total annual net N mineralization and were positively correlated with annual N mineralization (Mueller et al., 2013). Soil samples were extracted before and after the incubations with 1 M KCl solution, and the inorganic N (nitrate and ammonium) concentrations in the extracts were measured using an Alpkem auto-analyzer (Pulse Instrumentation, Saskatoon, Saskatchewan, Canada). The daily net N mineralization rate was calculated by subtracting the total inorganic N before incubation from that after incubation and then dividing by the number of days incubated (approximately one month). Soil nitrate and ammonium concentrations before incubation are also presented as inorganic N pools in soil solution. Root biomass was harvested in August, at peak biomass, from 1998 to 2014 for each plot. Root N concentrations were measured using an NA1500 C-N analyzer (Carlo-Erba Instruments, Milan, Italy).

Statistical analysis

Soil metrics and root traits were natural-logarithm transformed to fit the assumptions of a normal distribution. Soil nitrate and ammonium were not detected in some samples, which were assigned a value of zero, and 0.01 was added to all values prior to transformation (Mueller et al., 2013). The value of 0.01 is 1.2% and 0.5% of the average value of nonzero nitrate and ammonium values across all the plots selected in this study. Because the minimum value of net N mineralization was -1.00 in the whole dataset, 1.10 was added to all values prior to logarithm transformation. The outliers of soil metrics and root traits were assessed using Mahalanobis distance method (>4.5 viewed as outlier).

To test effects of the identity of the legume species, legume vs. non-legume or the numbers of legumes, and the effects of SR, CO_2, and N, and whether these effects changed over time, a nested, mixed-effects ANOVA was conducted by nesting plot within CO_2 and N as a random effect and including year as a continuous variable (Reich, Hobbie, Lee, & Pastore, 2018; Reich et al., 2004). Because N fixation rate varies greatly with legume species identity (Menge, Wolf, & Funk, 2015; West et al., 2005), we first examined whether legume species identity significantly affected the response of soil and root metrics to CO_2 or N with monocultures plots. Legume identity (ID), CO_2, N, year (Y) and their interactions were included in the model as independent variables. The results showed that most soil and root metrics were not affected by the interaction of legume identity with CO_2 and N (**Table S1**), thus legume identity was not included in the following examinations. For the monocultures and 4-species monogroups, functional group identity (L, legume vs. non-legume), SR, CO_2, N, year and their interactions were included in the model as independent variables to examine whether legumes have significant effects on soil and root metrics and whether such effects vary with SR, CO_2 or N. For 4-species communities with varying numbers of legume species (LS, 0-4), the LS, CO_2, N, year and their interactions were included in the model to test the effects of legume species numbers on soil and root metrics and whether such effects vary with CO_2 and N. A linear regression analysis was used to assess whether there were relationships between net N mineralization and root biomass and N concentration. All ANOVA and regression analyses were conducted using JMP 10.0 (SAS Institute, Cary, USA).

Results

Effects of legume species identity on soil N cycling and its response to CO_2 and N

Across all years, as hypothesized, all monocultures with legumes had higher soil net N mineralization rate and solution nitrate concentration than the average of the non-fixer monocultures (**Fig. 2a,c**), and higher net N mineralization rate than the highest non-fixer monoculture (*Anemone*, **Fig. S2**). *Amorpha* and *Lespedeza* had similar soil nitrate concentrations to that of the highest non-fixer (*Anemone*), likely due to very low N uptake by *Anemone* (**Fig. S2**). In addition, soil nitrate concentration and net N mineralization varied among legume species, with significantly higher nitrate in *Lupinus* and *Petalostemum* plots than the other two legumes ($P<0.0001$) and higher mineralization rate in *Lupinus* plots than the other three legumes ($P<0.0001$) (**Fig. 2a,c**, **Table S1**). Soil ammonium concentration was not affected by the identity of the legume species (**Fig. 2b**).

The identity of the legume species did not affect the response of soil N metrics to elevated CO_2 ($P>0.1$ for $ID\times CO_2$) or the response of nitrate and net N mineralization to enriched N ($P>0.1$ for $ID\times N$) (**Table S1**). Soil ammonium, however, was differentially increased by enriched N among the 4 species ($P=0.0108$ for $ID\times N$). Hereafter, the results for the individual legume species were pooled together to compare the effects of legumes on the responses of soil N cycling to elevated CO_2 and N.

The effects of functional group identity on soil N cycling across time and treatments

Across all years and the CO_2 and N treatments, soil nitrate concentration and net N mineralization were significantly higher in pure legume plots than non-legume plots both for monocultures and 4-species monogroups (**Table 1**, **Fig. 3a,e**). Soil nitrate concentration and net mineralization rate were also higher in plots with a larger number of legume species in 4-species communities (**Table 2**, **Figs. 3b,f and S3**). For example, soil nitrate and net N mineralization increased linearly, by 505% ($P<0.0001$) and 349% ($P<0.0001$), respectively, as the numbers of species that were legumes increased from 0 to 4 (**Fig. S3**). Furthermore, most of these positive effects of legumes were observed in each year (**Fig. 3a,b,e,f**), and were consistent across CO_2 and N treatments (**Tables 1 and 2**). Soil ammonium was not always higher in pure legume plots than in non-legume plots and did not always increase with the numbers of legume species in the plot (**Fig. 3c,d**).

Effects of species richness on the legume contribution to soil N cycling

Increasing SR from 1 to 4 decreased net N mineralization and inorganic N pools in non-legume plots, but increased them in pure legume plots (**Figs. 4a,e and S4**). Furthermore, higher SR significantly increased the difference in soil N metrics of legume vs. non-legume assemblages (**Fig. 4b,d,f**, **Table 1**). For example, across the CO_2 and N treatments, pure legume plots had 0.12 mg kg^{-1} d^{-1} (185%) and 0.92 mg kg^{-1} (129%) higher mineralization rate and nitrate concentration but 0.15 mg kg^{-1} (8%) lower ammonium concentration than non-legume plots when SR=1, but had 0.18 mg kg^{-1} d^{-1} (349%), 1.59 mg kg^{-1} (505%) and 0.15 mg kg^{-1} (9%) higher mineralization rate, nitrate and ammonium concentrations when

SR=4. These different responses of legumes vs. non-legumes to SR were similar regardless of CO_2 or N treatment (**Fig. 4**, **Table 1**) and among experimental years (**Table 1**).

Effects of elevated CO_2 and N supply on the response of soil N cycling to legumes

Elevated CO_2 did not affect soil nitrate and net N mineralization, but significantly increased soil ammonium in the ambient N treatment (**Figs. 4a,c,e and 5a,c,e**). The effects of functional group identity (pure legume vs. non-legume) or numbers of legume species on soil N metrics were similar between ambient and elevated CO_2 treatments (**Tables 1 and 2**).

Nitrogen enrichment increased soil N metrics, and the effects of legumes on soil N metrics depended on N supply. Averaged across the monocultures and 4-species monogroups, soil nitrate concentrations in the ambient and enriched N treatments were 0.77 mg kg^{-1} (482%) and 1.47 mg kg^{-1} (145%) higher in pure legume than in non-legume plots, while net mineralization rates were 0.14 mg kg^{-1} d^{-1} (271%) and 0.13 mg kg^{-1} d^{-1} (193%) higher, respectively (**Fig. 4b,f**). For the plots with varying legume species numbers, increasing legume species from 0 to 4 increased nitrate at the ambient and enriched N treatments by 0.98 mg kg^{-1} (1241%) and 2.13 mg kg^{-1} (367%), and N mineralization rate by 0.21 mg kg^{-1} d^{-1} (548%) and 0.16 mg kg^{-1} d^{-1} (229%), respectively (**Fig. 5b,f**). Similar to the effects on N mineralization, the differences of soil ammonium between pure legume plots and non-legume plots or among variable numbers of legume species were smaller or more negative in enriched than ambient N treatments (**Figs. 4c,d and 5c,d**). These effects were not influenced by CO_2, SR or experimental year (**Tables 1 and 2**). Therefore, N enrichment increased the absolute (but not relative) effect of legumes on soil nitrate concentrations, and modestly reduced relative (but not absolute) legume effects on mineralization rate and soil ammonium concentrations.

Legume influence swamps N enrichment effects on net N mineralization and its

The effect of legumes on soil N cycling was much greater than that of N enrichment in this N-limited grassland. For monoculture and 4-species monogroups, the effect of legumes (pure legume plots compared with non-legume plots in the ambient N treatment) on net N mineralization was greater than the effect of N enrichment (enriched N compared with ambient N in non-legume plots) in both ambient (+277% vs. +29%) and elevated CO_2 (+263% vs. +43%) treatments, and this pattern of influence was similar among experimental years and amplified as SR increased from 1 to 4 (**Fig. 6a,b**). Similarly, for 4-species communities, the effect of the numbers of legume species on mineralization was greater than the effect of N enrichment under both ambient and elevated CO_2 conditions either across or within experimental years (**Fig. 6c,d**).

Furthermore, legume effects were greater than those of N enrichment in alleviating potential negative effects of SR on mineralization. For monoculture and 4-species monogroups, in non-legume plots, increasing SR from 1 to 4 species reduced mineralization rates by -0.022 mg kg^{-1} d^{-1} (-37%) in ambient N; the negative SR effect was largely erased in enriched N treatments (-0.002 mg kg^{-1} d^{-1} or -3% effect). In contrast, in pure legume plots, those with 4 species had higher net N mineralization than monocultures; with +0.071 mg kg^{-1}

d^{-1} (+42%) and +0.027 mg kg^{-1} d^{-1} (+14%) higher rates at higher SR in ambient and enriched N treatments, respectively (P<0.05 for N×SR×L, **Figs. S4 and S5**). These trends were similar between ambient and elevated CO_2 treatments and among most experimental years (P>0.05 for CO_2×N×SR×L and Y×N×SR×L, **Table 1**, **Fig. S5**).

Effects of legumes and global change factors on root traits response to SR

Root biomass and N concentration relate to soil N turnover (Mueller et al., 2013; Reich, 2009; Wedin & Tilman, 1990) and thus may mediate the effects of legumes, SR and global change on N cycling (Wei, Reich, Hobbie, & Kazanski, 2017). In this study, for monoculture and 4-species monogroups, pure legume plots always had much higher root %N (by 53-142%) but much lower root biomass (by 55-73%) compared to non-legume plots across all sources of variation (P<0.0001, **Table 1**, **Fig. 7a,c**). Similarly, with increasing legume species numbers in 4-species functional group communities, root N increased but root biomass decreased, both in non-linear fashion (P<0.0001, **Table 2**, **Fig. 7b,d**). Root biomass increased modestly when legume species were 1 rather than 0 of 4-species assemblages, but decreased with further increases in legume species in the plot (**Fig. 7a,b**). The differences in root N concentration between pure legume and non-legume plots varied with SR (P<0.0001), with legumes increasing root N compared to non-legume plots more in 4-species monogroups (119%) than in monocultures (71%) (**Fig. S6**). However, N enrichment reduced the effect of legumes on root N (P<0.001), with greater effects of legume (+101%) or legume species numbers (+132%) in ambient N treatment than enriched N treatment (+71% and +106%, respectively) (**Fig. 7c,d**). Elevated CO_2 did not affect the response of root biomass and N to pure legume vs. non-legume or the numbers of legume species (P>0.05).

Root traits were significantly correlated with soil N mineralization, with mineralization rate decreasing with increased root biomass and increasing with increased root N concentration (**Fig. S7**). When root N and biomass were both included in a model, net N mineralization was only affected by root N, which explained more variance (36-67%) in N mineralization than root biomass (<2%) (**Table S2**). Therefore, root N was more important than root biomass in influencing and regulating the effects of legume and global change factors on net N mineralization. Our further examination within monocultures and 4-species monogroups showed that the slopes of relationship of net N mineralization to root N concentration was similar in pure legume plots (0.032±0.014, P=0.0211) and non-legume plots (0.037±0.006, P<0.0001).

Discussion

The presence of legumes influenced soil N cycling and its response to plant diversity and N supply, but not to CO_2. These results were consistent with some but not all of our hypotheses (**Fig. 1**). Pure legume plots had higher soil N cycling than non-legume plots, and the increasing proportion of legume species in a plot linearly increased soil N cycling, as predicted. However, although the species identity of legumes affected soil N cycling, it did not influence its response to elevated CO_2 and N supply. Legumes effects on N cycling were increased by increasing SR from 1 to 4, and reduced by N enrichment; both also consistent with our hypotheses. Inconsistent with our hypothesis, there was no interaction of elevated CO_2 and legume presence or abundance. As our hypotheses about dependency of legume effects on CO_2 and N treatments were largely based on similar stoichiometric theory, it is not clear why hypotheses were supported for N but not CO_2. Furthermore, the effect of legumes on net N mineralization and its response to SR were greater than that of N enrichment. Collectively, the results imply that legume influence on the response of soil N cycling to plant diversity, N supply and CO_2 are only partially consistent with current theoretical frameworks.

Effects of legume species identity on soil N cycling

The variations in soil N metrics among legume species identity could be attributed to species-specific differences in N fixation and root traits, which regulate soil N mineralization. However, an earlier study found symbiotic N fixation was not affected by the identity of legume species in this experiment (West et al., 2005). In contrast, root N was significantly higher while root biomass was significantly lower in *Lupinus* plots (**Table S1**, **Fig. S8**), explaining the higher mineralization rate in *Lupinus* plots than in other legume plots as root N regulates mineralization (**Fig. S7**; Fornara, Tilman, & Hobbie, 2009; Mueller et al., 2013; Parton et al., 2007).

We did not observe significant interactions between legume species identity and CO_2 or N treatment in this study, likely due to the lack of significant effects of legume species identity and CO_2 on N fixation (West et al., 2005) and the lack of the effects of CO_2 or N treatment on soil N mineralization or root N concentration (**Table S1**) in plots with each legume species. Although these results might be taken to indicate that legume species identity can be largely ignored among this particular group of legume species when assessing the response of N cycling to global change factors, N cycling did vary significantly with legume species identity *per se* and we are not confident that the results under complex combinations of global change factors found herein would be similar in other sites and other contexts.

Effects of legumes on soil N cycling

Effects of legumes on soil N cycling have been assessed previously (Cong et al., 2015; Lam, Chen, Norton, Armstrong, & Mosier, 2012; Mclaren & Turkington, 2010; Mulder et al., 2002; Spehn et al., 2002), but the effects of legume species number have scarcely been investigated (Li et al., 2015). We observed a linear increase in soil nitrate and net N mineralization with increasing numbers of legume species in the 4-species mixed plots, consistent with our hypotheses but inconsistent with a non-linear increase in N fixation with the proportion of legumes in a temperate steppe grassland in northeast China (Li et al., 2015). This inconsistency might be related to differences in soil available N levels among these studies. The inorganic N in our study was much lower (with a mean value of 2.45 mg kg^{-1} across all the treatments in 4-species plots with variable numbers of legume species) than that in soils from Li et al. (2015) (more than 20 mg kg^{-1}), even in our 4 legume species plots (3.77 mg kg^{-1}, **Fig. S3**). Therefore, our ecosystem is much more likely to be N limited even under higher legume abundance, and N supply less likely to be at levels high enough to suppress N fixation, resulting in a linear response to legume species numbers. These results further indicate that the response pattern of soil N cycling to legume abundance might relate to soil N supply, with a linear response in low N availability soils but non-linear response in relatively higher N availability conditions. Beyond soil N supply, the linear response of nitrate and mineralization could also be attributed to the linear response of root N concentrations to increased numbers of legume species (R^2=0.947, P=0.0271).

We expected that soil ammonium would be higher in pure legume plots than non-legume plots and would increase with number of legume species because symbiotic N fixation by legume supplies ammonium to soils (Stevenson, Stevenson, & Cole, 1999), and legumes enhance N mineralization, releasing nitrate and ammonium into soils (Carpenter-Boggs, Pikul, Vigil, & Riedell, 2000; Fornara et al., 2009; Mueller et al., 2013; Mulder et al., 2002). However, the sandy texture and the relatively lower soil moisture of the study soils provide an aerobic environment where soil ammonium was apt to be converted to nitrate. In the study soils, net nitrification dominated net mineralization during the same experiment (during 1998 to 2012, n=5565, Wei et al., 2017). Thus, soil nitrate, but not soil ammonium, was significantly increased by legumes in our study (**Figs. 3-5**).

Species richness enhances the effects of legume on soil N cycling

SR increased the effects of legumes on soil N cycling, primarily due to lower soil and root N metrics in non-legume plots that were more diverse (SR=4 vs. 1) and higher values in pure-legume plots that were more diverse (**Figs. 4a,e, 7c and S4**), supporting H1 (**Fig. 1**). In the non-legume plots, increased SR decreased soil solution N and mineralization, which is consistent with the findings from other experiments that increased species richness can have negative to neutral effects on soil N cycling (Fornara & Tilman, 2008; Knops et al., 2002; Oelmann et al., 2011; Tilman, Knops, Wedin, & Reich, 2002; Van Der Krift & Berendse, 2001), but different from observations by Reich et al., (2012) and Mueller et al., (2013) in separate BioCON sub-experiments with a larger SR gradient (1 vs. 16 species) than this study (1 vs. 4 species). The results showing positive long-term effects of SR (Mueller et al., 2013;

Reich et al., 2012), conflated SR with presence of legumes, as all 16 species plots included 4 legume species. The earlier and shorter-term negative effects of SR on N cycling from other studies (Fornara et al., 2009; Knops et al., 2002; Van Der Krift & Berendse, 2001) were primarily related to the enhanced N uptake and incorporation of more N into live or dead organic matter as well as to decreased root N concentration but increased root biomass (**Fig. 7a,c**), all of which decrease soil N mineralization (Fornara et al., 2009; Mueller et al., 2013; Parton et al., 2007). By contrast, in the pure legume plots, higher species richness was associated with greater growth and possibly N fixation by legumes (root biomass and root N were increased by 45% and 11% with increasing SR from 1 to 4) (**Fig. 7a,c**), which presumably released more soluble N into soils through leaching and mineralization, increasing soil solution N and mineralization rate (**Fig. 4a,e**). These opposite responses of soil N cycling to SR between non-legume and pure legume plots together indicate that legumes alleviate the negative effects of SR on soil N cycling, suggesting a major role of legumes in regulating the more general influence of plant diversity on soil biogeochemical cycles.

The effects of legumes on soil N cycling varied with N enrichment but not CO_2 elevation

The responses of soil N metrics to legumes were not affected by elevated CO_2 (**Figs. 4 and 5**, **Tables 1 and 2**), rejecting H2. Similarly, elevated CO_2 did not affect N fixation by legumes in the BioCON experiment (West et al., 2005) or in some other experiments (Hungate et al., 2004; Rogers et al., 2009). However, our results demonstrated that N enrichment enhanced the effects of legumes on soil nitrate (**Figs. 4a,b and 5a,b**). Because legumes can satisfy much of their own N needs and those of co-occurring non-legume species (Lee et al., 2003a; Menge et al., 2015; Mulder et al., 2002; Pirhofer-Walzl et al., 2012; Temperton et al., 2007; Vitousek et al., 2013), the uptake of added N in plots with higher numbers of legume species should be lower than in plots with lower numbers of legume species, leading to the greater accumulation of nitrate in soils after N enrichment where legumes were abundant. Therefore, legumes may result in the accumulation of nitrate from applied N in this sandy soil.

By contrast, the effects of legumes on ammonium and mineralization were smaller in enriched than in ambient N treatment (**Figs. 4c,d and 5c,d**), supporting H3. This is likely because N fixation by legumes increases the release of ammonium from plant tissues and soil N mineralization, while N enrichment inhibits symbiotic N fixation and thus reduces the response of soil ammonium and N mineralization to legumes (Lee et al., 2003b; Lu et al., 2011; Salvagiotti et al., 2008; Vitousek et al., 2013; West et al., 2005). Furthermore, N enrichment reduced the effects of legumes on root traits, with smaller (on root N) or more negative (on root biomass) effects of legume presence or numbers of legume species in enriched than ambient N treatment (**Figs. 7 and S6**). The response of N mineralization to legumes was diminished by N enrichment in turn, since root traits play an important role in regulating N mineralization (**Fig. S7**, **Table S2**; Mueller et al., 2013; Wedin & Tilman, 1990).

Our results further indicated that the effects of legume were greater than that of the N enrichment (+4 g N m^{-2} y^{-1}) level on net N mineralization and its response to SR, implying that the introduction of legume species could be an alternative to N addition in regulating soil N cycling in managed ecosystems (i.e., cropland). On the other hand, N deposition in natural ecosystems (i.e., grasslands or forests) may gradually decrease the abundance of legumes (Baez, Fargione, Moore, Collins, & Gosz, 2007; Reich, 2009; Suding et al., 2005), which may enhance the response of soil N cycling to N deposition.

Implications

Considering all of these results together, legumes influence soil N cycling and its response to N deposition and rising species diversity in this N-limited ecosystem. Hence, the effects of legumes should be considered when predicting the response of soil N cycling to some global change factors and when scaling up N biogeochemical cycling from the plot level to the landscape level in N-limited regions. Whether the relationships observed herein exist in N-rich ecosystems merits further examination so that a generalizable pattern might be derived, which would be helpful for understanding global N cycling in a changing world and for precisely predicting ecological processes.

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References

Baez, S., Fargione, J., Moore, D.I., Collins, S.L. & Gosz, J.R. (2007). Atmospheric nitrogen deposition in the northern Chihuahuan desert: Temporal trends and potential consequences. *Journal of Arid Environments, 68*, 640-651.

Blagodatskaya, E., Blagodatsky, S., Dorodnikov, M. & Kuzyakov, Y. (2010). Elevated atmospheric CO_2 increases microbial growth rates in soil: results of three CO_2 enrichment experiments. *Global Change Biology, 16*, 836-848.

Carpenter-Boggs, L., Pikul, J.L., Vigil, M.F. & Riedell, W.E. (2000). Soil nitrogen mineralization influenced by crop rotation and nitrogen fertilization. *Soil Science Society of America Journal, 64*, 2038-2045.

Cong, W.-F., Hoffland, E., Li, L., Janssen, B.H. & Van Der Werf, W. (2015). Intercropping affects the rate of decomposition of soil organic matter and root litter. *Plant and Soil, 391*, 399-411.

De Graaff, M.-A., Six, J. & Van Kessel, C. (2007). Elevated CO_2 increases nitrogen rhizodeposition and microbial immobilization of root-derived nitrogen. *New Phytologist, 173*, 778-786.

Ebersberger, D., Niklaus, P.A. & Kandeler, E. (2003). Long term CO_2 enrichment stimulates N-mineralisation and enzyme activities in calcareous grassland. *Soil Biology and Biochemistry, 35*, 965-972.

Fornara, D.A. & Tilman, D. (2008). Plant functional composition influences rates of soil carbon and nitrogen accumulation. *Journal of Ecology, 96*, 314-322.

Fornara, D.A., Tilman, D. & Hobbie, S.E. (2009). Linkages between plant functional composition, fine root processes and potential soil N mineralization rates. *Journal of Ecology, 97*, 48-56.

Gill, R.A., Polley, H.W., Johnson, H.B., Anderson, L.J., Maherali, H. & Jackson, R.B. (2002). Nonlinear grassland responses to past and future atmospheric CO_2. *Nature, 417*, 279-282.

Graham, P.H. & Vance, C.P. (2003). Legumes: importance and constraints to greater use. *Plant Physiology, 131*, 872-877.

Gruber, N. & Galloway, J.N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature, 451*, 293-296.

Hoogmoed, M., Cunningham, S., Baker, P., Beringer, J. & Cavagnaro, T. (2014). N-fixing trees in restoration plantings: Effects on nitrogen supply and soil microbial communities. *Soil Biology and Biochemistry, 77*, 203-212.

Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E., Hungate, B.A., Matulich, K.L., ... O'connor, M.I. (2012). A global synthesis reveals biodiversity loss as a major driver of ecosystem change. *Nature, 486*, 105-108.

Hoosbeek, M.R., Lukac, M., Van Dam, D., Godbold, D.L., Velthorst, E.J., Biondi, F.A., ... Scarascia - Mugnozza, G. (2004). More new carbon in the mineral soil of a poplar plantation under Free Air Carbon Enrichment (POPFACE): cause of increased priming effect? *Global Biogeochemical Cycles, 18*, 177-182.

Hu, G., Liu, H., Yin, Y. & Song, Z. (2016). The role of legumes in plant community succession of degraded grasslands in northern China. *Land Degradation and Development, 27*, 366-372.

Hu, S., Chapin III, F., Firestone, M., Field, C. & Chiariello, N. (2001). Nitrogen limitation of microbial decomposition in a grassland under elevated CO_2. *Nature, 409*, 188-191.

Hungate, B.A., Stiling, P.D., Dijkstra, P., Johnson, D.W., Ketterer, M.E., Hymus, G.J., ... Drake, B.G. (2004). CO_2 elicits long-term decline in nitrogen fixation. *Science, 304*, 1291.

Janssens, I.A., Dieleman, W., Luyssaert, S., Subke, J.A., Reichstein, M., Ceulemans, R., … Law, B.E. (2010). Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience, 3,* 315-322.

Jensen, E.S., Peoples, M.B., Boddey, R.M., Gresshoff, P.M., Hauggaard-Nielsen, H., Alves, B.J.R. & Morrison, M.J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for Sustainable Development, 32*, 329-364.

Knops, J., Bradley, K. & Wedin, D. (2002). Mechanisms of plant species impacts on ecosystem nitrogen cycling. *Ecology Letters, 5*, 454-466.

Lam, S.K., Chen, D., Norton, R., Armstrong, R. & Mosier, A.R. (2012). Nitrogen dynamics in grain crop and legume pasture systems under elevated atmospheric carbon dioxide concentration: A meta ‐ analysis. *Global Change Biology, 18*, 2853-2859.

Larsen, K.S., Andresen, L.C., Beier, C., Jonasson, S., Albert, K.R., Ambus, P., ... Stevnbak, K. (2011). Reduced N cycling in response to elevated CO_2, warming, and drought in a Danish heathland: Synthesizing results of the CLIMAITE project after two years of treatments. *Global Change Biology, 17*, 1884-1899.

Lee, T.D., Reich, P.B. & Tjoelker, M.G. (2003a). Legume presence increases photosynthesis and N concentrations of co-occurring non-fixers but does not modulate their responsiveness to carbon dioxide enrichment. *Oecologia, 137*, 22-31.

Lee, T.D., Tjoelker, M.G., Reich, P.B. & Russelle, M.P. (2003b). Contrasting growth response of an N_2-fixing and non-fixing forb to elevated CO_2: dependence on soil N supply. *Plant and Soil, 255*, 475-486.

Li, Q., Song, Y., Li, G., Yu, P., Wang, P. & Zhou, D. (2015). Grass-legume mixtures impact soil N, species recruitment, and productivity in temperate steppe grassland. *Plant and Soil, 394*, 271-285.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J., Hector, A., ... Schmid, B. (2001). Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science, 294*, 804-808.

Lu, M., Yang, Y., Luo, Y., Fang, C., Zhou, X., Chen, J., ... Li, B. (2011). Responses of ecosystem nitrogen cycle to nitrogen addition: a meta‐analysis. *New Phytologist, 189*, 1040-1050.

Makkonen, M., Berg, M.P., Handa, I.T., Hättenschwiler, S., Van Ruijven, J., Van Bodegom, P.M. & Aerts, R. (2012). Highly consistent effects of plant litter identity and functional traits on decomposition across a latitudinal gradient. *Ecology Letters, 15*, 1033-1041.

Mclaren, J.R. & Turkington, R. (2010). Plant functional group identity differentially affects leaf and root decomposition. *Global Change Biology, 16*, 3075-3084.

Menge, D.N.L., Wolf, A.A. & Funk, J.L. (2015). Diversity of nitrogen fixation strategies in Mediterranean legumes. *Nature Plants, 1*, 15064.

Milcu, A., Partsch, S., Scherber, C., Weisser, W.W. & Scheu, S. (2008). Earthworms and legumes control litter decomposition in a plant diversity gradient. *Ecology, 89*, 1872-1882.

Mueller, K.E., Hobbie, S.E., Tilman, D. & Reich, P.B. (2013). Effects of plant diversity, N fertilization, and elevated carbon dioxide on grassland soil N cycling in a long‐term experiment. *Global Change Biology, 19*, 1249-1261.

Mulder, C.P.H., Jumpponen, A., Högberg, P. & Huss-Danell, K. (2002). How plant diversity and legumes affect nitrogen dynamics in experimental grassland communities. *Oecologia, 133*, 412-421.

Oelmann, Y., Buchmann, N., Gleixner, G., Habekost, M., Roscher, C., Rosenkranz, S., ... Wilcke, W. (2011). Plant diversity effects on aboveground and belowground N pools in temperate grassland ecosystems: Development in the first 5 years after establishment. *Global Biogeochemical Cycles, 25*, doi.org/10.1029/2010GB003869.

Parton, W., Silver, W.L., Burke, I.C., Grassens, L., Harmon, M.E., Currie, W.S., ... Hart, S.C. (2007). Global-scale similarities in nitrogen release patterns during long-term decomposition. *Science, 315*, 361-364.

Pirhofer-Walzl, K., Rasmussen, J., Høgh-Jensen, H., Eriksen, J., Søegaard, K. & Rasmussen, J. (2012). Nitrogen transfer from forage legumes to nine neighbouring plants in a multi-species grassland. *Plant and Soil, 350*, 71-84.

Reich, P.B. (2009). Elevated CO_2 reduces losses of plant diversity caused by nitrogen deposition. *Science, 326*, 1399-1402.

Reich, P.B., Hobbie, S.E., Lee, T.D. & Pastore, M.A. (2018). Unexpected reversal of C_3 versus C_4 grass response to elevated CO_2 during a 20-year field experiment. *Science, 360*, 317-320.

Reich, P.B., Knops, J., Tilman, D., Craine, J., Ellsworth, D., Tjoelker, M., ... Bahauddin, D. (2001a). Plant diversity enhances ecosystem responses to elevated CO_2 and nitrogen deposition. *Nature, 410*, 809-812.

Reich, P.B., Peterson, D.W., Wedin, D.A. & Wrage, K. (2001b). Fire and vegetation effects on productivity and nitrogen cycling across a forest–grassland continuum. *Ecology, 82*, 1703-1719.

Reich, P.B., Tilman, D., Isbell, F., Mueller, K., Hobbie, S.E., Flynn, D.F. & Eisenhauer, N. (2012). Impacts of biodiversity loss escalate through time as redundancy fades. *Science, 336*, 589-592.

Reich, P.B., Tilman, D., Naeem, S., Ellsworth, D.S., Knops, J., Craine, J., ... Trost, J. (2004). Species and functional group diversity independently influence biomass accumulation and its response to CO_2 and N. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 10101-10106.

Riggs, C.E., Hobbie, S.E., Bach, E.M., Hofmockel, K.S. & Kazanski, C.E. (2015). Nitrogen addition changes grassland soil organic matter decomposition. *Biogeochemistry, 125,* 203-219.

Robertson, G.P., Wedin, D., Groffmann, P., Blair, J., Holland, E., Nadelhoffer, K. & Harris, D. (1999) Soil carbon and nitrogen availability: nitrogen mineralization, nitrification, and soil respiration potentials. In: *Standard soil methods for long-term ecological research* (pp 258-271). Oxford: Oxford University Press.

Rogers, A., Ainsworth, E.A. & Leakey, A.D. (2009). Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? *Plant Physiology, 151*, 1009-1016.

Rogers, A., Gibon, Y., Stitt, M., Morgan, P.B., Bernacchi, C.J., Ort, D.R. & Long, S.P. (2006). Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. *Plant, Cell & Environment, 29*, 1651-1658.

Salvagiotti, F., Cassman, K.G., Specht, J.E., Walters, D.T., Weiss, A. & Dobermann, A. (2008). Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crops Research, 108*, 1-13.

Soussana, J. & Hartwig, U. (1995). The effects of elevated CO_2 on symbiotic N_2 fixation: a link between the carbon and nitrogen cycles in grassland ecosystems. *Plant and Soil, 187*, 321-332.

Spehn, E., Scherer ‐ Lorenzen, M., Schmid, B., Hector, A., Caldeira, M., Dimitrakopoulos, P., ... Pereira, J. (2002). The role of legumes as a component of biodiversity in a cross ‐ European study of grassland biomass nitrogen. *Oikos, 98*, 205-218.

Stevenson, F.J., Stevenson, E. & Cole, M. (1999) *Cycles of soils: carbon, nitrogen, phosphorus, sulfur, micronutrients*. New York: John Wiley & Sons.

Suding, K.N., Collins, S.L., Gough, L., Clark, C., Cleland, E.E., Gross, K.L., ... Pennings, S. (2005). Functional-and abundance-based mechanisms explain diversity loss due to N fertilization. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 4387-4392.

Temperton, V.M., Mwangi, P.N., Scherer-Lorenzen, M., Schmid, B. & Buchmann, N. (2007). Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecologia, 151*, 190-205.

Tilman, D., Knops, J., Wedin, D. & Reich, P. (2002) *Plant diversity and composition: Effects on productivity and nutrient dynamics of experimental grasslands* (pp 21-35). Oxford: Oxford University Press.

Van Der Krift, T.A. & Berendse, F. (2001). The effect of plant species on soil nitrogen mineralization. *Journal of Ecology, 89*, 555-561.

Vitousek, P.M., Menge, D.N.L., Reed, S.C. & Cleveland, C.C. (2013). Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B-Biological Sciences, 368*, doi.org/10.1098/rstb.2013.0119.

Wardle, D.A., Bardgett, R.D., Callaway, R.M. & Van Der Putten, W.H. (2011). Terrestrial ecosystem responses to species gains and losses. *Science, 332*, 1273-1277.

Wedin, D.A. & Tilman, D. (1990). Species effects on nitrogen cycling: a test with perennial grasses. *Oecologia, 84*, 433-441.

Wei, X., Reich, P.B., Hobbie, S.E. & Kazanski, C.E. (2017). Disentangling species and functional group richness effects on soil N cycling in a grassland ecosystem. *Global Change Biology, 23*, 4717-4727.

West, J.B., Hillerislambers, J., Lee, T.D., Hobbie, S.E. & Reich, P.B. (2005). Legume species identity and soil nitrogen supply determine symbiotic nitrogen ‐ fixation responses to elevated atmospheric [CO_2]. *New Phytologist, 167*, 523-530.

Zak, D.R., Pregitzer, K.S., Curtis, P.S., Teeri, J.A., Fogel, R. & Randlett, D.L. (1993). Elevated atmospheric CO_2 and feedback between carbon and nitrogen cycles. *Plant and Soil, 151*, 105-117.

Zanetti, S., Hartwig, U.A., Luscher, A., Hebeisen, T., Frehner, M., Fischer, B.U., ... Nosberger, J. (1996). Stimulation of symbiotic N_2 fixation in *Trifolium repens* L. under elevated atmospheric pCO_2 in a grassland ecosystem. *Plant Physiology, 112*, 575-583.

Table 1. Mixed model results (P values) for soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, net N mineralization rate (N_{min}), root biomass and root N concentration (Root N) for the effects of elevated CO_2, enriched N, species richness (1-species monocultures vs. 4-species monogroups) and legume vs. non-legume.

Effects	NO_3^-	NH_4^+	N_{min}	Root biomass	Root N
CO_2	0.4602	**<0.0001**	0.2021	**<0.0001**	**0.0400**
N	**<0.0001**	**<0.0001**	0.0530	**0.0032**	**<0.0001**
SR	**<0.0001**	**0.0024**	**0.0477**	**<0.0001**	0.7616
L	**<0.0001**	0.0558	**<0.0001**	**<0.0001**	**<0.0001**
CO_2×N	0.1883	**0.0493**	0.0772	0.4323	0.5205
CO_2×SR	0.4825	0.7200	0.6974	0.1383	0.5012
CO_2×L	0.8237	0.0585	0.8209	0.6686	0.0798
N×SR	0.1592	**<0.0001**	0.1292	0.8857	0.1618
N×L	**<0.0001**	0.1858	0.6495	0.0514	**<0.0001**
SR×L	**<0.0001**	**0.0061**	**0.0020**	0.7518	**<0.0001**
CO_2×N×SR	0.6677	0.7601	0.1276	0.1339	0.2148
CO_2×N×L	0.0737	0.7481	0.0543	0.6203	0.9214
CO_2×SR×L	0.5531	0.8598	0.9354	0.6313	0.9916
N×SR×L	0.3480	0.3386	**0.0008**	0.1134	0.2324
CO_2×N×SR×L	0.7376	0.8875	0.3496	0.3703	0.2351
Y	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0013**
Y×CO_2	**0.0490**	0.5690	**0.0116**	0.8735	0.6242
Y×N	**<0.0001**	**<0.0001**	0.7588	0.3974	**0.0465**
Y×SR	0.9816	0.8899	**0.0447**	0.9255	0.3145
Y×L	**0.0041**	**<0.0001**	**0.0019**	**0.0010**	**0.0378**
Y×CO_2×N	0.7850	0.2329	0.4634	0.4836	0.7723
Y×CO_2×SR	0.9909	0.0766	0.8669	0.4079	0.8507
Y×CO_2×L	0.5664	**0.0090**	0.2899	0.9229	0.2127
Y×N×SR	0.8665	0.3995	0.4749	0.9546	0.8772
Y×N×L	**0.0060**	0.9177	0.4911	0.6196	0.2024
Y×SR×L	0.2040	**0.0068**	0.4721	0.0620	0.1747
Y×CO_2×N×SR	0.3337	0.6778	0.3070	0.8928	0.3263
Y×CO_2×N×L	0.9665	0.1598	0.2832	0.5751	0.3574
Y×CO_2×SR×L	0.7703	0.3308	0.7300	0.9037	0.8553
Y×N×SR×L	0.5253	0.9723	0.4509	0.5286	0.7676
Y×CO_2×N×SR×L	0.3960	0.4169	0.7053	0.8872	0.6438
R^2	0.467	0.262	0.144	0.239	0.443
RMSE	1.381	0.671	0.147	1.124	0.334
n	3256	3264	3244	3277	3256

R^2: coefficient of determination for the overall model; RMSE: root mean square error for the overall model; n: sampling size; CO_2: elevated CO_2; N: enriched N; SR: increased species from 1 to 4; L: pure legumes vs. non-legumes; Y: year.

Table 2. Mixed model results (P values) for soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, net N mineralization rate (N_{min}), root biomass and root N concentration (Root N) for the effects of elevated CO_2, enriched N and numbers of legume species (0 to 4) in 4-species communities.

Effects	NO_3^-	NH_4^+	N_{min}	Root biomass	Root N
CO_2	0.2767	**0.0018**	0.9016	**<0.0001**	**0.0001**
N	**<0.0001**	**<0.0001**	0.4434	**<0.0001**	**<0.0001**
LS	**<0.0001**	**0.0157**	**<0.0001**	**<0.0001**	**<0.0001**
CO_2×N	0.4736	**0.0209**	0.0753	**0.0099**	**0.0027**
CO_2×LS	0.5577	0.8103	0.3733	0.6253	0.2219
N×LS	0.0620	0.0596	**0.0121**	**0.0027**	**0.0001**
CO_2×N×LS	0.4281	0.9663	0.1065	**0.0255**	0.6270
Y	**<0.0001**	**<0.0001**	0.3922	**<0.0001**	**<0.0001**
Y×CO_2	0.6951	0.3418	0.0970	0.1458	**0.0297**
Y×N	**0.0001**	**<0.0001**	0.6386	**0.0403**	0.6810
Y×LS	0.0752	**<0.0001**	0.4826	0.5033	0.1891
Y×CO_2×N	0.7963	0.6904	**0.0138**	0.0989	0.0757
Y×CO_2×LS	0.5303	0.3292	0.8375	0.7708	0.4961
Y×N×LS	0.1864	0.6985	0.8234	0.6126	0.1677
Y×CO_2×N×LS	0.7616	0.1451	0.8727	0.4242	0.1887
R^2	0.396	0.241	0.102	0.211	0.408
RMSE	1.431	0.668	0.150	0.743	0.305
n	2035	2034	2025	2049	2057

R^2: coefficient of determination for the overall model; RMSE: root mean square error for the overall model; n: sampling size; CO_2: elevated CO_2; N: enriched N; LS: numbers of legume species; Y: year.

Figure captions

Fig. 1 Conceptual diagram outlining the effects of increased species richness (SR), elevated atmosphere CO_2 and nitrogen (N) supply on soil inorganic N pool and net N mineralization and their response to legumes. "+" indicates positive effects (e.g., increased soil N availability), "-" indicates negative effects (e.g., decreased soil N availability). The solid arrows indicate effects in communities without legumes; the dashed arrows indicate effects in communities with legumes, as mediated by effects on N fixation. **A**: Greater SR increases inorganic N uptake and soil microbes immobilization by increasing soil C inputs and plant and microbial demand for N; **B**: Increased N supply increases the inorganic N pool and promotes soil N mineralization by reducing soil C:N ratios; **C**: Elevated CO_2 enhances decomposition through priming and increases inorganic N release, by increasing labile C inputs to soils; **D**: Elevated CO_2 increases plant inorganic N uptake and soil microbes immobilization by increasing plant and microbial demand for N; **E**: Legume N fixation increases inorganic N inputs and N mineralization and decreases immobilization by reducing C:N ratios of plant inputs to soils; **F**: Greater SR decreases soil N availability and thus promotes legume N fixation, which will in turn offset the declines in inorganic N pools caused by higher SR; **G**: Increased N supply suppresses legume N fixation; **H**: Elevated CO_2 increases the availability of C relative to N and accelerates legume N fixation.

Fig. 2 Effects of legume species identity as well as non-legume species on nitrate (NO_3^-, **a**) and ammonium (NH_4^+, **b**) concentrations in soil solution, and net N mineralization rate (N_{min}, **c**) for monoculture plots averaged across all four combinations of ambient and elevated (560 μmol mol^{-1}) CO_2 and ambient and enriched (4 g m^{-2} year^{-1}) N treatments. The values for non-legumes were averaged across all monoculture plots of non-legume species. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean. The four legume species are *Amorpha canescens, Lespedeza capitata, Lupinus perennis* and *Petalostemum villosum*, respectively.

Fig. 3 Responses of soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, and net N mineralization rate (N_{min}) to legume vs. non-legume species, averaged across monocultures and 4-species monogroups (**a, c, e**), and to the numbers of legume species (0, 1, 2 and 4) in 4-species mixed functional group communities (**b, d, f**) over experimental years. Data were averaged across all four combinations of ambient and elevated (560 μmol mol^{-1}) CO_2 and ambient and enriched (4 g m^{-2} year^{-1}) N treatments. Error bars denote two standard errors of the mean.

Fig. 4 Nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, and net N mineralization rate (N_{min}) in pure legume and non-legume plots (**a, c, e**) and the differences of them between pure legume and non-legume plots (**b, d, f**) for 1-species monocultures and 4-species monogroups. Soil N metrics were averaged at four combinations of ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 and ambient (aN) and enriched (eN, 4 g m^{-2} year^{-1}) N treatments. The differences in N metrics were calculated by subtracting non-legume plots from pure-legume plots. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean.

Fig. 5 Nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, and net N mineralization rate (N_{min}) in 4-species mixed functional group communities with various numbers of legume species (**a, c, e**) and the differences of them between 4 legume species and 0 legume species plots (**b, d, f**). Soil N metrics were averaged at four combinations of ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 and ambient (aN) and enriched (eN, 4 g m^{-2} year^{-1}) N treatments. Data were averaged from 1998 to 2014. The differences in N metrics were calculated by subtracting values in four-species plots with 0 legume species from those in four-species plots with all legume species. Error bars denote two standard errors of the mean.

Fig. 6 Effects of N enrichment and legume on soil net N mineralization rate (N_{min}). Effects of N enrichment were compared between enriched (4 g m^{-2} year^{-1}) and ambient N treatment in non-legume plots in monocultures and monogroup plots or in 0 legume species plots of the 4-species mixed functional group communities. Effects of legume or the numbers of legume species were compared in ambient N treatment between pure legume vs. non-legume plots for 1-species monocultures and 4-species monogroups, and between plots with varying numbers of legume species (1, 2, and 4) vs. plots with 0 legume species for the 4-species mixed functional group communities. **a and c**: N treatment and legume effects were compared at ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 treatments averaged across all the years. **b and d**: N treatment and legume effects were compared at each year averaged across all four combinations of CO_2 and N treatments.

Fig. 7 Root biomass and root N concentration in pure legume vs. non-legume plots for 1-species monocultures and 4-species monogroups (**a, c**) and in 4-species mixed functional group communities with various number of legume species (**b, d**). Root biomass and root N concentration were averaged at four combinations of ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 and ambient (aN) and enriched (eN, 4 g m^{-2} year^{-1}) N treatments. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean.

Supporting Information captions

Table S1 Mixed model results (P values) for soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, net N mineralization rate (N_{min}), root biomass and root N concentration (Root N) for the effect of legume species identity in 4 legumes monocultures.

Table S2 Mixed model results for net N mineralization rate for the effect of root biomass and N concentration.

Fig. S1 Schematic of experimental design. Plots containing different diversity treatments are represented by black squares containing symbols with different colors and shape categories that represent different functional groups (blue squares - C4 grasses, green diamonds - legumes, orange stars - C3 grasses, pink circles - non-legume forbs). Different sizes and types of a particular shape represent different species within a functional group. Within the BioCON experiment there are plots planted with a single species (monocultures of 16 species, 4 from each of the 4 functional groups), plots planted with four different species, all from a single functional group (monogroups), and plots planted with four different species from at least two different functional groups (mixtures). All plots are replicated at two different levels of CO_2 (represented by the grey rectangles), ambient (hatched) and elevated (solid). Plots receive one of two levels of N treatment (represented by green rectangles), ambient (hatched) and enriched (solid). CO_2, N, and diversity treatments are replicated in a full factorial design.

Fig. S2 Effects of legume and non-legume species identity on nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, net N mineralization rate (N_{min}), and total plant N pool (Plant N, belowground plus aboveground) for monoculture plots averaged across all four combinations of ambient and elevated (560 μmol mol^{-1}) CO_2 and ambient and enriched (4 g m^{-2} $year^{-1}$) N treatments. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean. X axis is the species identity of four legumes, four C3 and C4 grasses, and four forbs. The four legumes species are *Amorpha canescens* (AC)*, Lespedeza capitata* (LC)*, Lupinus perennis* (LP) and *Petalostemum villosum* (PV), the four C3 grasses are *Agropyron repens* (AR), *Bromus inermis* (BI), *Koeleria cristata* (KC) and *Poa pratensis* (PP), the four C4 grasses are *Andropogon gerardii* (AG), *Bouteloua gracilis* (BG), *Schizachyrium scoparium* (SS) and *Sorghastrum nutans* (SN), the four forbs are *Achillea millefolium* (AM), *Anemone cylindrica* (AC), *Asclepias tuberosa* (AT) and *Solidago rigida* (SR).

Fig. S3 Response of nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, and net N mineralization rate (N_{min}) to the numbers of legume species in 4-species mixed functional group communities averaged across all four combinations of ambient and elevated (560 μmol mol^{-1}) CO_2 and ambient and enriched (4 g m^{-2} $year^{-1}$) N treatments. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean.

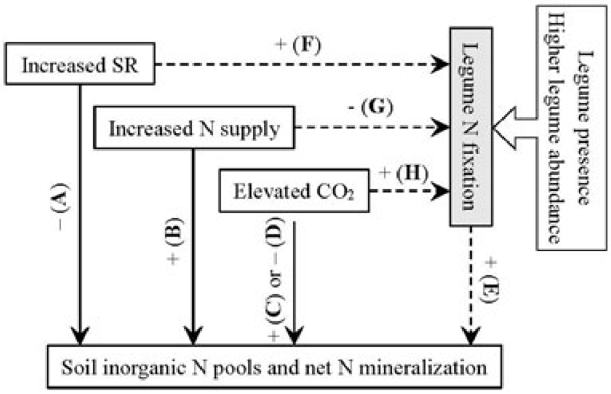
Fig. S4 Changes in nitrate (NO_3^-) and ammonium (NH_4^+) concentrations in soil solution, and net N mineralization rate (N_{min}) in response to increasing species richness from 1 to 4 in non-legume and pure legume plots. The changes in N metrics in response to species richness were compared between 4-species monogroups and 1-species monocultures at four combinations of ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 and ambient (aN) and enriched (eN, 4 g m^{-2} $year^{-1}$) N treatments. Data were averaged from 1998 to 2014. The changes in N metrics were always higher or less negative in legume plots than non-legume plots.

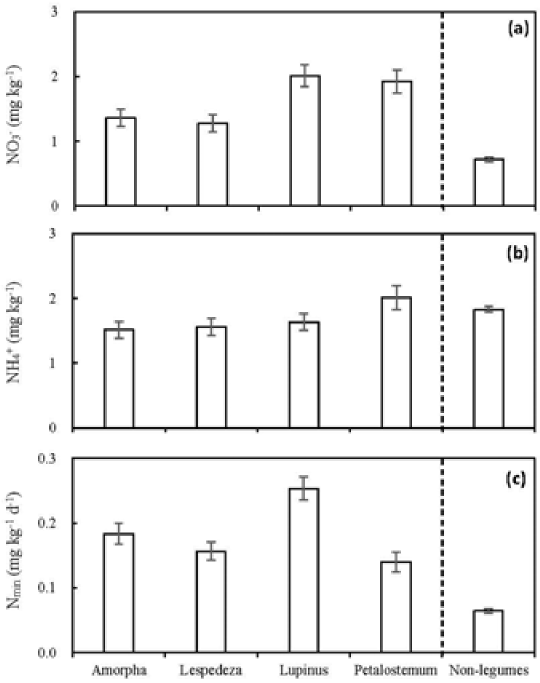
Fig. S5 Absolute changes in net N mineralization rate (N_{min}) in response to increasing species richness from 1 to 4 at each of four combinations of ambient (aN) and enriched (eN, 4 g m^{-2} $year^{-1}$) N treatments, and non-legume and pure legume plots over experimental years. The absolute changes in N_{min} were compared between 4-species monogroup plots and 1-species monoculture plots averaged across ambient and elevated CO_2 concentration (560 μmol mol^{-1}) treatments.

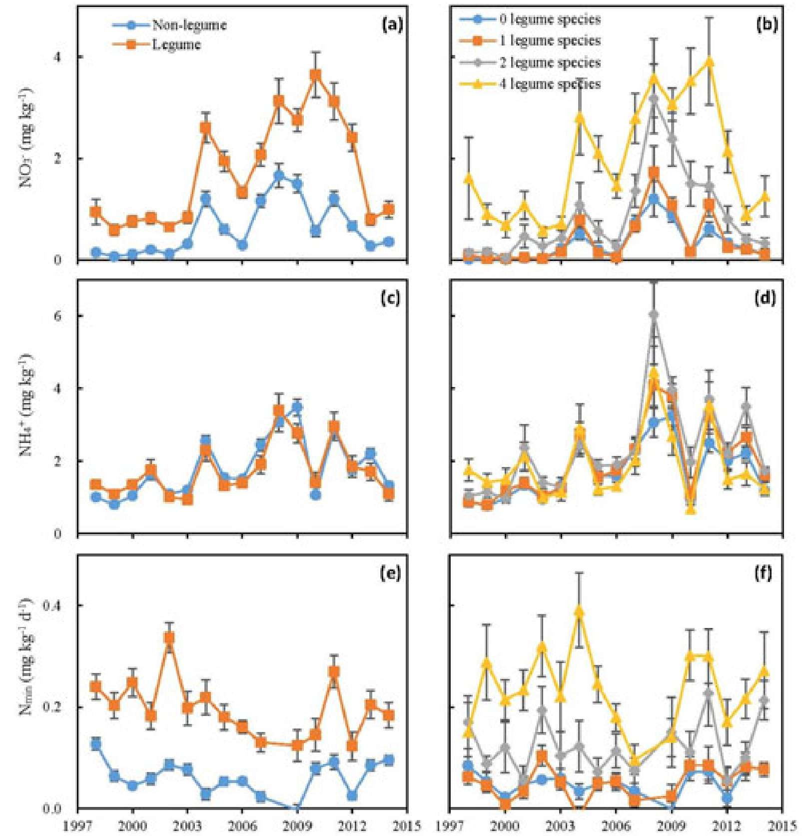
Fig. S6 Differences in root N concentration between pure legume and non-legume plots in 1-species monocultures and 4-species monogroups at four combinations of ambient (aCO_2) and elevated (eCO_2, 560 μmol mol^{-1}) CO_2 and ambient (aN) and enriched (eN, 4 g m^{-2} $year^{-1}$) N treatments. The differences in root N concentration were calculated by subtracting non-legume plots from pure-legume plots. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean.

Fig. S7 Relationships of net N mineralization (N_{min}) to root biomass (Left) and root N concentration (Right). The means were averaged across all the experimental years. The open circles were means for each combination of CO_2 treatment (ambient and elevated CO_2), N treatment (ambient and enriched N), and legume vs. non-legume treatments averaged across 1-species monocultures and 4-species monogroups. The closed circles were means for each combination of CO_2 treatment (ambient and elevated CO_2), N treatment (ambient and enriched N), and the numbers of legume species (0, 1, 2 and 4) in 4-species mixed functional group communities. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean.

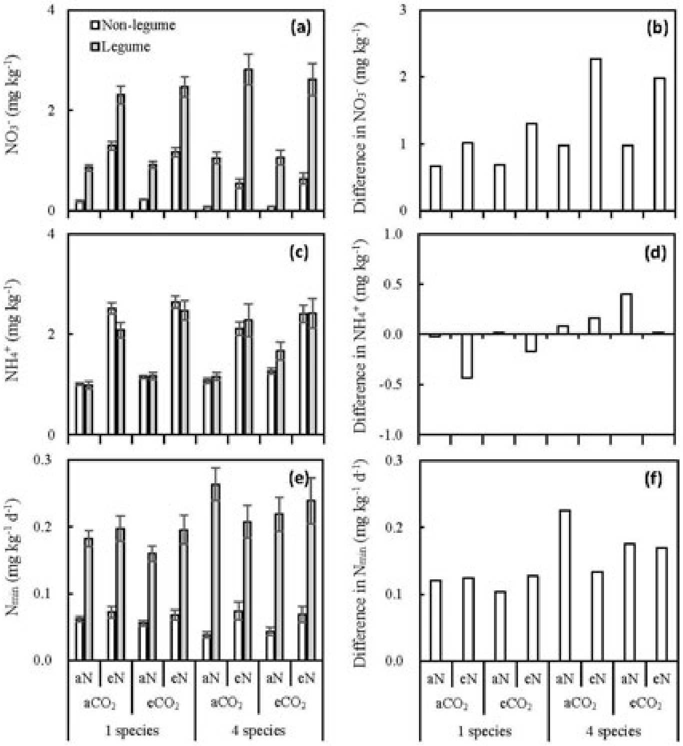
Fig. S8 Effects of legume species identity on root biomass and root N concentration for monoculture plots with legume species averaged across all four combinations of ambient and elevated (560 μmol mol^{-1}) CO_2 and ambient and enriched (4 g m^{-2} $year^{-1}$) N. Data were averaged from 1998 to 2014. Error bars denote two standard errors of the mean. X axis is the species identity of legumes.

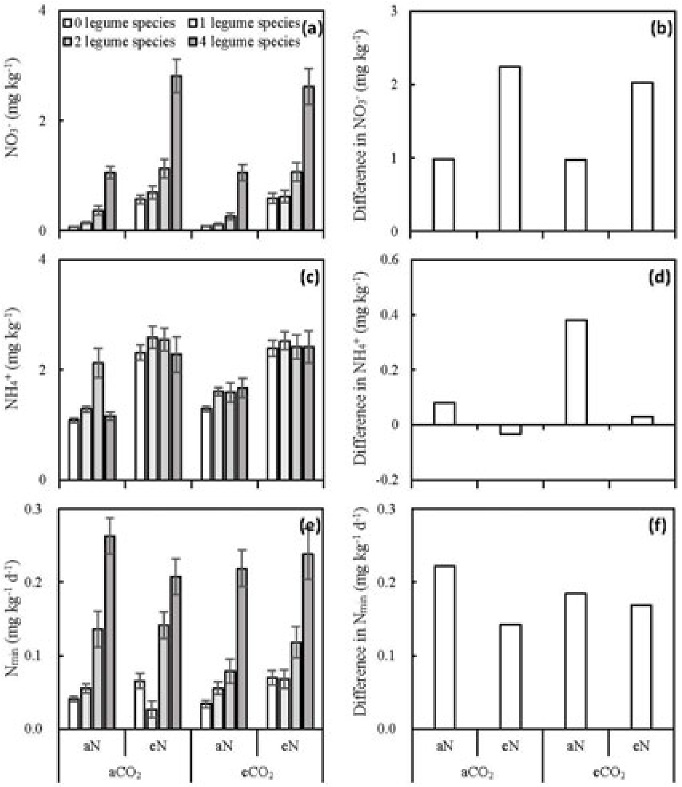

Increased SR
+ (F)
Increased N supply
- (G)
Elevated CO$_2$
+ (H)
Legume N fixation
Legume presence
Higher legume abundance
− (A)
+ (B)
+ (C) or − (D)
+ (E)
Soil inorganic N pools and net N mineralization

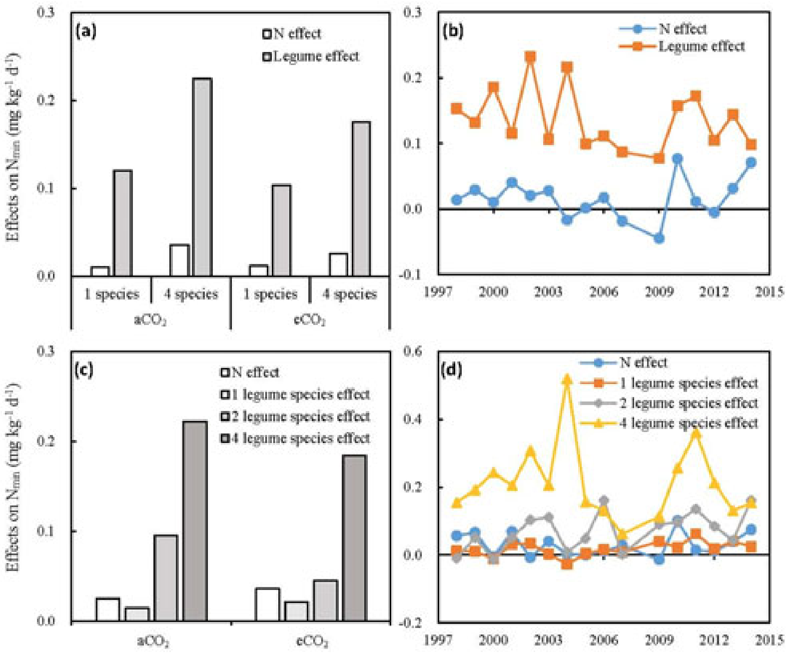

3
2
1
0
NO3- (mg kg-1)
(a)
3
2
1
0
NH4+ (mg kg-1)
(b)
0.3
0.2
0.1
0.0
Nmin (mg kg-1 d-1)
(c)
Amorpha
Lespedeza
Lupinus
Petalostemum
Non-legumes

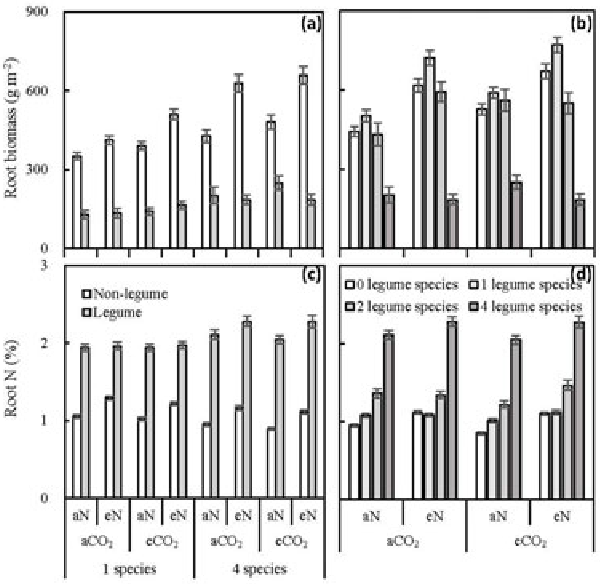


(a)
Non-legume
Legume
(b)
0 legume species
1 legume species
2 legume species
4 legume species
NO3- (mg kg-1)
0
2
4
(c)
(d)
NH4+ (mg kg-1)
0
2
4
6
(e)
(f)
Nmin (mg kg-1 d-1)
0.0
0.2
0.4
1997
2000
2003
2006
2009
2012
2015


(a)
Non-legume
Legume
NO3- (mg kg-1)
0
2
4
(b)
Difference in NO3- (mg kg-1)
0
1
2
3
(c)
NH4+ (mg kg-1)
0
2
4
(d)
Difference in NH4+ (mg kg-1)
-1.0
-0.5
0.0
0.5
1.0
(e)
Nmin (mg kg-1 d-1)
0.0
0.1
0.2
0.3
(f)
Difference in Nmin (mg kg-1 d-1)
0.0
0.1
0.2
0.3
aN
eN
aN
eN
aN
eN
aN
eN
aCO2
eCO2
aCO2
eCO2
1 species
4 species


0 legume species
1 legume species
2 legume species
4 legume species
(a)
NO3- (mg kg-1)
4
2
0
(b)
Difference in NO3- (mg kg-1)
3
2
1
0
(c)
NH4+ (mg kg-1)
4
2
0
(d)
Difference in NH4+ (mg kg-1)
0.6
0.4
0.2
0
-0.2
(e)
Nmin (mg kg-1 d-1)
0.3
0.2
0.1
0.0
(f)
Difference in Nmin (mg kg-1 d-1)
0.3
0.2
0.1
0.0
aN
eN
aN
eN
aCO2
eCO2


(a)
N effect
Legume effect
Effects on N_min (mg kg-1 d-1)
0.0
0.1
0.2
0.3
1 species
4 species
1 species
4 species
aCO2
eCO2
(b)
N effect
Legume effect
-0.1
0.0
0.1
0.2
0.3
1997
2000
2003
2006
2009
2012
2015
(c)
N effect
1 legume species effect
2 legume species effect
4 legume species effect
Effects on N_min (mg kg-1 d-1)
0.0
0.1
0.2
0.3
aCO2
eCO2
(d)
N effect
1 legume species effect
2 legume species effect
4 legume species effect
-0.2
0.0
0.2
0.4
0.6
1997
2000
2003
2006
2009
2012
2015


900
600
300
0
Root biomass (g m^{-2})
(a)
(b)
3
2
1
0
Root N (%)
(c)
Non-legume
Legume
(d)
0 legume species
1 legume species
2 legume species
4 legume species
aN
eN
aN
eN
aN
eN
aN
eN
aCO$_2$
eCO$_2$
aCO$_2$
eCO$_2$
1 species
4 species
aN
eN
aN
eN
aCO$_2$
eCO$_2$