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Role of N_2-fixation in Constructed Old-field Communities Under Different Regimes of [CO_2], Temperature, and Water Availability

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Abstract

Nitrogen fixation was measured in constructed old-field ecosystems that were exposed for 3 years to different combinations of elevated atmospheric [CO_2] and temperature (300 ppm and 3°C above ambient, respectively), and ambient or reduced soil moisture (corresponding to 25 or 2 mm rainfall per week). The old-fields included seven planted herbaceous annual and perennial species, including two legumes (*Trifolium pratense* and *Lespedeza cuneata*). Potential asymbiotic N_2-fixation by soils, measured in laboratory incubations, was significantly less under the ''dry'' treatment but was estimated to contribute little overall to annual ecosystem N budgets. Foliar N concentrations declined significantly under elevated [CO_2]. Effects of the three environmental factors on the mean (±SE) fraction of legume N derived from atmospheric N_2 (FN_{dfa}) varied from year-to-year, and FN_{dfa} ranged from 0.64 ± 0.05 to 0.94 ± 0.03 depending on species and growing season. High rates of symbiotic N_2-fixation (4.6–12 g N m^{-2} y^{-1}) that annually contributed from 44% to 51% to the aboveground N stock in the old-field community was an important process driving changes in species composition over the 3-year experiment. *Lespedeza* biomass increased over time at the expense of several other species, including the other N-fixer, *Trifolium*. The dominance of *Lespedeza* in our ecosystem was due to high symbiotic N_2-fixation rates, as well as shading effects on other species. The high symbiotic N_2-fixation rates were largely independent of manipulations of [CO_2], temperature, and water. The relatively high percentage of the aboveground N stock in this ecosystem contributed by symbiotic N_2-fixation suggests that non-legume species may have benefited indirectly via reduced community demands on soil N supplies. Species-specific traits were important in the constructed ecosystems, indicating that multi-species studies are required for understanding complex interactions among environmental factors and dynamic changes in community composition.

Key words: *Lespedeza*; legumes; ^{15}N natural abundance method; multi-factor experiments; climate change; constructed ecosystems.

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Introduction

Nitrogen fixation is an important biological process that moves N from the atmosphere into terrestrial ecosystems. Even in the absence of direct N transfer from legumes to non-legumes (for example, Gil and others 1997), increased N_2-fixation can indirectly benefit non-N_2-fixing plants through greater belowground inputs to soil N stocks (Ledgard and Steele 1992; Soussana and Hartwig 1996). This can be particularly important in ecosystems where net primary production is potentially constrained by soil N availability (Zanetti and others 1997; Cannell and Thornley 1998; Reich and others 2006).

Elevated [CO_2] can increase both the amount and percentage of host plant N derived *via* symbiotic N_2-fixation (Soussana and Hartwig 1996; Zanetti and Hartwig 1997), which may benefit the overall N economy of terrestrial ecosystems (Luo and Mooney 1995) and may be important in regulating ecosystem C and N balance under conditions of CO_2 enrichment (Hartwig and others 2000; Luscher and others 2000). For example, elevated [CO_2] can increase both the amount and percentage of host plant N derived via symbiotic N_2-fixation. Other environmental factors such as temperature or soil moisture availability can also impact the process separately or in combination with elevated [CO_2]. Although individual species responses can vary, increasing temperatures above 20°C generally decrease symbiotic N_2-fixation (McWilliam 1978; Lilley and others 2001; Bordeleau and Prevost 1994). Nitrogen fixation also exhibits a highly variable response to soil water availability (Serraj and Sinclair 1997; Serraj and others 1999) that depends, in part, on how fixed N is transported within the host legume (Serraj and others 2001). Although a decline in N_2-fixation in response to drought is not universal among legumes (Sinclair and Serraj 1995; Høgh-Jensen and Schjoerring 1997), numerous studies show a clear decline in nodulation, nodule mass, N_2-fixation, or legume production under conditions of plant water stress (Athar and Johnson 1996; Carranca and others 1999; Ohashi and others 1999; Kurdali and others 2002). In addition to legumes, free-living microorganisms (diazotrophs) are potential sources of N_2-fixation in terrestrial ecosystems. However, little is known about the response of asymbiotic N_2-fixation to altered [CO_2], temperature, and soil water availability.

The effects of environmental factors on N_2-fixation in herbaceous plant communities have been examined individually, but seldom from the perspective of potential multi-factor interactions or their indirect consequences for plant community composition and ecosystem N cycling. We therefore took advantage of a multi-factor experiment near Oak Ridge, Tennessee (USA), to better understand the effects and interactions of increased [CO_2], warming, and changes in soil moisture on N_2-fixation in a constructed old-field community that included C3 and C4 grasses, herbaceous dicots, and legumes. Little is known about the interactive effects of temperature, soil water, and elevated [CO_2] on N_2-fixation in old-fields and the significance of these processes for plant community dynamics under different climate regimes. Given the variable results seen in previous research on N_2-fixers to changes in [CO_2], temperature, and water availability, we hypothesized that: (1) the response of N_2-fixation by legumes to elevated [CO_2] would be modified by changes in temperature and soil water availability because [CO_2] and temperature or water availability have generally opposing effects on N_2-fixation, and (2) ecosystem-level responses, such as community composition and aboveground N stocks, would be controlled by interactions between component taxa and their individual and combined responses to changing environmental factors. In other words, legume production could be positively or negatively affected depending on how multi-factor interactions impacted N_2-fixation. The objectives of our research were to: (1) determine the effects of manipulated environmental factors on asymbiotic and symbiotic N_2-fixation, (2) quantify the role of N_2-fixation as it affects legume [N] and aboveground community N stocks, and (3) interpret the importance of N_2-fixation as a process contributing to observed changes in community composition over time.

Methods

Experimental Setup

The Old-Field Community, Climate, and Atmosphere Manipulation Experiment (OCCAM) was established in 2002 at Oak Ridge National Laboratory (35°54′12″N, 84°20′22″W) to study the interactive effects of multiple environmental factors on the structure and function of a constructed old-field community. The study site was used for row crops and pasture prior to 1942. From 1942 to 1978, the land was occasionally mowed and maintained in an old-field successional state. From 1978 to 1984, the site was cultivated for sorghum-sudan grass, soybean, and winter wheat (Johnston and Shriner 1986). Since 1984 the study area has

been maintained as a fescue field with no added fertilizer or other management other than occasional mowing. Old-fields in the immediate area are dominated by several *Panicum* species, *Andropogon virginicus*, *Festuca pratensis*, *Solidago gigantia*, *Lonicera japonica*, and *Rhus toxicodendron*.

In June 2002, four 4-m diameter experimental plots were established in each of three blocks on the site, the vegetation in the plots was killed with an application of glyphosate herbicide, and the dead plant biomass was extracted (to a depth of ≈1 cm) to remove aboveground meristems along with some of the seed bank. Seedlings of seven species were grown from seed and planted in the plots in July 2002 and April 2003. The species were: *Plantago lanceolata* L., an herbaceous, annual C3 dicot; *Andropogon virginicus* L., a cespitose C4 bunchgrass; *Festuca pratense* L. syn *F. elatior* L., a C3 bunchgrass; *Dactylis glomerata* L., a C3 bunchgrass; *Trifolium pratense* L.; *Solidago canadensis*, a perennial forb; and *Lespedeza cuneata* (Dum. Cours.) G. Don., a perennial N_2–fixing shrub. The plants were planted in a predetermined grid such that seedlings were approximately 18 cm apart and no individual neighbored a conspecific. Approximately 170 individuals were planted per plot. Both *Trifolium* and *Lespedeza* are N_2-fixers. The remaining five species are non-N_2-fixing and rely solely on root uptake of available soil N.

The combinations of ambient or elevated [CO_2] and ambient or elevated temperature were assigned at random to the plots in each block. Open-top chambers (4-m diameter × 2.2 m tall) were installed on top of four treatment plots in each block in March 2003 prior to the initiation of treatments. The experiment was based on a split-plot design with two levels (elevated and ambient) of [CO_2] and temperature (elevated and ambient) and three replicate chambers for each of the following treatment combinations: ambient CO_2–ambient temperature (ACAT), ambient CO_2–elevated temperature (ACET), elevated CO_2–ambient temperature (ECAT), and elevated CO_2–elevated temperature (ECET). The chambers were equipped to maintain ambient or elevated (+3°C) temperatures. Pure CO_2 was introduced into some chambers to achieve CO_2 enrichment of 300 ppm over ambient air (ambient daytime concentration was 390 ppm). Temperature and CO_2 control was achieved using methods described by Norby and others (1997) and Wan and others (2007). The treatments commenced in May 2003.

Each main plot was divided into two split-plots by lined trenches on a north–south axis through the center and assigned to either a ''wet'' or ''dry'' soil moisture treatment. Trenches were lined with insulating foam and 4-mil PVC film to provide a thermal and moisture barrier. To maintain differential soil moisture, we erected a clear plastic canopy (6-mil PVC, with 92% transmission of PAR) over the top of each chamber. Collected rain water was added to each plot weekly using a hose to add the equivalent of 25 and 2 mm precipitation to the wet and dry sides, respectively. Within each plot, the ''dry'' side had significantly lower (≈2–8%) soil water content than the ''wet'' side.

Measurement of Potential Asymbiotic N_2-fixation

Six surface (≈12 cm deep) soil cores were collected from each split-plot in mid-July 2004 and pooled to yield a single composite soil sample per split-plot. Samples were transported to the laboratory in a cooler and sieved to 6 mm. The smaller-than-6 mm fraction was refrigerated (5°C) to slow microbial activity until incubation and was used in all subsequent measurements of asymbiotic N_2-fixation. A portion of the smaller-than-6 mm soil fraction was oven dried (70°C) to determine the fresh weight-to-dry weight conversion factor. The dry soil was also analyzed for total C and N and ^{15}N abundance (see Soil and Plant Tissue Analysis).

Following methods that used by Limmer and Drake (1996), 2 g fresh soil from each composite sample were incubated in a 25-ml gas-tight serum bottle under both aerobic and anaerobic conditions, with the addition of 0.25 ml glucose (4%) or 0.25 ml deionized water (controls). Bottles were sealed with a butyl rubber stopper and a crimped aluminum cap. In anaerobic incubations, each serum bottle was thoroughly flushed with N_2 gas.

For the glucose-amended soil incubations, ^{15}N was introduced by replacing 20% of the bottle atmosphere with an equal volume (5 ml) of $^{15}N_2$ (98 atom %, Cambridge Isotope Laboratories, Andover, MA) using a gas-tight syringe. Each bottle was then injected with acetylene (C_2H_2) to give a final concentration of approximately 4% v/v. Bottles were incubated in the dark at 20°C and 100 µl gas samples were periodically withdrawn to determine ethylene (C_2H_4) production over time according to the C_2H_2 reduction method (Hardy and others 1968, 1973). At the end of the incubations, soils were removed from the bottles, oven-dried (70°C), and analyzed for total [N]. Soils from the glucose-amended incubations were analyzed for ^{15}N to determine the fraction of soil N derived from the $^{15}N_2$ injected into each bottle (F_a) according to:

$$F_a = (E - S)/(A - S), \quad (1)$$

where E is the final atom % ^{15}N in incubated soils, S is the initial atom % ^{15}N in incubated soils, and A is the ^{15}N concentration for N_2 in the incubation bottle (19.9 atom %). The mass of ^{15}N-fixed (M, g ^{15}N-fixed g^{-1} soil) was calculated from:

$$M = (F_a)(B), \quad (2)$$

where B is the final soil [N] (g N g^{-1}).

Ethylene concentrations over the course of the incubations were measured using a Hewlett-Packard 5980 Series II gas chromatograph equipped with flame ionization detector and a stainless-steel alumina column (50 m, 0.54 mm ID) from Agilent Technologies, Inc. (Palo Alto, CA). Calibration curves were developed from an analysis of C_2H_4 gas standards (from Alltech Associates, Inc., Deerfield, IL) and used to convert C_2H_4 peak area to ppm. Ethylene production (nmol C_2H_4 produced g^{-1} soil) was calculated based on the oven dry soil mass in each incubation bottle. The molar ratio of C_2H_2 reduction-to-N_2-fixation was calculated for each soil sample in the incubations amended with glucose. The measurement of asymbiotic N_2-fixation was based on laboratory incubations of unamended and amended, fresh, surface soils at optimal temperature and, as such, represents a maximum potential rate.

Aboveground Sampling

Aboveground green leaf samples from each species were collected each year (2003–2005) from dry and wet split-plots within each chamber. Samples were collected from two or more individuals of each species (≈1 g total), ground, and oven-dried (70°C) prior to elemental and isotopic analysis. Green tissue in 2004 and in 2005 was collected throughout the growing season from plants at their peak. Plant tissue samples from 2003 were collected too late in the growing season (that is, after partial senescence) for accurate determinations of green leaf [N], however $\delta^{15}N$ was analyzed and reported because N reabsorption during leaf senescence does not affect $\delta^{15}N$ (Kolb and Evans 2002). Data from 2004 and 2005 were averaged to estimate tissue [N] in 2003 for the purpose of calculating aboveground N stocks. Nitrogen stocks in each year were calculated by multiplying N concentrations (g N g^{-1}) by peak aboveground biomass (g m^{-2}). Because peak biomass occurred at different times for different species, the calculated community-level N stocks for each growing season represent maximum values. Determination of plant biomass will be described in other publications; briefly, in 2003, the aboveground biomass in a 0.4-m^2 area of each plot comprising two individuals of each species was harvested and the plants were oven-dried and weighed. In 2004 and 2005, estimates were made nondestructively based on allometric relationships between dry mass and leaf and stem length, which were determined for each species. Leaf and stem lengths of each plant in two 0.5-m^2 subplots per experimental plot were measured when biomass was close to its annual peak.

Measurement of Symbiotic N_2-fixation

The fraction of tissue N derived from atmospheric N_2-fixation (FN_{dfa}) by *Trifolium* or *Lespedeza* was calculated using the ^{15}N-natural abundance method (Shearer and Kohl 1986):

$$FN_{dfa} = (X - Y)/(X - C), \quad (3)$$

where, X = the ^{15}N abundance of N derived from soil by reference plants (neighboring non-N_2-fixing plants), Y = the ^{15}N abundance of N_2-fixing plants grown under the same conditions as the reference plants, and C = the ^{15}N abundance of N_2-fixing plants grown in a N-free substrate. In each year of the study, $\delta^{15}N$ values from four non-N_2-fixing species (*Festuca*, *Plantago*, *Dactylis*, and *Andropogon*) were averaged in each split-plot to estimate the ^{15}N abundance of N derived from soil (that is, reference plants).

The isotopic fractionation of N_2-fixation by *Trifolium* and *Lespedeza* was determined by growing both legumes separately under N-free conditions in a greenhouse. Seeds from each species were mixed with Royal Peat™ inoculant (Becker Underwood, Inc., Ames, IA) and planted in clean, washed sand in three plastic pots. *Trifolium* seeds were mixed with an ''alfalfa/clover'' inoculant and *Lespedeza* seeds were mixed with a ''cowpea–peanut–*Lespedeza*'' inoculant. The pots were watered regularly with N-free nutrient solution (for recipe, see Garten 1990). Following 7 weeks of growth, leaves with petioles were harvested at different times from several individual plants in each pot over a period of approximately 27 weeks. The tissue samples (≈1 dry g per pot) were pooled by pot, ground to a powder in liquid N_2, oven-dried, and analyzed for ^{15}N-abundance.

Soil and Plant Tissue Analysis

Soils and plant tissue samples were analyzed for total C and N using a LECO CN-2000 (LECO Corporation, St. Joseph, MI). The instrument was cal-

ibrated using secondary standards traceable to the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Samples were analyzed for stable N isotope ratios using an Integra-CN, continuous flow, isotope ratio mass spectrometer (PDZ-Europa, Cheshire, United Kingdom). Ammonium sulfate ($\delta^{15}N = -0.4‰$), traceable to NIST, was used as an internal standard for the stable isotope measurements.

Calculations and Statistical Analysis

Results from the laboratory analysis of the asymbiotic N_2-fixation were analyzed using StatView® software (SAS Institute, Cary, NC). Amounts of N in legumes derived from the atmosphere (S_{dfa}) by N_2-fixation in each of the 24 split-plots were calculated as the product of legume N stock (g N m^{-2}) and FN_{dfa} for both *Lespedeza* and *Trifolium*. Data from the field experiment were compiled and analyzed for treatment differences using a split-plot analysis of variance (ANOVA) with a combined random and fixed effects model including the Kenward-Rogers adjustment for degrees of freedom (PROC MIXED, The SAS System, Cary, NC). Wet and dry split-plots within the main treatment combinations (ACAT, ACET, ECAT, and ECET) imposed on the open-top chambers were treated as repeated measures (that is, each chamber was a split-plot). The species composition in the constructed old-fields changed markedly from 2003 to 2005 (see Results). Therefore, the analysis of $\delta^{15}N$, FN_{dfa}, and tissue [N] was performed by year and we looked for repeatable effects of species or treatments across different years. Unless indicated otherwise, means (±SE) were used to summarize the results, statistical significance was indicated by $P \leq 0.05$, and the Scheffe's test was used in the post hoc comparison of mean values.

Results

Potential Asymbiotic N_2-fixation

Acetylene Reduction by Incubated Soils. The mean (±SE) C_2H_4 production by glucose-amended soils under anaerobic and aerobic conditions was 1,499 ± 104 and 40 ± 14 nmol g^{-1} d^{-1}, respectively, with most of the activity occurring between day 1 and day 8 of the 15-day incubations. The potential daily rate was calculated as the difference in C_2H_4 production over the first 7 days. Watering treatment significantly ($P \leq 0.05$) increased C_2H_2 reduction rates whereas [CO_2] and temperature effects were not statistically significant. Soils from the dry and wet treatment plots exhibited mean (±SE) potential C_2H_4 production rates of 1,311 ± 123 and 1,688 ± 153 nmol g^{-1} d^{-1}, respectively, in anaerobic incubations.

Under aerobic conditions, most of the C_2H_2 reduction in glucose-amended soils occurred between day 1 and day 16 of the 23-day incubations. The potential daily rate was calculated as the difference in C_2H_4 production over the first 16 days. There was measurable C_2H_2 reduction by soils amended with glucose in aerobic incubations, but no statistically significant treatment effects on the daily rate of C_2H_4 production. Acetylene reduction by glucose-amended soils in anaerobic incubations was approximately 37 times greater than that in aerobic incubations.

Both anaerobic and aerobic incubations of control soils (that is, soils amended with water) resulted in little C_2H_2 reduction over 57-day incubations. There was no detectable effect of [CO_2], temperature, or moisture regime on C_2H_4 production by control soils. Mean (±SE) total C_2H_4 production in anaerobic and aerobic incubations was 2.9 ± 0.2 and 1.3 ± 0.2 nmol g^{-1}, respectively. Mean (±SE) potential daily rates of C_2H_4 production by control soils, over 57 days, were 0.033 ± 0.003 and 0.023 ± 0.003 nmol g^{-1}, respectively, in anaerobic and aerobic incubations.

Asymbiotic $^{15}N_2$–fixation by Soils. The final atom % ^{15}N in soil was significantly greater than the initial atom % ^{15}N for soils incubated in a $^{15}N_2$-labeled atmosphere (Table 1). The watering treatment significantly ($P \leq 0.05$) increased the amount of N fixed in anaerobic incubations of soils amended with glucose. Potential N_2-fixation by soil from the wet treatment plots was 67% greater than that in the dry treatment plots. Over the duration of the 15-day incubations, the mean daily rate of N_2-fixation by soils from wet and dry moisture regimes was 20 and 12 nmol g^{-1} during the anaerobic incubation. There were no detectable differences in N_2-fixation by soils from different [CO_2] and temperature treatments.

The amount of $^{15}N_2$-fixation in aerobic incubations was consistently less than that in anaerobic incubations (Table 1). Due to a large degree of variation in measurements (0.4–424 nmol g^{-1}), the experimental treatment effects on N_2-fixation under aerobic conditions were not statistically significant. The overall mean (±SE) amount of N_2 fixed by soils amended with glucose in aerobic incubations was 47 (±17) nmol g^{-1} and the mean daily rate was 1.3 nmol g^{-1}.

The mean daily amount of $^{15}N_2$ fixed by glucose-amended soils, from wet treatment plots, under anaerobic (20 nmol g^{-1} d^{-1}) or aerobic (1.9 nmol

Table 1. Mean (±SE) N_2-fixation by Glucose-amended Soils during 15 day Anaerobic and 36 day Aerobic Incubations

Incubation	Soil moisture	mg N g^{-1} soil	Initial atom %	Final atom %	F_a	N_2-fixed (nmol g^{-1})
Anaerobic	Dry	1.97 ± 0.07	0.3679	0.4197 ± 0.0088	0.00212 ± 0.00036	178 ± 23
	Wet	1.83 ± 0.08	0.3678	0.4644 ± 0.0187	0.00395 ± 0.00076	297 ± 42
Aerobic	Dry	2.12 ± 0.07	0.3679	0.3744 ± 0.0014	0.00027 ± 0.00006	25 ± 5
	Wet	2.03 ± 0.07	0.3678	0.3893 ± 0.0116	0.00088 ± 0.00047	69 ± 33

F_a is the fraction of soil N originating from asymbiotic fixation of atmospheric $^{15}N_2$. Each mean is based on 12 measurements.

g^{-1} d^{-1}) conditions was multiplied by the mass of soil to a 12 cm sampling depth (1.8×10^5 g m^{-2}) and extrapolated to 1 year to estimate maximum annual asymbiotic N_2-fixation by soils at the study site. Resulting annual estimates for maximum N_2-fixation by soils were 36 and 3.5 g N m^{-2} y^{-1}, respectively. However, the ratio of N_2-fixation in glucose-amended to control soils was 45,424 and 1,739, respectively, in anaerobic and aerobic incubations. When the preceding factors were accounted for, the estimated maximum amount of asymbiotic N_2-fixation in unamended soils was 2.0 or less mg N m^{-2} y^{-1}. Measurements of asymbiotic N_2-fixation were discontinued after the first year of the study (2003) because the foregoing potential rate was negligible relative to symbiotic N_2-fixation rates in the constructed old-fields.

Symbiotic N_2-fixation

Greenhouse Studies. Foliar ^{15}N abundance in *Trifolium* and *Lespedeza* grown under N-free conditions in a greenhouse first declined and then leveled off after 120 days (Figure 1), indicating that the potential isotopic fractionation associated with N_2-fixation in maturing plants was −1.62 ± 0.23 and −2.72 ± 0.12‰ in *Trifolium* and *Lespedeza*, respectively. These values were used in equation (3) for the $\delta^{15}N$ value of N_2-fixing plants grown under N-free conditions.

Field Measurements of $\delta^{15}N$. All seven species were present during the 2003 growing season, but only six species were analyzed for $\delta^{15}N$ during the 2004 and 2005 growing seasons (Table 2). There were significant differences in $\delta^{15}N$ among species in 2003 ($P \leq 0.001$), 2004 ($P \leq 0.001$), and 2005 ($P \leq 0.05$). In 2003, there was a significant ($P \leq 0.05$) interaction between [CO_2] and watering regime on $\delta^{15}N$ values in *Lespedeza*. In this case, EC decreased $\delta^{15}N$ values by 0.6‰ in the wet treatment whereas $\delta^{15}N$ values were unaffected by EC in the dry treatment. In addition, there was a significant ($P \leq 0.001$) interaction among species,

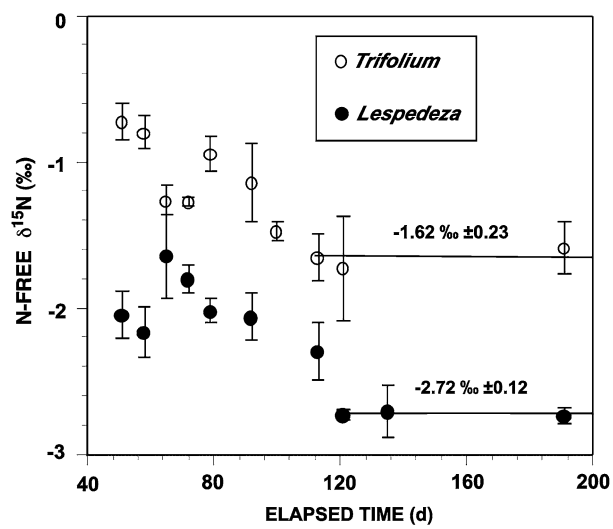

Figure 1. Mean (±SE) foliar $\delta^{15}N$ in *Trifolium* and *Lespedeza* grown under N-free conditions in a greenhouse. Isotopic fractionation associated with N_2-fixation (horizontal line for each species) was based on $\delta^{15}N$ values measured between 120 and 190 days of plant growth. Mean values were calculated based on samples of leaf tissue (≈1 dry g per pot) collected from three pots at each sampling time.

[CO_2], and watering regime in 2004. This interaction, caused by increasing $\delta^{15}N$ under EC in the dry treatment and declining $\delta^{15}N$ under EC in the wet treatment, was observed in only one species (*Dactylis*). Across all six species in 2004, ET caused a 0.6‰ decline in $\delta^{15}N$ ($P \leq 0.05$) relative to AT, and EC caused a 0.4‰ decline in $\delta^{15}N$ ($P \leq 0.05$) relative to AC. In 2005, there were no significant treatment effects or significant interactions in the analysis of plant $\delta^{15}N$ values; only species differences were statistically significant as noted above. Under field conditions, *Lespedeza* and *Trifolium* (when present) consistently had more negative $\delta^{15}N$ values than non-legumes (Table 2).

Calculated FN_{dfa}. In 2003 and 2004, ANOVA to test for the effect of species, temperature, [CO_2], and soil moisture regime indicated a complicated

Table 2. Mean (±SE) $\delta^{15}N$ (‰) in Different Plant Species in 2003, 2004, and 2005

Year	Species						
	FES	PLA	DAC	AND	SOL	TRI	LES
2003	$1.20^{a} \pm 0.20$	$1.05^{ab} \pm 0.21$	$0.92^{ab} \pm 0.17$	$0.51^{ab} \pm 0.21$	$0.30^{b} \pm 0.18$	$-1.14^{c} \pm 0.06$	$-2.14^{d} \pm 0.09$
2004	$0.33^{a} \pm 0.22$	$0.19^{a} \pm 0.19$	$1.18^{a} \pm 0.41$	$0.67^{a} \pm 0.19$	–	$-0.92^{b} \pm 0.09$	$-2.40^{c} \pm 0.14$
2005	$1.56^{a} \pm 0.35$	$1.52^{a} \pm 0.35$	$1.61^{a} \pm 0.21$	0.61 ± 0.18	$1.05^{a} \pm 0.28$	–	$-2.48^{b} \pm 0.11$

Each mean is based on 20–24 values. Means in the same row with different alphabetic superscripts are significantly different ($P \leq 0.05$). Abbreviations are as follows: FES = Festuca elatior, PLA = Plantago lanceolata, DAC = Dactylis glomerata, AND = Andropogon virginicus, SOL = Solidago canadensis, TRI = Trifolium pratense, and LES = Lespedeza cuneata. Values without alphabetic superscripts were omitted from statistical analysis due to missing values.

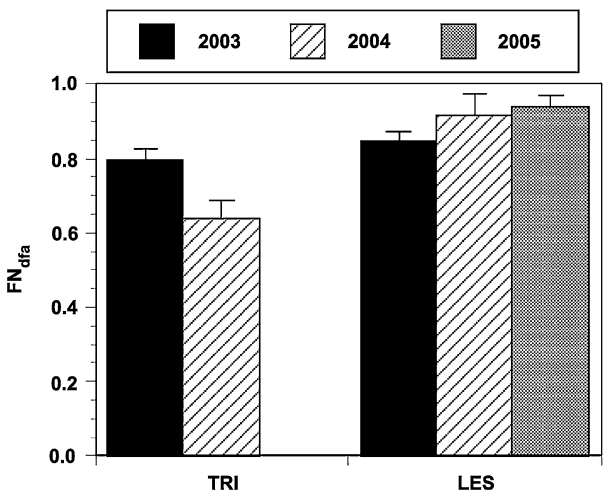

Figure 2. Mean (±SE) fraction of tissue N derived from atmospheric N_2-fixation (FN_{dfa}) by *Trifolium* (TRI) and *Lespedeza* (LES) in 2003, 2004, and 2005. Mean values are based on 23 or 24 measurements.

pattern of response for FN_{dfa}. In 2003, there was a significant ($P \leq 0.05$) interaction between [CO_2] and watering treatment as well as a significant ($P \leq 0.05$) temperature by species interaction. FN_{dfa} was greatest under elevated [CO_2] in the wet treatment. The opposite was true under ambient [CO_2] although the absolute differences were small (all mean estimates of FN_{dfa} in the comparison ranged from 0.78 to 0.85). Also, FN_{dfa} responded differently to elevated temperature in the two legumes ($P \leq 0.05$) although, again, the differences were small (<15%). The difference in FN_{dfa} between the legumes increased from 2003 to 2004, and *Lespedeza* derived significantly ($P \leq 0.05$) more tissue N from the atmosphere in 2004 than did *Trifolium* (Figure 2). There was also a significant ($P \leq 0.05$) effect of [CO_2] on FN_{dfa} in 2004 (mean ± SE FN_{dfa} under elevated and ambient [CO_2] was 0.85 ± 0.06 and 0.73 ± 0.04, respectively). In 2005, *Lespedeza* was the only legume remaining in the old-fields and ANOVA indicated no significant main effects or interactions among treatments in the analysis of FN_{dfa}. Similar to results in prior years, a relatively large proportion of plant tissue N in *Lespedeza* was derived from the atmosphere via N_2-fixation in 2005 (Figure 2).

Tissue N Concentrations. For both 2004 and 2005, analysis of variance indicated that species ($P \leq 0.001$) and [CO_2] ($P \leq 0.01$) had a significant effect on plant tissue [N]. In 2004, mean (±SE) tissue [N] was 2.23 ± 0.08 and 2.58 ± 0.10%, respectively, under elevated and ambient [CO_2]. In 2005, tissue [N] was 1.84 ± 0.064% under EC and 2.08 ± 0.072% under AC. Warming and soil moisture had no significant effect in either year. There was, however, a significant ($P \leq 0.05$) moisture × [CO_2] interaction in 2004 and a significant ($P \leq 0.05$) [CO_2] × species interaction in 2005. In the former case, the interaction between moisture and [CO_2] was caused by contrasting responses to watering regimes under AC and EC in a single species (*Dactylis*). In the latter case, reductions in tissue [N] were observed in all species under EC, except *Andropogon* and *Solidago*. Also, in 2004, statistically significant reductions in plant tissue [N] in CO_2-enriched plots were observed in all species (declines ranged from 13% to 24%), except for *Andropogon*. The latter exception gave rise to a significant ($P \leq 0.01$) species × [CO_2] interaction. A complex species × temperature × soil moisture interaction ($P \leq 0.05$) for [N] in 2005 was attributable to opposite changes in response to temperature in the dry and wet treatment plots in a single species (*Plantago*). In both 2004 and 2005, legumes and *Solidago* had the highest tissue [N] (Table 3).

Changes in Aboveground Biomass. There was a progressive increase in the relative importance of *Lespedeza* in the old-field communities from 13% of total aboveground biomass in 2003 to 46% in 2005 (Table 4). This change was accompanied by a decline in *Trifolium* biomass from 27% in 2003 to 0% in 2005. The relative contribution of *Dactylis* to total

Table 3. Mean (±SE) Foliar N Concentrations (%) in Different Plant Species in 2004 and 2005

Year	Species						
	FES	PLA	DAC	AND	SOL	TRI	LES
2004	$2.01^{ab} \pm 0.073$	$2.01^{ab} \pm 0.058$	$2.04^{b} \pm 0.063$	$1.65^{a} \pm 0.056$	$2.67^{c} \pm 0.088$	$4.03^{d} \pm 0.113$	$2.43^{c} \pm 0.098$
2005	$1.53^{b} \pm 0.073$	$2.12^{cd} \pm 0.111$	$2.03^{c} \pm 0.058$	$1.20^{a} \pm 0.044$	$2.43^{de} \pm 0.056$	–	$2.44^{e} \pm 0.074$

Each mean is based on 20–24 values. Means in the same row with different alphabetic superscripts are significantly different ($P \leq 0.05$). Abbreviations for species are listed in Table 2.

Table 4. Mean (±SE) Aboveground Biomass in Different Plant Species and Total Community Aboveground Biomass in 2003, 2004, and 2005

Year	Unit	SOL	AND	FES	PLA	LES	TRI	DAC	Total
2003	g m^{-2}	$11^{a} \pm 1.9$	$64^{a} \pm 18$	$77^{a} \pm 8.3$	$113^{ab} \pm 17$	$139^{ab} \pm 27$	$277^{bc} \pm 69$	$363^{c} \pm 32$	1056 ± 54
	Fraction*	0.01	0.06	0.07	0.11	0.13	0.26	0.34	0.98
2004	g m^{-2}	$14^{a} \pm 2.3$	$45^{ab} \pm 13$	$0.91^{a} \pm 0.23$	$6.3^{a} \pm 1.2$	$122^{c} \pm 20$	$92^{bc} \pm 28$	$119^{c} \pm 23$	400 ± 37
	Fraction*	0.04	0.11	0.00	0.02	0.31	0.23	0.30	1.01
2005	g m^{-2}	$36^{a} \pm 6.0$	$126^{a} \pm 42$	$21^{a} \pm 7.4$	$28^{a} \pm 7.5$	$275^{b} \pm 53$	0^{a}	$110^{a} \pm 17$	596 ± 64
	Fraction*	0.06	0.21	0.04	0.05	0.46	0.00	0.18	1.00

**Fraction of total aboveground biomass; the total may not add to exactly 1.00 due to rounding errors*
Each mean is based on 24 values. Means in the same row with different alphabetic superscripts are significantly different ($P \leq 0.05$). Abbreviations for species are listed in Table 2.

aboveground biomass also declined whereas two other minor non-N_2-fixing species, *Solidago* and *Andropogon*, increased in relative abundance over the same time frame (Table 4). Analysis of variance indicated a significant ($P \leq 0.001$) effect of species and a significant ($P \leq 0.05$) species × temperature interaction for measurements of aboveground biomass in the constructed old-fields in 2003 and 2004. Elevated [CO_2] had no significant effect on biomass. Biomass was greater in some species under ET, but the species affected were not consistent between different growing seasons. In 2003, the biomass of *Dactylis*, *Plantago*, and *Solidago* was greater under ET, relative to AT, and the biomass of *Trifolium*, *Andropogon* and *Lespedeza* was less under ET. In 2004, the biomass of *Andropogon*, *Lespedeza*, and *Solidago* was greater under ET, relative to AT, whereas the biomass of *Dactylis* and *Trifolium* was less under ET. In both 2004 and 2005, plant species ($P \leq 0.001$) and watering regime ($P \leq 0.05$) had a significant effect on measured aboveground biomass. In 2004, mean (±SE) biomass of the community under the dry and wet treatments was 310 ± 17 and 491 ± 63 g m^{-2}, respectively. In 2005, aboveground biomass in the dry treatment was 596 ± 64 g m^{-2} and that in the wet treatment was 757 ± 97 g m^{-2}.

Contribution of Legumes to Aboveground N Stocks. Inter-annual variation in aboveground N stocks was strongly influenced by inter-annual variation in aboveground biomass. Aboveground N stocks in the newly established old-fields in 2003 were markedly higher than those measured during the 2004 and 2005 growing season (Table 5). Nitrogen stocks were significantly different among species in 2003 ($P \leq 0.001$), 2004 ($P \leq 0.001$), and 2005 ($P \leq 0.001$). In addition, in 2003 there was a significant ($P \leq 0.05$) species × temperature interaction in the analysis of N stocks due to interspecific variation in the response of aboveground N stocks to elevated [CO_2]. In 2005, watering regime also had a significant ($P \leq 0.05$) effect on aboveground N stocks with 61% higher plant N stocks under the wet treatment. In 2003 and 2004, *Trifolium* had the greatest N stocks and this single species comprised approximately 36–41% of aboveground old-field N stocks in both years. By 2005, *Lespedeza* accounted for most (54%) of the aboveground N (Table 5). Over the 3 years of the study, legumes derived from 4.6 to 12.3 g N m^{-2} from symbiotic N_2-fixation, and N derived from the atmosphere contributed from 46% to 51% to the old-field aboveground N stock. Despite the significant effect of EC on tissue [N], there was no effect

Table 5. Mean (±SE) Aboveground N Stocks in Different Plant Species in 2003, 2004, and 2005

Nitrogen stock	Species	g N m^{-2}		
		2003	2004	2005
Total*	SOL	$0.27^{a} \pm 0.05$	$0.39^{ab} \pm 0.07$	$0.86^{a} \pm 0.14$
	AND	$0.89^{a} \pm 0.26$	$0.71^{ab} \pm 0.21$	$1.57^{a} \pm 0.57$
	FES	$1.41^{a} \pm 0.18$	$0.02^{a} \pm 0.01$	$0.32^{a} \pm 0.12$
	PLA	$2.26^{ab} \pm 0.33$	$0.12^{a} \pm 0.02$	$0.60^{a} \pm 0.17$
	LES	$3.47^{ab} \pm 0.76$	$2.95^{bc} \pm 0.51$	$6.59^{b} \pm 1.21$
	DAC	$7.40^{bc} \pm 0.66$	$2.46^{abc} \pm 0.46$	$2.24^{a} \pm 0.36$
	TRI	$10.9^{c} \pm 2.45$	$3.76^{c} \pm 1.13$	0
	All species	26.6 ± 2.0	10.4 ± 1.3	12.2 ± 1.3
N derived from the atmosphere	LES	2.94 ± 0.63	2.69 ± 0.46	6.28 ± 1.19
	TRI	9.35 ± 2.12	1.91 ± 0.51	0

Represents N derived from only soil N (non-legumes) or soil N and atmospheric N_2–fixation (legumes)
Means in the same column with different alphabetic superscripts are significantly different ($P \leq 0.05$). Each mean is based on 24 values. Abbreviations for species are listed in Table 2.

of EC (or other climate change factors) on aboveground N stocks. The absence of an effect was not attributable to a compensatory response between EC effects on [N] and biomass because there was no correlation between them.

Discussion

Our research resulted in four major findings: (1) higher soil moisture increased potential asymbiotic N_2-fixation, but the process was biologically insignificant in this system when compared to symbiotic N_2-fixation rates; (2) plant tissue [N] in legumes and some non-legumes declined significantly under elevated [CO_2], but there was no response to warming or altered soil moisture; (3) symbiotic N_2-fixation was an important process in the N budget of the constructed old-fields and contributed 44–51% of the aboveground community N stocks, and (4) significant interactions between treatment effects and plant species indicate that single-species experiments may overlook complex interactions among environmental drivers and plant community composition.

The constructed old-field communities were a good experimental system for investigating the direct and indirect effects of [CO_2] enrichment, warming, and changes in soil moisture on N_2-fixation in a dynamic, mixed, plant community. Old fields have been a model ecosystem for testing theory related to succession and community dynamics since, at least, Gleason's pioneering work (Gleason 1928). Old fields also serve as test beds for understanding the impact of multiple global change factors on community and ecosystem processes (for example, Reich and others 2004). The constructed ecosystem we investigated is typical of local old fields. For example, a recent survey of 250 1-m^2 plots across 17 old fields near the study site (Souza, unpublished data) indicated that the species included here are among the most frequently detected. *Lespedeza cuneata* is the second most common, occurring in 174 out of the 250 quadrats. In addition, it is often negatively correlated with the biomass and cover of other species especially N_2-fixing species. *Lespedeza cuneata* was recently classified as a ''Rank 1'' (Severe Threat) pest plant species by the Southeast Exotic Pest Plant Council (http://www.se-eppc.org), because it can alter the composition and structure of communities of native old-field plants (Eddy and Moore 1998; Brandon and others 2004). Thus, it is not surprising that our experiments demonstrate a potential negative effect of *L. cuneata* on other species.

Asymbiotic N_2-fixation Increased Under Elevated Soil Moisture, but Contributed Little to Ecosystem N Budgets

Elevated soil moisture increased asymbiotic N_2-fixation in anaerobic incubations of old-field soils amended with glucose. These results are consistent with other studies (Limmer and Drake 1996). Asymbiotic N_2-fixation is stimulated when a readily available C supply, like glucose, is provided for soil microbial activity (Fehr and others 1972; Limmer and Drake 1996). For unamended soils, the potential maximum rates (≤ 2 mg N m^{-2} y^{-1}) were less than reported median rates of nonalgal asymbiotic N_2-fixation (19 mg N m^{-2} y^{-1}) in grasslands (Cleveland and others 1999), however our reported rates may be somewhat underestimated due to the

presence of C_2H_2 in the incubations and the greater affinity of nitrogenase for C_2H_2 than N_2. By comparison, symbiotic N_2-fixation by legumes in the constructed old-fields was estimated to contribute 4.6–12 g N m^{-2} y^{-1} to aboveground plant N stocks. Although the latter range is beyond estimates of symbiotic N_2-fixation in natural grasslands (Cleveland and others 1999), it is substantially less than annual N_2-fixation rates reported for perennial forage legume systems (Carlsson and Huss-Danell 2003).

Symbiotic N_2-fixation was Largely Influenced by Plant Community Composition and not by Environmental Treatments

Inter-specific differences, not treatment effects, were the primary cause of variability in plant $\delta^{15}N$ values and calculations of FN_{dfa} using the ^{15}N natural abundance method. When significant interactions were found between species and treatment effects, they were usually caused by one species responding differently to a treatment than the other six species. Although elevated [CO_2] increased FN_{dfa} in some years, over the entire study legumes consistently derived most of their aboveground N content through symbiotic N_2-fixation. In all 3 years, *Lespedeza* had the lowest $\delta^{15}N$ values and consistently the $\delta^{15}N$ values for samples from the field were similar to $\delta^{15}N$ values measured in plants grown under N-free conditions in the greenhouse. Initially, in 2003, symbiotic N_2-fixation accounted for most (80%) of the aboveground N stock in *Trifolium*. However, the FN_{dfa} in *Trifolium* declined in 2004 prior to the near disappearance of this species from the constructed old-fields in 2005.

Plant tissue N concentration has important influences on many biotic and abiotic processes in terrestrial ecosystems and can be an important mediator of ecosystem responses to atmospheric and climatic change (Reich and others 2006). Plant tissue [N] responses to warming have been inconsistent in previous studies, with positive effects observed in cold ecosystems and negative effects in warm ecosystems; the inconsistencies of these observed patterns may reflect the effects of warming on soil N availability (Reich and others 2006). There were no effects of warming (or other treatments) on soil N availability in this study (Classen, unpublished data), which may explain why there was no observed foliar [N] response to warming. In contrast, tissue [N] was reduced by approximately 12–14% under elevated [CO_2]. Reductions of this magnitude in foliar [N] are a common response to elevated [CO_2] (Cotrufo and others 1998; Luo and others 2006), and they are most often associated with an increase in leaf mass per unit leaf area (in part related to carbohydrate accumulation) more than an absolute decline in leaf N content (Ainsworth and Long 2005). Consistent with previous work, total plant N content was not altered by elevated CO_2 in 2004 or 2005. Foliar [N] across the whole community declined over time (from 2.5% in 2004 to 2.1% in 2005) which can be attributed to the loss of N-rich *Trifolium* (Table 3). *Lesepedeza* also had elevated tissue [N] that was significantly greater than tissue [N] measured in non-legumes; a difference that was likely linked to *Lespedeza's* high N_2-fixing abilities (Lynd and Ansman 1993).

Community composition in the constructed old-fields changed dramatically over the first 3 years of the experiment with increasing *Lespedeza* at the expense of *Trifolium* and other non-legumes like *Dactylis* and *Plantago*. Some field experiments indicate that greater aboveground biomass and N stocks of legumes relative to non-legumes in response to elevated [CO_2] is directly attributable to symbiotic N_2-fixation (Luscher and others 2000). Caution is required when evaluating this hypothesis from the standpoint of *Lespedeza's* success in our system. *Lespedeza* is a tall-growing, invasive species that has a propensity to shade-out potential competitors on nutrient-poor soils (Brandon and others 2004). Shading effects by *Lespedeza* on *Trifolium* were probably important in the decline and virtual disappearance of *Trifoilum* from the oldfields in 2005. In addition, *Lespedeza* has been shown to inhibit the growth or development of other plant species through allelopathic effects (Kalburtji and Mosjidis 1992, 1993a, b; Kalburtji and others 2001). High levels of symbiotic N_2-fixation, shading, and potential allelopathy (primarily species specific attributes) likely acted together, and apparently independent of [CO_2], temperature, and water, as factors contributing to the success of *Lespedeza* and the increasingly important contribution of this N_2-fixing species to aboveground old-field biomass from 2003 to 2005.

High Rates of Symbiotic N_2-fixation Reduce Ecosystem-level Demands on soil N Supplies

Calculations based on FN_{dfa} indicated that both *Lespedeza* and *Trifolium* met a high proportion of their aboveground N requirements through symbiotic N_2-fixation and these results support N_2-fixation rates measured in other legumes (Gil and others 1997; Dear and others 1999; Carlsson and

Huss-Danell 2003). The soil N inputs obtained via this process can be significant to the N budget of mixed plant communities, such as oldfields. Unpublished data from this study on *Lespedeza* (labeled in 2006 by leaf feeding with 99 atom % ^{15}N-enriched urea) indicated that some transfer of ^{15}N to neighboring non-N_2-fixing plants is possible (Garten, unpublished data). Rhizodeposition of N fixed by legumes potentially benefits the entire community by increasing soil N supplies and transferring N to non-legume species in N-poor environments (Ledgard and Steele 1992; Viera-Vargas and others 1995; Soussana and Hartwig 1996; McNeill and others 1997; Høgh-Jensen and Schjoerring 2000, 2001; Mayer and others 2003). Non-legumes can also benefit from an overall lower community demand for soil N supplies when neighboring legumes derive a high proportion of their N requirements via symbiotic N_2-fixation (Temperton and others 2006). Although some non-legumes (that is, *Solidago*) appeared to have δ^{15}N values that were intermediate between N_2-fixing and reference plants, we cannot unequivocally quantify, based on the ^{15}N natural abundance method or our limited ^{15}N-tracer experiments, the importance of legume N rhizodeposition to non-N_2-fixing species in the constructed oldfields. However, the relatively high percentage of aboveground N in the old-field communities contributed by symbiotic N_2-fixation suggests that non-legume species in this experiment did benefit indirectly via reduced community demands on soil N availability.

Conclusion

Symbiotic N_2-fixation was an important process in the N budget of the constructed old-field ecosystems, contributing 44–51% to aboveground community N stocks. Asymbiotic N_2-fixation contributed much less to the old-field N budget. Symbiotic N_2-fixation was influenced more by temporal, internal changes in plant community structure than by external treatments that included changes in temperature, [CO_2], and soil moisture. Plant species effects were important and indicated that single-species experiments may overlook complex interactions among environmental factors and changing community composition. Legume N_2-fixation may have benefited non-N_2-fixing species by reducing community demands on N supplies. Nevertheless, N_2-fixation was one factor that conveyed an overall advantage to *Lespedeza* in competition with other plant species and led to a progressive increase in the importance of *Lespedeza* as a contributor to total aboveground biomass and aboveground N budgets irrespective of manipulated environmental factors. Changes in species composition over time in the old-field community had larger impacts on the N-budget than the response of N_2 fixers to elevated [CO_2], temperature, and differences in watering regime, and although seemingly unresponsive to the experimental manipulations, symbiotic N_2-fixation was a primary driver in changing old-field species composition during the 3-year experiment.

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References

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. New Phytol 165:351–71.

Athar M, Johnson DA. 1996. Nodulation, biomass production, and nitrogen fixation in alfalfa under drought. J Plant Nutr 19:185–99.

Bordeleau LM, Prevost D. 1994. Nodulation and nitrogen-fixation in extreme environments. Plant Soil 161:115–25.

Brandon AL, Gibson DJ, Middleton BA. 2004. Mechanisms for dominance in an early successional old field by the invasive non-native *Lespedeza cuneata* (Dum. Cours.) G. Don. Biol Invasions 6:483–93.

Cannell MGR, Thornley JHM. 1998. N-poor ecosystems may respond more to elevated [CO_2] than N-rich ones in the long term. A model analysis of grassland. Glob Change Biol 4:431–42.

Carlsson G, Huss-Danell K. 2003. Nitrogen fixation in perennial forage legumes in the field. Plant Soil 253:353–72.

Carranca C, Varennes AD, Rolston D. 1999. Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the ^{15}N isotope dilution technique. Eur J Agron 10:49–56.

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO, Perakis SS, Latty EF, Von Fischer JC, Elseroad A, Wasson MF. 1999. Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. Global Biogeochem Cy 13:623–45.

Cotrufo MF, Ineson P, Scott A. 1998. Elevated CO_2 reduces the nitrogen concentration of plant tissues. Glob Change Biol 4:43–54.

Dear BS, Cocks PS, Peoples MB, Swan AD, Smith AB. 1999. Nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.) growing in pure culture and in mixtures with varying densities of lucerne (*Medicago sativa* L.) or phalaris (*Phalaris aquatica* L.). Aust J Agr Res 50:1047–58.

Eddy TA, Moore CM. 1998. Effects of sericea lespedeza (*Lespedeza cuneata* (Dumont) G. Don) invasion on oak savannas in Kansas. Trans Wisconsin Acad Sci Arts Lett 86:57–62.

Fehr PI, Cho CM, Pang PC, Hedlin RA. 1972. Some factors affecting asymbiotic nitrogen fixation in soils as measured by ^{15}N enrichment. Agron J 64:251–4.

Garten CT. 1990. Multispecies methods of testing for toxicity: use of the *Rhizobium*-legume symbiosis in nitrogen fixation and correlations between responses by algae and terrestrial plants. In: Wang W, Gorsuch JW, Lower WR, Eds. Plants for toxicity assessment, ASTM STP 1091. Philadelphia: American Society for Testing and Materials. pp 69–84.

Gil JL, Guenni O, Espinoza Y. 1997. Biological N_2-fixation by three tropical forage legumes and its transfer to *Brachiaria humidicola* in mixed swards. Soil Biol Biochem 29:999–1004.

Gleason HA. 1928. The individualistic concept of the plant association. B Torrey Bot Club 53:7–26.

Hardy RWF, Burns RC, Holsten RD. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol Biochem 5:47–81.

Hardy RWF, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. Plant Physiol 43:1185–207.

Hartwig UA, Luscher A, Daepp M, Blum H, Soussana JF, Nosberger J. 2000. Due to symbiotic N_2-fixation, five years of elevated atmospheric pCO_2 had no effect on the N concentration of plant litter in fertile, mixed grassland. Plant Soil 224:43–50.

Høgh-Jensen H, Schjoerring JK. 1997. Effects of drought and inorganic N form on nitrogen fixation and carbon isotope discrimination in *Trifolium repens*. Plant Physiol Bioch 35:55–62.

Høgh-Jensen H, Schjoerring JK. 2000. Below-ground nitrogen transfer between different grassland species: direct quantification by ^{15}N leaf feeding compared with indirect dilution of soil ^{15}N. Plant Soil 227:171–83.

Høgh-Jensen H, Schjoerring JK. 2001. Rhizodeposition of nitrogen by red clover, white clover and ryegrass leys. Soil Biol Biochem 33:439–48.

Johnston JW, Shriner DS. 1986. Yield response of Davis soybeans to simulated acid rain and gaseous pollutants in the field. New Phytol 103:695–707.

Kalburtji KL, Mosjidis JA. 1992. Effects of sericea lespedeza residues on warm-season grasses. J Range Manage 45:441–4.

Kalburtji KL, Mosjidis JA. 1993. Effects of sericea lespedeza root exudates on some perennial grasses. J Range Manage 46:312–5.

Kalburtji KL, Mosjidis JA. 1993. Effects of sericea lespedeza residues on cool-season grasses. J Range Mange 46:315–9.

Kalburtji KL, Mosjidis JA, Mamolos AP. 2001. Allelopathic plants. 2. *Lespedeza cuneata*. Allelopathy J 8:41–9.

Kolb KJ, Evans RD. 2002. Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues. New Phytol 156:57–64.

Kurdali F, Al-Ain F, Al-Shamma M. 2002. Nodulation, dry matter production, and N_2-fixation by fababean and chickpea as affected by soil moisture and potassium fertilizer. J Plant Nutr 25:355–68.

Ledgard SF, Steele KW. 1992. Biological nitrogen-fixation in mixed legume grass pastures. Plant Soil 141:137–53.

Lilley JM, Bolger TP, Peoples MB, Gifford RM. 2001. Nutritive value and the nitrogen dynamics of *Trifolium subterraneum* and *Phalaris aquatica* under warmer, high CO_2 conditions. New Phytol 150:385–95.

Limmer C, Drake HL. 1996. Non-symbiotic N_2-fixation in acidic and pH-neutral forest soils: aerobic and anaerobic differentials. Soil Biol Biochem 28:177–183.

Luo YQ, Hui DF, Zhang DQ. 2006. Elevated CO_2 stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. Ecology 87:53–63.

Luo YQ, Mooney HA. 1995. Long-term CO_2 stimulation of carbon influx into global terrestrial ecosystems: issues and approaches. J Biogeogr 22:797–803.

Luscher A, Hartwig UA, Suter D, Nosberger J. 2000. Direct evidence that symbiotic N_2-fixation in fertile grassland is an important trait for a strong response of plants to elevated atmospheric CO_2. Glob Change Biol 6:655–62.

Lynd JQ, Ansman TR. 1993. Symbiotic tripartite nitrogen-fixation effectual in eroded soil restoration with 20 year-age *Sericea lespedeza*. J Plant Nutr 16:149–64.

Mayer J, Buegger F, Jensen ES, Schloter M, Heb J. 2003. Estimating N rhizodeposition of grain legumes using a ^{15}N *in situ* stem labelling method. Soil Biol Biochem 35:21–8.

McNeill AM, Zhu CY, Fillery IRP. 1997. Use of *in situ* ^{15}N-labeling to estimate the total belowground nitrogen of pasture legumes in intact soil-plant systems. Aust J Agr Res 48:295–304.

McWilliam JR. 1978. Response of pasture plants to temperature. In: Wilson JR, Eds. Plant relations in Pastures. Australia: Commonwealth Scientific and Industrial Research Organization, East Melbourne. pp 17–34.

Norby RJ, Edwards NT, Riggs JS, Abner CH, Wullschleger SD, Gunderson CA. 1997. Temperature-controlled open-top chambers for global change research. Glob Change Biol 3:259–67.

Ohashi Y, Saneoka H, Matsumoto K, Ogata S, Premachandra GS, Fujita K. 1999. Comparison of water stress effects on growth, leaf water status, and nitrogen fixation activity in tropical pasture legumes *Siratro* and *Desmodium* with soybean. Soil Sci Plant Nutr 45:795–802.

Reich PB, Hungate BA, Luo YQ. 2006. Carbon-nitrogen interactions in terrestrial ecosystems in response to rising atmospheric carbon dioxide. Annl Rev Ecol Evol Syst 37:611–36.

Reich PB, Tilman D, Naeem S, Ellsworth D, Knops J, Craine J, Wedin D, Trost J. 2004. Species and functional diversity independently influence biomass accumulation and its response to CO_2 and N. Proc Natl Acad Sci USA 101:10101–6.

Serraj R, Sinclair TR. 1997. Variation among soybean cultivars in dinitrogen fixation response to drought. Agron J 89:963–9.

Serraj R, Sinclair TR, Purcell LC. 1999. Symbiotic N_2-fixation response to drought. J Exp Bot 50:143–55.

Serraj R, Vadez V, Sinclair TR. 2001. Feedback regulation of symbiotic N_2-fixation under drought stress. Agronomie 21:621–6.

Shearer G, Kohl DH. 1986. N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Aust J Plant Physiol 13:699–756.

Sinclair TR, Serraj R. 1995. Legume nitrogen-fixation and drought. Nature 378:344 .

Soussana JF, Hartwig UA. 1996. The effects of elevated CO_2 on symbiotic N_2-fixation: a link between the carbon and nitrogen cycles in grassland ecosystems. Plant Soil 187:321–32.

Temperton VM, Mwangi PN, Scherer-Lorenzen M, Schmid B, Buchmann N. 2006. Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. Oecologia 151:190–205.

Viera-Vargas MS, Souto CM, Urquiaga S, Boddey RM. 1995. Quantification of the contribution of N_2-fixation to tropical forage legumes and transfer to associated grass. Soil Biol Biochem 27:1193–200.

Wan S, Norby RJ, Ledford RJ, Weltzin JF. 2007. Responses of soil respiration to elevated CO_2, air warming, and changing soil water availability in a model old-field grassland. Glob Change Biol 1 13:1–14.

Zanetti S, Hartwig UA. 1997. Symbiotic N_2-fixation increases under elevated atmospheric pCO_2 in the field. Acta Oecol 18:285–90.

Zanetti S, Hartwig UA, Van Kessel C, Luscher A, Hebeisen T, Frehner M, Fischer BU, Hendrey GR, Blum H, Nosberger J. 1997. Does nitrogen nutrition restrict the CO_2 response of fertile grassland lacking legumes?. Oecologia 112:17–25.