Maintenance of leaf N controls the photosynthetic CO$_2$ response of grassland species exposed to 9 years of free-air CO$_2$ enrichment

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Abstract
Determining underlying physiological patterns governing plant productivity and diversity in grasslands are critical to evaluate species responses to future environmental conditions of elevated CO$_2$ and nitrogen (N) deposition. In a 9-year experiment, N was added to monocultures of seven C$_3$ grassland species exposed to elevated atmospheric CO$_2$ (560 μmol CO$_2$ mol$^{-1}$) to evaluate how N addition affects CO$_2$ responsiveness in species of contrasting functional groups. Functional groups differed in their responses to elevated CO$_2$ and N treatments. Forb species exhibited strong down-regulation of leaf N mass concentrations (−26%) and photosynthetic capacity (−28%) in response to elevated CO$_2$, especially at high N supply, whereas C$_3$ grasses did not. Hence, achieved photosynthetic performance was markedly enhanced for C$_3$ grasses (+68%) in elevated CO$_2$, but not significantly for forbs. Differences in access to soil resources between forbs and grasses may distinguish their responses to elevated CO$_2$ and N addition. Forbs had lesser root biomass, a lower distribution of biomass to roots, and lower specific root length than grasses. Maintenance of leaf N, possibly through increased root foraging in this nutrient-poor grassland, was necessary to sustain stimulation of photosynthesis under long-term elevated CO$_2$. Dilution of leaf N and associated photosynthetic down-regulation in forbs under elevated [CO$_2$], relative to the C$_3$ grasses, illustrates the potential for shifts in species composition and diversity in grassland ecosystems that have significant forb and grass components.

Keywords: C$_3$ grass species, carboxylation rate, FACE, free-air CO$_2$, Nitrogen, photosynthesis, species functional groups

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Introduction
With increasing CO$_2$ emissions from human activities driving increases in mean global atmospheric [CO$_2$], there are concerns over the capacity of natural ecosystems to continue to serve as sinks for atmospheric CO$_2$ over decades to come (Canadell et al., 2007). During the 20$^{th}$ century, the sink capacity of native grasslands was related to climate, atmospheric CO$_2$, and nitrogen (N) deposition, but our understanding of the interactions among these factors and the mechanisms driving these interactions remains incomplete (Schimel et al., 2001). Because ecosystem C and N cycles are strongly coupled, interactive effects of elevated CO$_2$ and N availability are likely, potentially reducing the magnitude of photosynthetic enhancement under elevated CO$_2$ (McMurtrie & Comins, 1996; Rastetter et al., 1997; Luo et al., 2004). In addition, species-specific responses to environmental conditions influence the rate of C and N cycling in ecosystems and interactions between species and elevated CO$_2$ or N addition. Long-term field experiments in which multiple factors are manipulated simultaneously therefore provide an important tool for untangling ecological interactions (Hunter, 2001; Mikkelsen et al., 2008). Moreover, it is important to link...
physiological responses to whole plant biomass accumulation to understand the underlying controls and their effect on plant productivity in ecosystems under expected climatic and atmospheric change (Körner, 2003).

Plant N stocks and photosynthesis-leaf N relationships couple ecosystem C and N cycles (Lee et al., 2001, 2003; Ellsworth et al., 2004). Long-term elevated CO\(_2\) typically causes a reduction in leaf N (Yin, 2002; Ainsworth & Long, 2005) and hence potentially in plant productivity per unit C availability, particularly when root N uptake is not enhanced to support increased growth demands in elevated CO\(_2\) (Field et al., 1992; Luo et al., 1994). In contrast to the reduction of leaf N when [CO\(_2\)] is enriched, increased N supply to soils would be expected to increase leaf N (Field et al., 1992). Thus, leaf N and its impacts on leaf physiology can help us better understand the interactions between N availability and atmospheric CO\(_2\) concentration that are critical to predicting how plant productivity and diversity are affected in an increasingly eutrophic biosphere (Vitousek, 1994).

Plant species can vary in their divergent responses to environmental change, including rising atmospheric [CO\(_2\)] and N addition (Zanetti et al., 1997; Joel et al., 2001; Lee et al., 2001; Poorter & Perez-Soba, 2001; Reich et al., 2004). Many grassland studies have found that increased plant growth under elevated CO\(_2\) can only be sustained with sufficient N supply (Grünzweig & Körner, 2003; Lüscher et al., 2004; Schneider et al., 2004; Dukes et al., 2005; Reich et al., 2006a). However, such a response may not typify all species or functional groups (Poorter & Navas, 2003). Several studies have shown that forbs might be more sensitive to elevated CO\(_2\) than other functional groups (Reich et al., 2001b; Teyssonneraye et al., 2002; Polley et al., 2003). Recent reports have shown that C\(_3\) forbs in grasslands are negatively impacted by increased N deposition (Zavaleta et al., 2003b; Stevens et al., 2006; Clark & Tilman, 2008) but it is unclear how or why this functional group responds differently than other functional groups. A negative response to N addition in C\(_3\) forbs could be related to water availability (Morgan et al., 2004), competition for light (Mohan et al., 2007) or soil resources, altered soil processes (Niklaus et al., 2003; West et al., 2006) or altered allocation between plant C pools (Poorter, 1993). Differential responses to elevated CO\(_2\) and N deposition among plant groups can lead to changes in species composition and diversity, and the structure and function of ecosystems (Potvin et al., 2007).

Grasslands constitute 40% of global land area (Morgan et al., 2007) and are often relatively species-rich. Broad functional groups could be useful for capturing the aggregated responses of different types of species and their responses to changing environmental conditions (Zavaleta et al., 2003a). It has been hypothesized that a number of intrinsic physiological leaf traits, such as photosynthetic rates, specific leaf area (SLA) and foliar nitrogen, central to how species functional groups are depicted, also determine the response of species to elevated [CO\(_2\)] (Woodward & Cramer, 1996; Lavorel et al., 1997). These key functional traits shared by species in functional groups could be represented in models predicting community responses to environmental change (Suding et al., 2008).

This study aims to provide insight into mechanisms that drive species responses to atmospheric change for two contrasting functional groups under a combination of elevated CO\(_2\) and N addition. This is particularly important in light of recent reports of species losses in grassland ecosystems under climatic and atmospheric change (Joel et al., 2001; Zavaleta et al., 2003a; Suding et al., 2005). Our goal was to examine how trait differences between C\(_3\) forbs and C\(_3\) grasses yield different responses to the combination of elevated CO\(_2\) and N addition. We investigated physiological mechanisms underlying species responses to both elevated CO\(_2\) and N deposition, and the multiple interactions between these environmental factors and species. Functional group responses were also examined to assess whether these groupings could represent species responses within their respective functional group. We studied C\(_3\) grass and forb species across the sixth to ninth years of elevated [CO\(_2\)] exposure and chronically low levels of N addition in a nutrient-poor prairie grassland in Minnesota, USA to address the following hypotheses:

H.1. Long-term reductions in foliar N under elevated CO\(_2\) are reflected in declining photosynthetic capacity such that the instantaneous CO\(_2\) enhancement effect is offset by photosynthetic down-regulation. This would result in little or no enhancement of realized photosynthetic rates in elevated CO\(_2\) in a nutrient-poor grassland.

H.2. Nutrient addition can compensate for reduced foliar N under elevated CO\(_2\) such that photosynthetic capacity of C\(_3\) grassland species remains unchanged or increased with CO\(_2\) enrichment.

We examined these hypotheses for multiple C\(_3\) grassland species in a long-term grassland free-air CO\(_2\) enrichment (FACE) experiment where atmospheric [CO\(_2\)] and soil N were manipulated (Reich et al., 2001a). Collectively, these hypotheses are used to explain differences in species performance in elevated CO\(_2\) and N addition, and test for commonality of species and functional group responses, thereby
improving our capacity to generalize global change responses in grassland ecosystems.

Materials and methods

Site description and experimental design

The BioCON (Biodiversity, CO₂ and N) FACE experiment is part of the U.S. National Science Foundation Long-term Ecological Research network and is located in central Minnesota, USA (45°24′13.5″N, 93°11′08″W). The site is located in a humid continental climate on glacial outwash comprised of loamy sand soils with low nutrient availability (Grigal et al., 1976). The mean annual precipitation is 660 mm yr⁻¹ and the mean maximum July temperature is 28.3 °C.

The BioCON FACE experiment consists of six circular plots of 20 m diameter, three of which control atmospheric [CO₂] to 560 μmol mol⁻¹ while three plots remain at ambient [CO₂]. Daytime exposure of plots to elevated [CO₂] proceeds continuously from the beginning of the growing season in April until the end of the growing season in October. One-minute average [CO₂] in FACE rings were within 10% of the target concentration >95% of the time during the years of this study. The plants were planted in 1997, with the first season of CO₂ fumigation in 1998.

A subset of plots from the complete FACE experiment (see Reich et al., 2001a, b) was used for the analyses here, specifically the 56 2 m × 2 m plots within the six FACE rings with monocultures of our seven target C₃ grass or nonleguminous forb species. Monocultures were used to assess species responses rather than mixtures since the emphasis was on independent species responses to the treatment factors. Among these plots, soil N addition treatments had been randomly assigned in two replicates in a split-plot design since the start of the experiment in 1998. N addition consisted of 4 g N m⁻² yr⁻¹ in the form of solid ammonium nitrate applied each year across May, June and July. There were eight monoculture subplots of each of the seven species equally divided across the four combinations of CO₂ and N-addition treatments. Above-, belowground and total biomass of these plots were determined each year in June by harvest of a subsample area of the main plot (Reich et al., 2001b). Belowground harvests were conducted by means of three 5-cm diameter cores to 20 cm depth. Fine roots were defined as <2 mm diameter and were separated manually from the larger roots.

The species chosen for this study were four C₃ grasses: Poa pratens L., Koeleria cristata Pers., Bromus inermis Leyss. and Agropyron repens L. and three forb species: Solidago rigida L. and Anemone cylindrica A. Gray and Achillea millefolium L. These species are referred to in figures by a combination of the first three letters of the genus and the first two letters of the species name.

Gas exchange and leaf nitrogen

Measurements in this study were made during the sixth through ninth growing seasons of the experiment (2003–2006) to assess the long-term effects of elevated CO₂ and nitrogen additions and potential interactions between them. Species composition, biomass and physiological responses to CO₂ and N were relatively stable at this stage of the experiment. Gas exchange measurements were conducted with a portable infrared gas analyzer system (LiCOR 6400, Li-Cor Inc., Lincoln NE, USA) during the main portion of the season when each species was active (May–June of each growing season). To assess instantaneous and long-term (up to 9 years) effects of elevated CO₂ on photosynthetic capacity, photosynthetic CO₂ response curves (A–Cᵢ) were measured on leaves of each plant species with a minimum of seven different CO₂ concentrations between 60 and 1500 μmol mol⁻¹, using saturating light conditions (photon flux density of 1800 μmol m⁻² s⁻¹) and controlled temperatures (leaf temperatures of 28–30 °C) in the leaf cuvette. Per species, plants in monoculture plots were measured with two replicates for each CO₂ and N treatment. All grass measurements were from the top-most fully expanded leaf adjacent to the flag leaf to ensure similar leaf ages. Leaves were collected and placed on ice after each A–Cᵢ response curve to determine projected leaf area in the chamber (IMAGE J v1.37, National Institutes of Health, Bethesda, MD, USA). In the laboratory, leaves were dried at 70 °C, weighed, and finely ground. A subsample was analyzed for total nitrogen and carbon content using an elemental analyzer (Carlo-Erba Strumentazione, Milan, Italy) with appropriate reference standards for herbaceous leaves in each analysis run (National Institute of Standards and Technology, Boulder, CO, USA).

Physiological variables were fitted from the A–Cᵢ response curves using the Farquhar photosynthesis model (Farquhar et al., 1980) according to the procedure laid out in Ellsworth et al. (2004). To evaluate changes in photosynthetic capacity and assess potential down-regulation of photosynthesis, we analyzed the variables maximum carboxylation rate (Vₕmax) and the maximum electron transport rate (Jₕmax) as well as the measured net photosynthesis in current growth conditions (either ambient or elevated [CO₂]) (Aₙet) and net photosynthesis at a common CO₂ level of 365 μmol mol⁻¹. Comparing photosynthesis at a common measurement CO₂ level allows for assessing the long-term effects of elevated [CO₂] and N on intrinsic photosynthetic capacity.
(Lee et al., 2001; Ellsworth et al., 2004; Ainsworth & Rogers, 2007). Net photosynthesis at a common CO₂ level was analyzed both on a mass basis (Aₘ₃₆₅) and area basis (Aₙ₃₆₅) concurrent with leaf N expressed on a mass basis (Nₘ₃₆₅) and on an area basis (Nₐ₃₆₅). A slight increase in LMA (leaf mass per area) was observed in elevated CO₂ (P = 0.07). Despite this, results were generally similar whether expressed on mass or area bases. We also analyzed net photosynthesis at a common CO₂ level of 560 μmol mol⁻¹ corresponding to the CO₂ concentration of the elevated CO₂ treatment, however, those results strongly paralleled the results for Aₘ₃₆₅ and hence are not shown. These variables help to evaluate the basic physiological mechanisms behind changes in plant growth and productivity in long-term elevated CO₂ and N addition, and facilitate the comparison of those mechanisms in different C₃ species.

### Statistical analyses

Since we are interested in the long-term effects of elevated CO₂ and N addition rather than interannual variation, species averages were from photosynthetic CO₂ response curves conducted across the four years when biomass responses to treatments were constant. Averaging across years resulted in similar sample sizes for each species per treatment combination, and represented average responses of each species to long-term elevated CO₂ and N addition. Seasonal variation was minimized because the same species were measured during the same time of the year across years, just before when species achieved peak biomass. Year effects were tested via a full factorial three-way ANOVA using [CO₂], N and year as main effects. There was no significant year effect for any variables of interest or any significant interactions of [CO₂] and N with year. All further analyses of variance described below were conducted on variables averaged across years by species, plot, CO₂ and N treatment.

The BioCON experiment was designed as a split-plot with N addition nested within atmospheric CO₂ treatment (Reich et al., 2001b). Treatment effects were assessed using the appropriate whole-plot random effect of atmospheric CO₂ or within-plot error variances against the residual error in the F-test. The whole-plot random effect was not significant (P > 0.1) in any case. Since our goal was to investigate species within functional groups responses to the experimental treatments, as well as responses of functional groups themselves, we conducted ANOVA using main effects CO₂ level, N level, and Functional group and Species identity within functional group [denoted Spp(FunctGr)] in Tables 1 and 2 to test for effects and interactions in the experiment (Table 1). The statistical significance of the functional group factor as well as the interactions involving this factor were assessed using the Spp(FunctGr) term in the denominator of the F-test. Post-hoc Tukey’s tests were used to examine differences among the different species. Because species responded differently to elevated CO₂ (Table 1), we further analyzed differences

### Table 1  
_P*-values, whole-model error mean squares (MS) and goodness of fit for an ANOVA with CO₂ treatment (CO₂), N addition treatment (N), Functional Group (Funct gr) and species within functional group [Spp(FunctGr)] as main effects, including degrees of freedom (df), for the following variables: maximum carboxylation rate (Vₙ₃₆₅), maximum electron transport rate (lₙ₃₆₅), net photosynthesis in respective growth conditions e.g., either ambient or elevated [CO₂] (Aₙ₃₆₅), net photosynthesis at a common CO₂ level of 365 μmol mol⁻¹ on an area basis (Aₙ₃₆₅) and mass basis (Aₘ₃₆₅) and foliage N on a mass basis (Nₘ₃₆₅) and area basis (Nₐ₃₆₅)_

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Vₙ₃₆₅</th>
<th>lₙ₃₆₅</th>
<th>Aₙ₃₆₅</th>
<th>Aₘ₃₆₅</th>
<th>Nₘ₃₆₅</th>
<th>Nₐ₃₆₅</th>
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<tr>
<td>CO₂</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>–</td>
<td>&lt;0.0001</td>
<td>–</td>
<td>&lt;0.0001</td>
<td>–</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>0.016</td>
<td>0.023</td>
<td>0.067</td>
<td>–</td>
<td>–</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CO₂ × N</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>0.043</td>
<td>0.001</td>
<td>0.021</td>
<td>–</td>
</tr>
<tr>
<td>Funct gr</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.042</td>
<td>–</td>
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<tr>
<td>Spp (Funct gr)</td>
<td>5</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.003</td>
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<tr>
<td>CO₂ × Funct gr</td>
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<td>0.011</td>
<td>0.068</td>
<td>0.001</td>
<td>0.006</td>
<td>0.006</td>
<td>0.010</td>
</tr>
<tr>
<td>N × Funct gr</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.015</td>
<td>–</td>
</tr>
<tr>
<td>CO₂ × Spp(Funct gr)</td>
<td>5</td>
<td>0.039</td>
<td>0.060</td>
<td>–</td>
<td>–</td>
<td>0.021</td>
<td>0.005</td>
</tr>
<tr>
<td>N × Spp(Funct gr)</td>
<td>5</td>
<td>0.016</td>
<td>0.008</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.008</td>
</tr>
<tr>
<td>CO₂ × N × Spp(Funct gr)</td>
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<td>0.022</td>
<td>0.016</td>
<td>0.081</td>
<td>0.059</td>
<td>0.001</td>
<td>–</td>
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<td>Error MS</td>
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<td>128.0</td>
<td>398.7</td>
<td>8.69</td>
<td>7.44</td>
<td>1465.0</td>
<td>3.02</td>
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<tr>
<td>Whole model R²</td>
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<td>0.73</td>
<td>0.81</td>
<td>0.69</td>
<td>0.81</td>
<td>0.85</td>
<td>0.84</td>
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</table>

1. Denotes that results were not significant (P > 0.1).
2. Transformation used to meet normality assumption: Log(Nₐ₃₆₅−0.2).

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between ambient and elevated CO₂ for each species separately, including the whole-plot random effect.

Species and functional group responses to elevated CO₂ were further explored for N-addition plots because there were significant CO₂ × N interactions (alone or in combination with functional group) for all key metrics (see Table 1), with the largest differences in responses seen in the N-addition plots. All statistical analyses were conducted in 
JMP 5.0.1 software, SAS Institute, Cary, NC, USA.

Results
Since the BioCON FACE experiment was designed with [CO₂] and N as the two central experimentally manipulated factors, we first focus on the main and interactive effects of these factors. We then present species and functional group effects as well as higher-order interactions of elevated CO₂ and N with these factors.

Effects of elevated CO₂ and N treatments on leaf nitrogen and photosynthesis across species
A number of photosynthetic and nitrogen-related traits varied significantly with CO₂ treatment, N addition treatment and their interaction across all seven grassland species (Table 1). As expected, foliar N concentration was increased 23% with N addition across all species (both area- and mass-based N, *P* < 0.0001, Table 1). Long-term elevated CO₂ exposure significantly decreased foliar N on a mass basis (−11%, *P* < 0.0001, Table 1) more than on an area basis (N.S. in Table 1). However, there was a significant CO₂ × N interaction in N_{mass} (*P* = 0.021, Table 1) because there was a much larger decline in N_{mass} due to elevated CO₂ treatment at high than at low N levels. There were similar trends for N_{area} to those for N_{mass}, but CO₂ × N was not statistically significant for this parameter (*P* > 0.10, Fig. 1b).

With a +200 μmol mol⁻¹ enrichment in [CO₂], there was a significant enhancement in realized net photosynthesis (+41% response in A_{net}, *P* < 0.0001, Table 1) across all species and functional groups. In contrast, net photosynthesis in growth [CO₂] conditions (A_{net}) responded weakly to N addition (+8%, *P* = 0.067, Table 1). There was no significant CO₂ × N interaction for A_{net} across species. The long-term CO₂ treatment had no significant main effect on photosynthesis at a common CO₂ level (A_{365}), but showed a significant CO₂ × N interaction (Table 1; Fig. 1c and d). Both area-based (A_{a365}) and mass-based (A_{m365}) photosynthesis at a common measurement [CO₂] showed CO₂ treatment-induced down-regulation under added N but not under ambient N.

As with A_{a365} and A_{m365}, V_{cmax} and J_{max} did not differ significantly between CO₂ treatments when pooled across the different species (Table 1), in these cases because species or functional groups differed in their response to elevated CO₂ or elevated CO₂ and N. Both V_{cmax} and J_{max} increased significantly with N

![Figure 1](https://example.com/figure1.png)

**Fig. 1** Effects of elevated CO₂ and N addition treatments on (a) foliar N on a mass basis (N_{mass}), (b) foliar N on an area basis (N_{area}), (c) photosynthesis at a common CO₂ level on a mass basis, A_{m365} and (d), photosynthesis at a common CO₂ level on an area basis, A_{a365} across seven grassland species. Means in ambient CO₂ (open bars) and elevated CO₂ treatment (black bars) are shown. 0N denotes unamended plots and +N denotes N-addition plots. Sample sizes associated with the means and standard error bars in this figure varied between 6 and 8. **Represents a significant *t*-test within N-treatment of *P* < 0.01.
addition (+11% and +10%, respectively, Table 1) across species.

Species effects and higher-order interactions

Species differed significantly in all measures of photosynthetic capacity and leaf N ($P \leq 0.003$, Table 1). All grass species had higher $N_{mass}$ values than forb species, resulting in a significant functional group difference ($P = 0.042$). Across CO$_2$ and N treatments, species ranked similarly in $V_{cmax}$, $J_{max}$, $A_{m365}$ and $A_{net}$. $S$. rigida had consistently the highest photosynthetic capacity, and $A$. inermis and $P$. pratensis always represented the lowest three values (in descending order).

For some variables, there were significant treatment $\times$ species interactions (Table 1). There were several CO$_2$ $\times$ N species interactions, because only $S$. and $B$. showed significantly reduced $V_{cmax}$, $J_{max}$ or $N_{mass}$ in elevated CO$_2$. The same physiological variables also showed significant N $\times$ Species interactions (Table 1). Species consistently responded to N addition with a significant increase in $N_{mass}$ (11–45% increase, $P < 0.04$), except $A$. For $V_{cmax}$ and $J_{max}$ only $P$. and $A$. showed a significant increase with N-addition.

These species differences were often consistent with functional group differences in response to elevated CO$_2$. Forbs reduced photosynthetic capacity and leaf N in elevated CO$_2$ by at least 15% ($P = 0.042$), whereas grasses did not show significant reductions in elevated CO$_2$. Moreover, there were significant three-way interactions of CO$_2$ $\times$ N $\times$ Functional group for variables reflecting photosynthetic capacity: $V_{cmax}$ ($P = 0.022$), $J_{max}$ ($P = 0.016$), $A_{m365}$ ($P = 0.001$) and $A_{a365}$ ($P = 0.059$) (Table 1). These measures of photosynthetic capacity showed significantly reduced $V_{cmax}$, $J_{max}$ or $N_{mass}$ in elevated CO$_2$.

![Fig. 2](image-url)

Fig. 2 Species-specific responses and standard error bars (left panels) to elevated CO$_2$ in N-addition plots for maximum carboxylation rate, $V_{cmax}$ (a, b) and maximum electron transport rate, $J_{max}$ (c, d). The aggregate functional groups responses of $V_{cmax}$ and $J_{max}$ to elevated CO$_2$ are shown at right in (b) and (d). Open bars represent the ambient CO$_2$ treatment and black bars are the elevated CO$_2$ treatment. Significant differences between CO$_2$ treatments within each species or functional group are represented by *$P < 0.1$, **$P < 0.05$, and ***$P < 0.01$. Samples sizes ranged from one to three for species effects (a, c) and six to eight for functional group effects (b, d).
were generally reduced by elevated CO$_2$ more in forbs than grasses (Table 1, two-way interactions). Moreover, these reductions were more pronounced in N addition treatments (Table 1: three-way interactions, Fig. S1 in Supporting information), and hence were examined in more detail. Also, examining the CO$_2$ responses of different functional groups in the N-addition plots provides insight into CO$_2$ × functional group interactions that are difficult to visualize as three-way interactions with N.

**Elevated CO$_2$ responses of functional groups under N addition**

Under added N conditions, the response of photosynthetic capacity to elevated CO$_2$ varied among functional groups (significant CO$_2$ × Functional group interactions). The three variables that best reflect photosynthetic capacity ($V_{\text{cmax}}$, $I_{\text{max}}$ and $A_{\text{m30}}$) were all reduced in all forb species in response to elevated CO$_2$ (by >25%), whereas grasses showed no change in these variables in elevated CO$_2$ (Fig. 2). These effects were generally consistent among species within each group (Fig. 2a) and hence represent functional group differences (Fig. 2b and d). *Poa, Koeleria* and *Agropyron* did not show down-regulation of photosynthetic capacity in elevated CO$_2$ ($V_{\text{cmax}} = +39\%$, $+2\%$ and $+4\%$ enhancement respectively) under N addition (Fig. 2a) while *Bromus* showed a $-10\%$ change in $V_{\text{cmax}}$ (Fig. 2a).

In contrast, *Achillea*, *Anemone* and *Solidago* all had lower $V_{\text{cmax}}$ in elevated CO$_2$ ($P<0.1$ across all forb species; $-33\%$, $-21\%$, $-30\%$, respectively) in N addition plots.
(Fig. 2a). In the N-enriched treatment, the larger magnitude of down-regulation in forbs vs. grasses resulted in no significant enhancement of net photosynthesis in elevated CO\textsubscript{2} for forbs in contrast to a sizeable enhancement for the grasses (Fig. 3b).

We found similar trends in \textit{N}\textsubscript{mass} to those for photosynthetic capacity. A significant \textit{CO\textsubscript{2}} × functional group interaction on leaf \textit{N}\textsubscript{mass} (\textit{P} = 0.01) showed that elevated CO\textsubscript{2} negatively affected the leaf N concentration in forbs but not in grasses (Table 1). Under added N, \textit{N}\textsubscript{mass} in forb leaves was 26% lower in elevated CO\textsubscript{2} (\textit{P} = 0.0004) compared with ambient CO\textsubscript{2} (range among species within this group of −18% to −35%; Fig. 3c and d). In contrast, there was no consistent CO\textsubscript{2} effect on \textit{N}\textsubscript{mass} among grass species (Fig. 3c and d), though \textit{Bromus} did in fact show a decrease of 19% (\textit{P} = 0.013). Thus, leaf N concentrations were reduced strongly in forbs when exposed to elevated CO\textsubscript{2}, whereas grasses were more generally able to maintain leaf N concentrations in elevated CO\textsubscript{2}.

\textbf{Root biomass allocation patterns in forb and grass species}

In an attempt to gain perspective on the observed elevated CO\textsubscript{2} responses in N-addition plots, we examined biomass allocation patterns in the seven species in this study because non N-fixing species gain most required N from the soil. Root mass fraction (total root biomass/total biomass) was calculated for each species and treatment across the sixth to ninth growing season of elevated CO\textsubscript{2} exposure (Table 2). Although there were no significant CO\textsubscript{2} treatment differences in total root biomass, fine root biomass and root mass fraction, all increased in N-addition plots compared with un-amended plots across species (\textit{P} = 0.0009, 0.0008 and 0.046, respectively, Table 2). In addition, there was a strong functional group effect in which \textit{C}\textsubscript{3} grasses showed higher total root biomass (Fig. 4a, \textit{P} = 0.028, Table 2) and fine root biomass (\textit{P} = 0.050, Fig. 4b) compared with the forb species, and this was especially pronounced in N-addition plots (Table 2, Fig. 4a and b). Root mass fraction also showed a significant interaction between elevated CO\textsubscript{2} and functional group (\textit{P} = 0.027, Table 2). Whereas root mass fraction was no different between forbs and grasses in ambient CO\textsubscript{2}, forbs decreased root mass fraction significantly (−28%) in response to elevated CO\textsubscript{2} and grasses did not (Fig. 4c). Grasses always had higher fractional distribution of biomass to roots compared with forbs (Fig. 4d), and these patterns remained regardless of elevated CO\textsubscript{2} treatments or N addition.

\textbf{Discussion}

We investigated differential responses in photosynthetic capacity of \textit{C}\textsubscript{3} species from two grassland functional groups to elevated atmospheric CO\textsubscript{2} and N addition to understand their long-term responses to global eutro-

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![Table 2](image-url)
Such differential responses could be ecologically important if they influence species productivity and subsequent species dynamics (Joel et al., 2001; Zavaleta et al., 2003a; Niklaus et al., 2007). C_3 forb responses to elevated CO_2 have been compared previously with those of grasses in a number of experiments (Knapp et al., 1996; Anderson et al., 2001; Morgan et al., 2001). However, few of these experiments have examined such responses under combinations of elevated CO_2 and enhanced N supply, or for time periods longer than a few years. In our study, after 6–9 years of elevated CO_2 exposure, significant CO_2 × N interactions were observed across species for photosynthetic capacity (A_{m365}) and leaf nitrogen (N_{mass}). These interactions indicated that elevated CO_2 induced stronger down-regulation of photosynthetic capacity and its related variables when N availability was higher (Fig. 1). Moreover, we observed strong differences in photosynthetic responses to elevated CO_2 between functional groups, and these were especially pronounced under higher N supply. Forbs showed strong and consistent photosynthetic down-regulation that eliminated the elevated CO_2 fertilization effect on photosynthesis. In contrast, C_3 grasses maintained a substantial photosynthetic stimulation even after 9 years of elevated CO_2 exposure.

In our study, reduced rates of carboxylation and electron transport, and less realized photosynthetic enhancement in elevated CO_2, correlated with a reduction in leaf N concentration in elevated CO_2. In species or functional groups in which reductions in leaf N were observed under elevated CO_2, photosynthetic down-regulation followed. The leaf N responses to long-term elevated CO_2 appear to drive down-regulation of photosynthetic capacity. Because most nitrogen is invested in photosynthetic components, the CO_2-induced reduction in N_{mass} resulted in no significant CO_2-induced enhancement of realized photosynthesis in forb species receiving N addition. Based on these results, we conclude that our first hypothesis – that down-regulation is of a similar magnitude as, and therefore can eliminate, the CO_2 fertilization effect – was supported. However, it was only true for one functional group, the C_3 forbs, and was not supported at all for the C_3 grasses. In contrast, the second hypothesis – that the down-regulatory process would be ameliorated by enhanced N supply – was surprisingly countered by the data, in opposition to general support for enhanced productivity responses to elevated CO_2 with greater N supply (Reich et al., 2006b). Our observation of less photosynthetic enhancement from elevated CO_2 under N enrichment than ambient N supply was observed in forbs but not in grasses.

Despite the photosynthetic down-regulation responses to elevated CO_2 reported here, positive biomass responses (and predominantly for roots) to elevated CO_2 and N addition were reported early in this experiment by Reich et al. (2001a, b) in C_3 species, including both C_3 grasses and perennial forbs. However, the present study provides evidence of a strong increase in root biomass in response to N addition for the grasses but not for the forb species (Table 2). Even though these species share the same photosynthetic pathway, they have different growth forms and rooting patterns which have implications for resource uptake and allocation. It has also been argued that responses to N deposition and
N addition to soils are different between these species groups (Stevens et al., 2006), consistent with our results. Our results showing reduced photosynthesis in forbs in response to elevated CO₂ and N addition (Fig. 3) are also consistent with Reich et al. (2004) where forbs showed a 12% biomass reduction to N addition but grasses showed larger biomass increases (by 20%). The total plant N pool increased strongly in grasses and there was no response in forbs, suggesting forbs did not take up the additional N supply (Reich et al., 2004). Our findings are also consistent with species-specific data, in which two of the three forb species (Achillea and Anemone) showed greater biomass enhancement due to elevated CO₂ in ambient N than in N-addition plots across a larger set of grassland species and species mixtures (data not shown).

Although stimulation of photosynthesis in elevated CO₂ is still possible with reduced leaf N concentrations (Ainsworth & Long, 2005), this N-redistributing mechanism likely does not provide all plant growth demands for N in elevated CO₂ (Bassirirad et al., 2001; Hungate et al., 2003). This may even be the case when additional N is supplied, if the plants cannot take advantage of the additional resources. Plants may not be able to take up the additional N due to increased immobilization of N in elevated CO₂ (de Graaff et al., 2006; Finzi et al., 2006; Holmes et al., 2006; Hungate et al., 2006; Knops et al., 2007), lack of mycorrhizal colonization of roots (Hartnett & Wilson, 1999), or increased N leaching in N addition plots (Hobbie, 1992; Dijkstra et al., 2007). Leaching of dissolved inorganic nitrogen was especially apparent in forb and legume monocultures at our site (Dijkstra et al., 2007).

We observed a strong increase in leaf N and a weaker increase in net photosynthesis to N addition across species. Grasses responded to N addition with a 15% increase in net photosynthesis whereas forbs did not respond to N addition. This is consistent with a study at the same site that found that soil solution N concentration was close to zero underneath grass monocultures, whereas it was about 60% underneath forb species (Reich et al., 2004), indicating that grasses forage strongly for available soil N whereas forb monocultures do not.

If plant N demand exceeds N supply, then the stimulated growth response in elevated CO₂ is likely not sustainable (Lu et al., 2004; Gill et al., 2006) as indicated by a recent review of CO₂ × N interactions in long-term field studies (Reich et al., 2006b). Increased fine root growth is a potential way to access more soil nitrogen in elevated CO₂ (Bassirirad et al., 2001). Fine root biomass as well as specific root length was significantly smaller in forbs compared with grasses grown under a combination of elevated CO₂ and N addition (Craine et al., 2002). Typical interpretations of specific root length would suggest that forbs do not have as much root absorption or N acquisition capacity as grasses. Smaller root biomass combined with smaller specific root length suggests that forbs might not be able to exploit the soil as efficiently as grasses do. Forbs have a greater fraction of biomass in aboveground components (>50%) whereas in grasses more than 75% of total biomass is found in the roots. Therefore, differences in access to soil resources due to different root morphology, root biomass distribution, and total root biomass between forbs and grasses (Fig. 4d) likely affected the CO₂ responsiveness in these functional groups, in particular the ability to maintain leaf N and avoid down-regulation of photosynthetic capacity by grasses but not by forbs.

Another way to increase soil exploitation is via mycorrhizal symbiosis. In a grassland study, Hartnett & Wilson (1999) observed large increases in forb biomass with mycorrhizal symbiosis, compared with no increase in biomass with mycorrhizae in C₃ grasses. While mycorrhizal colonization is generally increased in elevated CO₂ (Gamper et al., 2004), many studies have clearly found lower mycorrhizal colonization in N-fertilized plots (Högberg et al., 2003; Johnson et al., 2003; Blanke et al., 2005; Egerton-Warburton et al., 2007). Therefore, it is possible that these antagonistic effects may be more detrimental to forbs than grasses, accounting for the reductions in leaf N in forbs and down-regulation of photosynthesis that was observed.

Grasses, which are less dependent on mycorrhizal symbiosis due to their large root systems (Wilson & Hartnett, 1998; Craine et al., 2002), may be able to maintain their leaf N and photosynthetic enhancement in elevated CO₂ and N-added plots. Different root morphology as well as reliance on mycorrhizal colonization between C₃ grasses and forbs may provide insight into relationships between plant community structure, species diversity and ecosystem functioning in species-diverse grasslands (van der Heijden et al., 2006).

Resource differentiation is a mechanism underlying niche complementarity (Tilman, 1986) which helps maintain biodiversity because species with similar resource requirements access resources differently in space or time. The biodiversity of grasslands may not be maintained in conditions of elevated CO₂ and N deposition. Although earlier studies found increased forb biomass in response to elevated CO₂ (Leadley et al., 1999; Teyssemyerre et al., 2002; Polley et al., 2003), reduced biomass and relative abundance in forbs has been found in elevated CO₂ (Reich et al., 2001b; Zavaleta et al., 2003b; Niklaus & Korner, 2004) and with N...
deposition (Stevens et al., 2006; Clark & Tilman, 2008). The significant three-way interaction in this study (Table 1) shows that forbs were negatively affected in elevated CO$_2$ compared with grasses, and this was exacerbated in high N conditions.

Zavaleta et al. (2003b) reported reduced species richness at the Jasper Ridge grassland site with a combination of elevated CO$_2$ and N deposition, due largely to poor performance of the forbs. Thus, if these kinds of shifts in the competitive balance of grasses and forbs commonly occur, it may lead to less diverse grasslands dominated by graminoids in the elevated [CO$_2$] of the future. Lower grassland diversity could also be a consequence of reduced association with mycorrhizae in the forbs grown in high N conditions, also be a consequence of reduced association with mycorrhizae in the forbs grown in high N conditions, inducing a shift towards more C$_3$ dominated grasslands (Egerton-Warburton et al., 2007). However, empirical evidence for such shifts is still sparse (but see Thomas et al., 2004) and other grasslands might respond differently due to resource limitations other than nitrogen, particularly in low-rainfall zones (Morgan et al., 2004).

Although species responses to elevated CO$_2$ and N addition treatments in this study were individualistic, there were also strong functional group responses (Figs 2 and 3). Our goal was to examine whether and how differences between forbs and C$_3$ grasses yield different functional group responses to a combination of elevated CO$_2$ and N addition. Our results suggest that differences in resource acquisition might drive differences in CO$_2$ responsiveness in these temperate grassland species. Therefore, functional group responses to climate change perturbations could be useful for modeling responses and feedbacks to ecosystem C-cycling, even when not predictive of species-specific responses (Zavaleta et al., 2003b). That species and functional group traits like leaf N and functional group photosynthetic characteristics and whole-plant responses may be related to community-level responses argues for further work evaluating mechanistic links between ecophysiological and community level processes in order to predict the direction and magnitude of environmental change to ecosystem functioning and composition (Suding et al., 2008).

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References


LEAF N CONTROLS PHOTOSYNTHETIC RESPONSES IN GRASSES


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Functional group responses and standard error bars to elevated CO₂ in ambient N plots (Natural N, left panels) and N-addition plots (Amended N, right panels) for maximum carboxylation rate, V_{cmax} and maximum electron transport rate, J_{max}, net photosynthesis in respective growth conditions, A_{net} and mass-based foliage nitrogen concentration, N_{mass}. Significant differences between CO₂ treatments within species or functional group are represented by * for P<0.1, ** for P<0.05, *** for P<0.01 and **** for P<0.001.

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