



# Carbon isotope discrimination and bundle sheath leakiness in three C<sub>4</sub> subtypes grown under variable nitrogen, water and atmospheric CO<sub>2</sub> supply

A. Fravolini<sup>1,3</sup>, D. G. Williams<sup>1</sup> and T. L. Thompson<sup>2</sup>

<sup>1</sup> School of Renewable Natural Resources, University of Arizona, Tucson, AZ 85721, USA

<sup>2</sup> Department of Soil, Water and Environmental Science, PO Box 210038, University of Arizona, Tucson, AZ 85721, USA

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## Abstract

The changes in composition and productivity of semi-arid C<sub>4</sub> grassland, anticipated with rising atmospheric CO<sub>2</sub>, will depend on soil water and nutrient availability. The interactive effects of soil resource limitation and elevated CO<sub>2</sub> on these grasses, furthermore, may vary among C<sub>4</sub> biochemical subtypes (NADP-ME, NAD-ME, PCK) that differ in bundle sheath leakiness ( $\Phi$ ) responses to drought and nitrogen supply. To address C<sub>4</sub> subtype responses to soil resource gradients, the carbon isotope discrimination ( $\Delta$ ), bundle sheath leakiness ( $\Phi$ ), leaf gas exchange ( $A$ ,  $g_s$ ,  $c_i/c_a$ ) and above-ground biomass accumulation were measured on three dominant grasses of semi-desert grassland in south-eastern Arizona. *Bouteloua curtipendula* (PCK), *Aristida glabrata* (NADP-ME) and the non-native *Eragrostis lehmanniana* (NAD-ME) were grown in controlled-environment chambers from seed under a complete, multi-factorial combination of present ambient (370 ppm) and elevated (690 ppm) CO<sub>2</sub> concentration and under high and low water and nitrogen supply. *E. lehmanniana* (NAD-ME) had the highest photosynthetic rate ( $A$ ) and lowest  $\Phi$  compared to the other two grasses when grown under low nitrogen and water availability. However, favourable water and nitrogen supply and elevated atmospheric CO<sub>2</sub> enhanced photosynthetic performance and above-ground biomass production of *B. curtipendula* (PCK) to a greater extent than in *A. glabrata* and *E. lehmanniana*. Contrary to previous studies,  $\Phi$  and  $\Delta$  in the NADP-ME subtype (*A. glabrata*) were most affected by changing environ-

mental conditions compared to the other subtypes; deviations from the classic NADP-ME anatomy in *Aristida* could have accounted for this result. Overall, response of semi-arid grasslands to rising atmospheric CO<sub>2</sub> may depend more on species-specific responses to drought and nitrogen limitation than on general C<sub>4</sub> subtype responses.

Key words: Bundle sheath leakiness, carbon isotope discrimination, CO<sub>2</sub> supply, grasses, nitrogen, semi-arid, water.

## Introduction

There is often a close relationship between the amount of precipitation and the distribution of grasses representing different C<sub>4</sub> biochemical subtypes (Hattersley, 1992; Schulze *et al.*, 1996). The NAD-ME subtype occurs predominantly in environments receiving comparatively lower average rainfall, whereas the NADP-ME and the PCK subtypes are dominant in regions with higher rainfall. Rising atmospheric CO<sub>2</sub> concentrations potentially could alter these distributional hierarchies if the biochemical subtypes responded differently to enhancements of this trace gas. For example, the reduced stomatal conductance observed in C<sub>4</sub> grasses grown under elevated atmospheric CO<sub>2</sub> (Rogers *et al.*, 1983; Imai and Okamoto-Sato, 1991; Knapp *et al.*, 1993; Owensby *et al.*, 1993; Morgan *et al.*, 1994) could potentially alter water-use efficiency to a different extent among subtypes, either exacerbating or attenuating their differences and their distribution across aridity gradients. Different responses in transpiration could

<sup>3</sup> To whom correspondence should be addressed. E-mail: alefravo@u.arizona.edu

be decisive in terms of competition among or between grasses and other lifeforms, especially in semi-arid ecosystems where water is the most limiting factor to plant growth. The enhancing effect of rising atmospheric CO<sub>2</sub> concentration on plant growth will endure as long as nutrients, such as nitrogen, are available to maintain sufficient enzyme production to sustain biomass production.

The long-term discrimination against <sup>13</sup>CO<sub>2</sub> ( $\Delta$ ) that occurs in leaves during CO<sub>2</sub> assimilation can be measured to assess variation in photosynthetic metabolism in C<sub>4</sub> plants. This parameter depends on both the ratio of intercellular to ambient CO<sub>2</sub> concentration ( $c_i/c_a$ ) and bundle sheath leakiness ( $\Phi$ ) (Farquhar, 1983). Calculations of  $\Delta$  and  $\Phi$  based on stable isotope ratio analysis have been used to characterize C<sub>4</sub> photosynthetic responses to changing environmental conditions (Ranjith *et al.*, 1995; Saliendra *et al.*, 1996; Schulze *et al.*, 1996; Buchmann *et al.*, 1996; Meinzer and Zhu, 1998; Williams *et al.*, 2001). However, few studies have focused on how environmental factors such as water and nitrogen availability influence  $\Phi$  and  $\Delta$  in diverse C<sub>4</sub> subtypes under elevated atmospheric CO<sub>2</sub> (Williams *et al.*, 2001). Grasses of the NADP-ME subtype are affected less by changing environmental variables because they maintain a more stable and lower  $\Phi$  compared to the PCK and the NAD-ME subtypes (Hattersley, 1982; Buchmann *et al.*, 1996). However, it is still poorly understood how and if these patterns would continue under higher atmospheric CO<sub>2</sub>. In C<sub>4</sub> plants, the efficiency of the CO<sub>2</sub> concentrating mechanism in the bundle sheath depends on biochemical and anatomical features, which vary among biochemical subtypes. These differences influence the efficiency of carbon assimilation by varying the synchronization between PEP carboxylase in the mesophyll and Rubisco in the bundle sheath, respectively. At the community level, the success of one species over another may depend on its superiority in co-ordinating these enzyme systems under fluctuating environmental conditions.

Bundle sheath leakiness ( $\Phi$ ), the fraction of CO<sub>2</sub> released by the decarboxylation of C<sub>4</sub> compounds in the bundle sheath, which subsequently leaks out to the mesophyll, can be used to measure the extent to which the carboxylation of PEP exceeds RuBP (Henderson *et al.*, 1992). Bundle sheath conductance to CO<sub>2</sub> and the CO<sub>2</sub> gradient between bundle sheath and mesophyll are two major factors that determine  $\Phi$ . In the NADP-ME and PCK subtypes, where the presence of a suberized lamella surrounding the bundle sheath limits CO<sub>2</sub> diffusion back to the mesophyll,  $\Phi$  is usually lower than in the NAD-ME subtype (Hatch *et al.*, 1995; Hattersley *et al.*, 1982). Lacking a suberized lamella, the NAD-ME subtype could be more sensitive to increasing CO<sub>2</sub> concentrations because of increasing CO<sub>2</sub> bundle sheath leakiness (LeCain and Morgan, 1998). However, the relationship

between bundle sheath anatomy, and  $\Phi$  is not always so clear. Henderson *et al.* (1992) found that C<sub>4</sub> grass subtypes with and without a suberized lamella can have high  $\Phi$  values.

Under nitrogen-limiting conditions Rubisco activity can decrease to a greater extent than PEP carboxylase activity (Ranjith *et al.*, 1995). Therefore, if nitrogen is limiting, CO<sub>2</sub> fixation in the bundle sheath may diminish, resulting in a greater portion of the unfixed CO<sub>2</sub> leaking back into the mesophyll. Although several studies reported differences in photosynthetic nitrogen-use efficiency (PNUE; photosynthesis per unit leaf nitrogen) between C<sub>3</sub> and C<sub>4</sub> plants (Brown, 1978; Bolton and Brown, 1980; Sage and Pearcy, 1987), the relationship between nitrogen and C<sub>4</sub> grasses within different subtypes is still unclear. Partitioning of nitrogen into Rubisco and PEP carboxylase may vary among species and subtypes (Evans and Seemann, 1984; Makino *et al.*, 1987; Evans and Austin, 1986; Makino and Osmond, 1991). It has been assumed that the higher enzymatic complexity of the NAD-ME pathway may require more nitrogen for enzyme synthesis compared to the other two subtypes, resulting in lower nitrogen-use efficiency (Bowman, 1991). However, recent studies have not found a clear pattern between subtypes (Taub and Lerda, 2000), suggesting that adaptation to low nitrogen availability may depend more on differences at the species level rather than in biochemical subtypes.

Can C<sub>4</sub> grasses be lumped into a few functional groupings based on biochemical subtypes, based upon growth and photosynthetic responses to rising atmospheric CO<sub>2</sub> concentrations under conditions of soil resource limitation? Alternatively, do responses depend entirely on unique species traits? carbon isotope discrimination ( $\Delta$ ), bundle sheath leakiness ( $\Phi$ ), and their relationship with photosynthetic gas exchange and above-ground biomass production were measured in *Bouteloua curtipendula* (PEP-CK), *Aristida glabrata* (NADP-ME) and *Eragrostis lehmanniana* (NAD-ME) under ample and limited soil resource supply and ambient and elevated atmospheric CO<sub>2</sub> concentrations. These species share similar life histories and co-occur in the semi-arid grasslands of south-eastern Arizona. *B. curtipendula* and *A. glabrata* are native to southern Arizona, but *E. lehmanniana* was introduced from South Africa to this region in the 1940s.

## Materials and methods

C<sub>4</sub> grasses representing the three decarboxylation subtypes were used in the experiment: *Aristida glabrata* Vasey (NADP-malic enzyme), *Bouteloua curtipendula* Michx. (PEP-carboxykinase), and *Eragrostis lehmanniana* Nees. (NAD-malic enzyme). Caryopses of each species (source: Wildlands Restoration, Tucson, Arizona) were sown in 6.0 l pots constructed from PVC and filled with a sandy loam soil collected from the Santa Rita Experimental Range south of Tucson. Soils were packed to a uniform bulk density of 1.60 g cm<sup>-3</sup>. Seedlings were allowed to develop and grow in a growth chamber

for 20 d under ample water supply. After 20 d, plants were thinned and clipped to a 4 cm height and four replicates of each species were randomly assigned to a complete factorial combination of ambient and elevated CO<sub>2</sub> concentration, ample and limited nitrogen supply and dry and wet irrigation treatments.

Plants grown with ample water supply ('wet') were watered to field capacity (500 ml) every 2 d, while plants under the 'dry' condition received 500 ml of water every 3 d. Plants were fertilized every three waterings with 500 ml of a complete Hoagland's nutrient solution containing 15 mmol l<sup>-1</sup> N as NH<sub>4</sub>NO<sub>3</sub>. Plants grown under low nitrogen conditions ('-N') were fertilized with the same nutrient solution, but without NH<sub>4</sub>NO<sub>3</sub>. A nitrification inhibitor (Nitrapyrin, N-Serve®) was added to the '+N' solution in order to block the primary ammonium oxidase enzyme action and maintain N in the NH<sub>4</sub><sup>+</sup> form.

Experiments were conducted in two growth chambers (CONVIRON, PGW 36, Canada). The relative humidity in the chambers was kept near 30% during the day and 60% at night. The photoperiod was 14 h with a midday photosynthetic photon flux density of 600±50 μmol m<sup>-2</sup> s<sup>-1</sup>. The air temperature was 30–35 °C during the day and 23 °C at night. A CO<sub>2</sub>-injection system maintained a CO<sub>2</sub> concentration of 370 ppm in one chamber, and 690 ppm in the other. Plants were randomized with respect to position in the chambers and plants and their respective CO<sub>2</sub> levels were switched between chambers weekly to minimize chamber effect.

The CO<sub>2</sub> assimilation rate (*A*), stomatal conductance (*g<sub>s</sub>*), and the ratio of intercellular to ambient CO<sub>2</sub> concentration *c<sub>i</sub>/c<sub>a</sub>*, were monitored weekly following rewetting using a portable gas exchange system (CIRAS-1, PP Systems, Hertz, UK). One young fully expanded leaf blade on each plant was used for gas exchange measurements. Gas exchange was measured under the respective treatment CO<sub>2</sub> concentrations (either 370 or 690 ppm) by adjusting the flow rate of a high CO<sub>2</sub> source into the leaf chamber. After 45 d the leaves used for gas exchange measurements were cut, immersed in liquid nitrogen, freeze-dried, and then ground to a fine powder. A 0.2 mg sample was combusted in a continuous-flow isotope ratio mass spectrometer (Fison Isochrome, Manchester, UK) at the Columbia University Biosphere 2 center in Oracle, AZ. The stable carbon isotope composition (δ<sup>13</sup>C) was expressed relative to the Pee Dee belemnite standard. The carbon isotope composition of air (δ<sub>a</sub>) in each chamber was measured by-weekly using 2.0 l glass flasks. The CO<sub>2</sub> from the flasks was cryogenically extracted and analysed on a mass spectrometer (Finnigan Mat Delta S, Germany) in the Department of Geosciences at the University of Arizona in Tucson. Carbon isotope discrimination (Δ) was calculated according to Farquhar (1983):

$$\Delta = (\delta_a - \delta_p) \delta_a / (1 + \delta_p) \quad (1)$$

where δ<sub>p</sub> is the isotopic composition of the leaf dry matter and δ<sub>a</sub> is the isotopic composition of the air. The average δ<sub>a</sub> measured in each chamber (-10.4±0.6‰ ambient, -7.6±0.3‰ elevated CO<sub>2</sub> treatment) over the course of the experiment was used to calculate Δ. Bundle sheath leakiness (Φ) was calculated according to Farquhar (1983) and Henderson *et al.* (1992):

$$\Phi = [\Delta - a + (a - b_4)c_i/c_a] / [(b_3 - s)(c_i/c_a)] \quad (2)$$

where *a* (4.4‰) is the fractionation during diffusion of CO<sub>2</sub> in air, *b<sub>4</sub>* (-5.7‰) is the isotopic fractionation that occurs during dissolution of HCO<sub>3</sub><sup>-</sup> and fixation by PEP carboxylase, *b<sub>3</sub>* (29‰) is the fractionation during the carboxylation of Rubisco, and *s* (1.8‰) is the fractionation during leakage of CO<sub>2</sub> from the bundle sheath to the mesophyll. At the end of the experiment (day 66) above-ground biomass was harvested. All materials was oven-dried at 70 °C for 1 week and then weighed.

### Statistical analysis

The experiment was a completely randomized factorial design with three treatments (CO<sub>2</sub>, water, N), two levels per treatment, and four replicates. Because chambers were alternated weekly and pot location was randomized within the chambers, CO<sub>2</sub> was regarded as an independent treatment. Multivariate analysis for repeated measures (MANOVA) was conducted for *A*, *g<sub>s</sub>* and *c<sub>i</sub>/c<sub>a</sub>* in each species. Analysis of variance (ANOVA) was performed for Δ and Φ. Species effect was not included in the analysis of variance (ANOVA) because the large number of results would be difficult to interpret. Data are presented and discussed only at the highest level of significance (generally *P* ≤ 0.05). JMP software for Macintosh (SAS Institute, 1995) was used to perform the analyses.

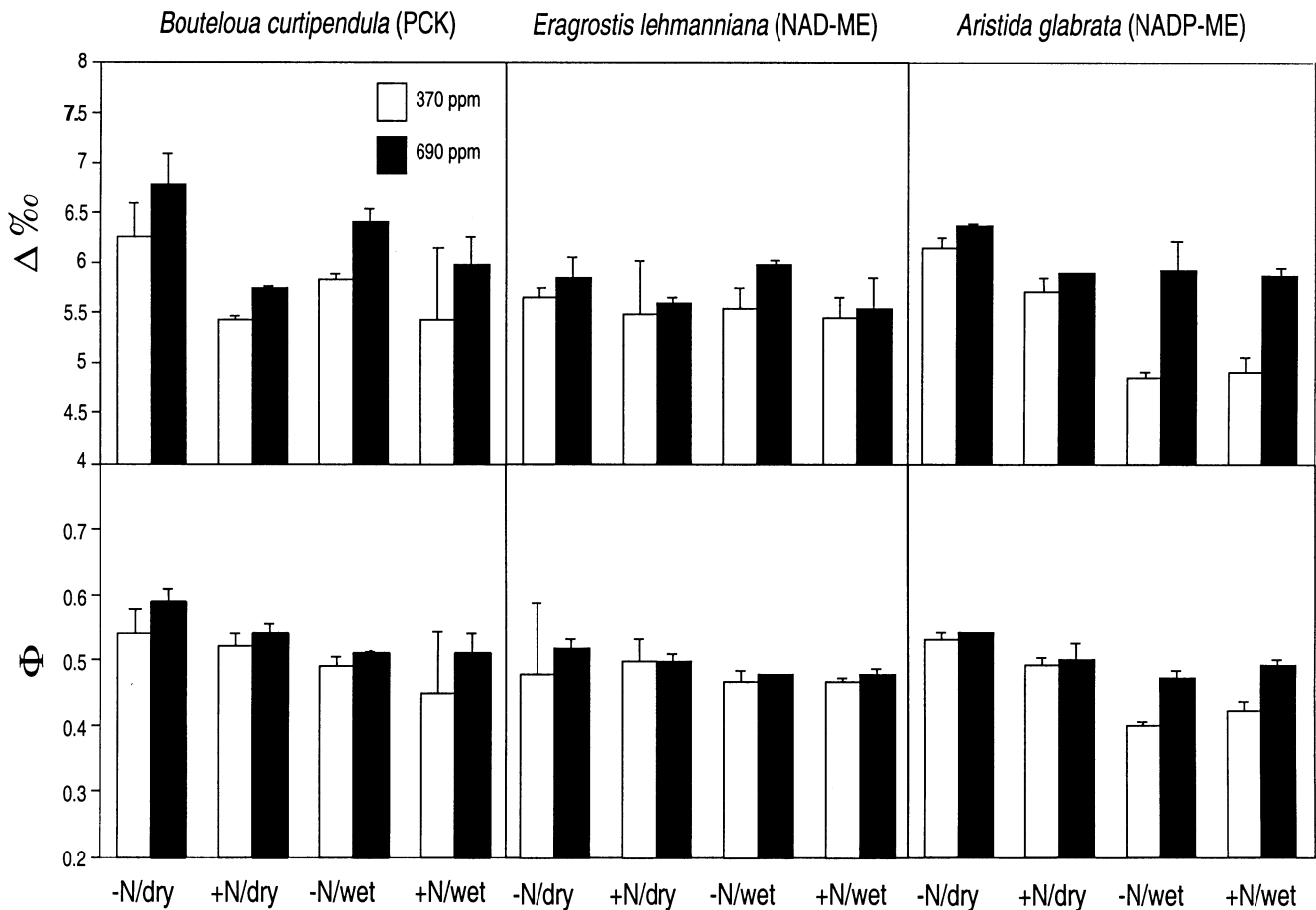
### Results

Carbon isotope discrimination (Δ) was higher under elevated atmospheric carbon dioxide concentration in all three species (Fig. 1, Table 1). In *A. glabrata*, however, the response of Δ to the CO<sub>2</sub> treatment depended on water supply (significant water by CO<sub>2</sub> interaction) (Table 1). Under high water availability *A. glabrata* had higher Δ and Φ when grown under elevated compared to ambient CO<sub>2</sub> conditions. Likewise, the enhancement of Δ under elevated CO<sub>2</sub> conditions in *E. lehmanniana* depended on nitrogen supply; CO<sub>2</sub> had a prominent effect on Δ only under low nitrogen conditions. Low nitrogen supply enhanced Δ in *B. curtipendula* and *A. glabrata* while low water supply led to higher Δ only in *A. glabrata*. No significant change in Δ was apparent in *E. lehmanniana* grown under different water supplies (Table 1).

Elevated atmospheric CO<sub>2</sub> resulted in higher values of Φ in *B. curtipendula* and *A. glabrata*, but not in *E. lehmanniana* where Φ remained fairly stable (Fig. 1). Under ambient CO<sub>2</sub> conditions bundle sheath leakiness was 0.39–0.58 for all three species. Low water supply increased Φ in *B. curtipendula* and *A. glabrata*, but not significantly in *E. lehmanniana*. *A. glabrata* had the lowest Φ values under high water availability compared to the other two species (Fig. 1). In this species bundle sheath leakiness increased 20% from wet to dry treatments under ambient atmospheric CO<sub>2</sub>, but only 7% under elevated CO<sub>2</sub> conditions. Under ambient CO<sub>2</sub> conditions *E. lehmanniana* had intermediate values of Φ ranging from 0.43 to 0.50.

Values of Φ for grasses grown under elevated CO<sub>2</sub> were between 0.43 and 0.62. Bundle sheath leakiness was higher in plants grown with low water and nitrogen availability, among them *E. lehmanniana* had the lowest values of Φ. High values of Φ were found in *B. curtipendula* grown under low nitrogen and water supply.

To determine the extent to which Δ was influenced by Φ and *c<sub>i</sub>/c<sub>a</sub>*, Δ was regressed against these two parameters individually (Fig. 2). This analysis showed that Φ was the major factor contributing to variations in Δ for *B. curtipendula* and *A. glabrata*. Conversely, in *E. lehmanniana* changes in *c<sub>i</sub>/c<sub>a</sub>* influenced Δ more than did Φ.



**Fig. 1.** Carbon isotope discrimination ( $\Delta$ ) and bundle sheath leakiness ( $\Phi$ ) in *Bouteloua curtipendula* (PCK), *Eragrostis lehmanniana* (NAD-ME) and *Aristida glabrata* (NADP-ME), 37 d after the beginning of water, nitrogen and CO<sub>2</sub> treatments. Error bars indicate standard deviation.

All three grasses showed a moderate increase (from 3% to 31%) in photosynthetic assimilation rate ( $A$ ) under enriched CO<sub>2</sub> conditions (Fig. 3), however, this trend was significant for *A. glabrata* and *B. curtipendula*, but not for *E. lehmanniana*. With high water and nitrogen supply,  $A$  in the PEP-CK subtype, *B. curtipendula*, was enhanced by elevated CO<sub>2</sub> to a greater extent than in the other subtypes. This species was sensitive to both water and nitrogen treatments (Table 2). The water  $\times$  nitrogen interaction was significant for *B. curtipendula* in which nitrogen supply increased  $A$  under low water availability. The NAD-ME subtype, *E. lehmanniana*, had higher  $A$  under low nitrogen and water availability compared to the other two subtypes (Fig. 3). Favourable conditions did not appear to enhance  $A$  in this species. Likewise  $A$  responses in *E. lehmanniana* to elevated CO<sub>2</sub> were not significant. *A. glabrata* showed the lowest photosynthetic rates under all treatments (Fig. 3). Like *E. lehmanniana* this species was not stimulated by higher water and nitrogen supply. However, assimilation rates in *A. glabrata* increased significantly under elevated atmospheric CO<sub>2</sub> (Table 2). In all species grown under

elevated CO<sub>2</sub> conditions,  $g_s$  decreased while  $c_i/c_a$  showed the opposite trend (Fig. 3).

Both  $g_s$  and  $c_i/c_a$  changed during the experiment (Table 2). Increases in  $c_i/c_a$  were observed in *E. lehmanniana* when grown under higher water availability. However, toward the end of the study those differences became less pronounced. In *B. curtipendula* high nitrogen supply reduced  $c_i/c_a$ . Differences in stomatal conductance ( $g_s$ ) were present among species and treatments. Under ambient CO<sub>2</sub>, *E. lehmanniana* had, in general, higher  $g_s$  compared to the other two species except under high nitrogen and water availability (Fig. 3). Stomatal conductance in *B. curtipendula* increased significantly with nitrogen availability especially in the presence of water (Fig. 3). *A. glabrata* had lower  $g_s$  under dry conditions and elevated CO<sub>2</sub> compared to ambient CO<sub>2</sub>. Differences in  $g_s$  among species were attenuated by elevated CO<sub>2</sub> conditions.

Despite the moderate responses in assimilation rates, above-ground biomass of all three subtypes was highly stimulated by elevated CO<sub>2</sub> (Fig. 3). At high water and

**Table 1.** Statistical significance of carbon isotope discrimination ( $\Delta$ ) and bundle sheath leakiness ( $\Phi$ ) responses to water, nitrogen and CO<sub>2</sub> treatments in *Bouteloua curtipendula* (PCK), *Eragrostis lehmanniana* (NAD-ME) and *Aristida glabrata* (NADP-ME) at day 37 of the experiment

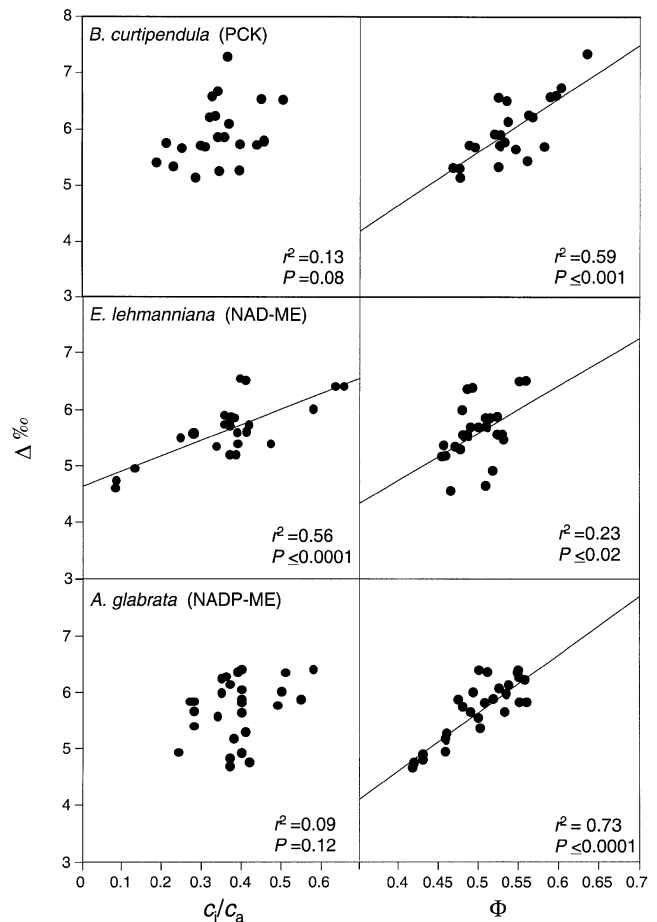
|                                    | Source of variation <sup>a</sup> |        |
|------------------------------------|----------------------------------|--------|
|                                    | $\Delta$                         | $\Phi$ |
| <i>B. curtipendula</i> (PCK)       |                                  |        |
| Water                              | ns                               | ***    |
| Nitrogen                           | ***                              | *      |
| CO <sub>2</sub>                    | ***                              | **     |
| Water×nitrogen                     | ns                               | ns     |
| Water×CO <sub>2</sub>              | ns                               | ns     |
| CO <sub>2</sub> ×nitrogen          | ns                               | ns     |
| CO <sub>2</sub> ×nitrogen×water    | ns                               | ns     |
| <i>E. lehmanniana</i> (NAD-ME)     |                                  |        |
| Water                              | ns                               | *      |
| Nitrogen                           | ns                               | ns     |
| CO <sub>2</sub>                    | **                               | ns     |
| Water×nitrogen                     | ns                               | ns     |
| Water×CO <sub>2</sub>              | ns                               | ns     |
| CO <sub>2</sub> ×nitrogen          | ***                              | ns     |
| CO <sub>2</sub> ×nitrogen×water    | *                                | ns     |
| <i>Aristida glabrata</i> (NADP-ME) |                                  |        |
| Water                              | ***                              | ***    |
| Nitrogen                           | ***                              | ns     |
| CO <sub>2</sub>                    | **                               | ***    |
| Water×nitrogen                     | **                               | ***    |
| Water×CO <sub>2</sub>              | ***                              | ***    |
| CO <sub>2</sub> ×nitrogen          | ns                               | ns     |
| CO <sub>2</sub> ×nitrogen×water    | ns                               | ns     |

<sup>a</sup>ns, \*\*, \*\*\*, and ns for  $P \leq 0.1$ ,  $P \leq 0.05$ ,  $P \leq 0.01$  and not significant, respectively. The actual  $P$ -values are reported when  $P > 0.1$  and  $P < 0.2$ .

nitrogen availability, above-ground biomass was stimulated more by elevated CO<sub>2</sub> in *B. curtipendula* (50%) than *A. glabrata* (40%) or *E. lehmanniana* (10%). As resources became more limiting, especially nitrogen, above-ground biomass became considerably lower in *B. curtipendula* and *A. glabrata*, but not in *E. lehmanniana*. In all three species the above-ground biomass, under low water availability, was enhanced by elevated atmospheric CO<sub>2</sub>. The differences in above-ground biomass, due to nitrogen treatments within each species, became more pronounced when those plants were grown under elevated atmospheric CO<sub>2</sub> conditions.

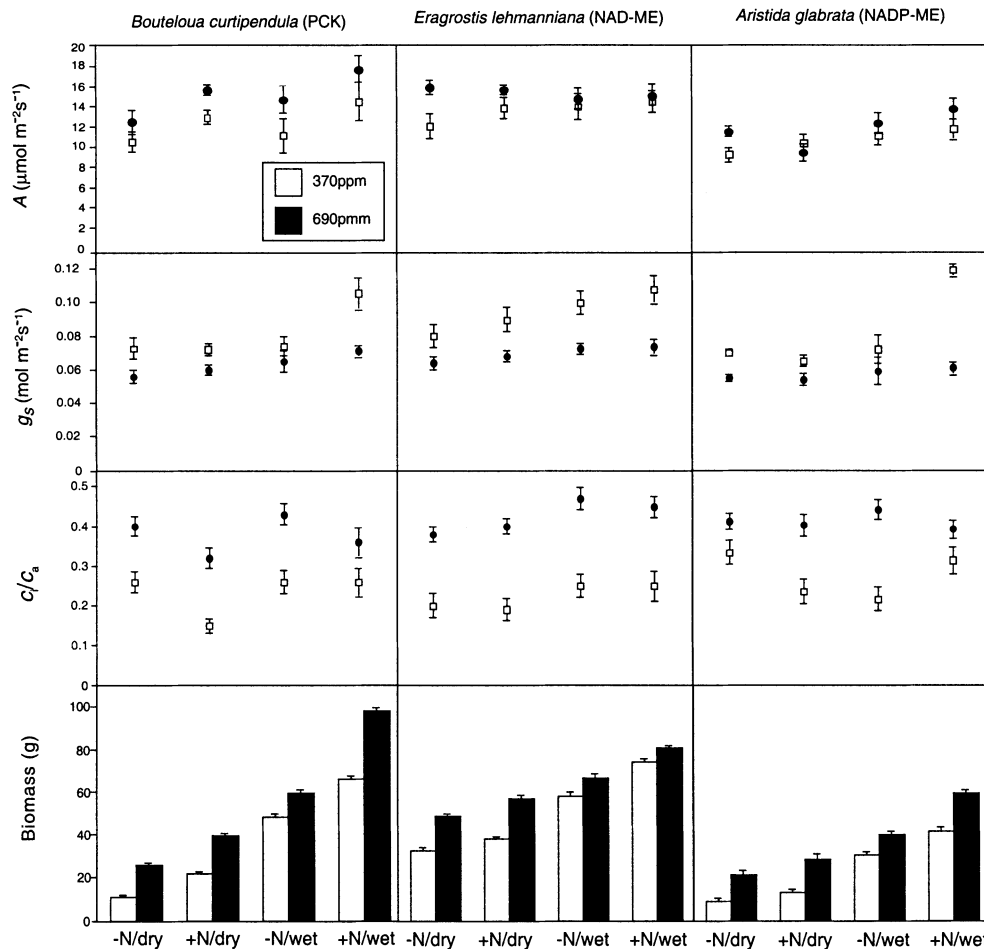
## Discussion

Carbon isotope discrimination and bundle sheath leakiness of NADP-ME grasses are often less influenced by changes in environmental conditions compared to the other two biochemical subtypes (Buchmann *et al.*, 1996). However, in this study *E. lehmanniana* (NAD-ME), rather than *A. glabrata* (NADP-ME), exhibited a stable  $\Phi$  among treatments. Furthermore, despite its lower leakiness under wet



**Fig. 2.** Relationship between carbon isotope discrimination ( $\Delta$ ) and the ratio of internal to ambient CO<sub>2</sub> partial pressure ( $c_i/c_a$ ) and bundle sheath leakiness ( $\Phi$ ) in *Bouteloua curtipendula* (PCK), *Eragrostis lehmanniana* (NAD-ME) and *Aristida glabrata* (NADP-ME), 37 d after the beginning of water, nitrogen and CO<sub>2</sub> treatments. Data from all treatments were pooled from these regression analyses.

conditions, *A. glabrata* had only moderate enhancements in A and above-ground biomass, contrary to the hypothesis that this subtype would be more responsive to water supply based on the general tendency for this subtype to be dominant in the wetter part of aridity gradients (Hattersley, 1992; Schulze *et al.*, 1996). Deviations from the classical NADP-ME bundle sheath anatomy in the genus *Aristida* might explain this observation. The genus *Aristida* forms a morphologically and anatomically distinct group because of its lack of suberized lamellae (Clayton, 1981; Watson and Dallwitz, 1989). To confirm this anomaly Schulze *et al.* (1996) reported a wide range of  $\delta^{13}\text{C}$  values in species of *Aristida*, that spanned the NAD-ME and the NADP-ME values. The high sensitivity to environmental conditions observed in *A. glabrata* in this study suggests that, in this species, the atypical bundle sheath anatomy may have overcome and masked the classic characteristics of the NADP-ME subtype.



**Fig. 3.** Photosynthetic assimilation ( $A$ ), stomatal conductance ( $g_s$ ), ratio of internal to ambient  $\text{CO}_2$  partial pressure ( $c_i/c_a$ ) and above-ground biomass in *Bouteloua curtipendula* (PCK), *Eragrostis lehmanniana* (NAD-ME) and *Aristida glabrata* (NADP-ME) grown under different water and nitrogen treatments and under two atmospheric  $\text{CO}_2$  levels. Each data point represents the average of  $A$  along the entire time-course of the experiment (66 d). Error bars indicate standard deviation.

Carbon isotope discrimination ( $\Delta$ ) of all three subtypes changed substantially in response to elevated atmospheric  $\text{CO}_2$  concentration (Fig. 1). Plants grown under elevated  $\text{CO}_2$  had higher  $\Delta$  compared to plants grown in ambient  $\text{CO}_2$  conditions. In  $\text{C}_4$  grasses carbon isotope discrimination is influenced by  $\Phi$  and  $c_i/c_a$  (equation 1). The extent to which these two parameters affect  $\Delta$  is still a subject of debate. Previous studies reported that  $\Delta$  is mostly influenced by changes in  $c_i/c_a$  due to variations in stomatal conductance (Madhavan *et al.*, 1991; Henderson *et al.*, 1998). Conversely,  $c_i/c_a$  was fairly stable in sugarcane (*Saccharum* spp, NADP-ME) under changing environmental conditions, suggesting that variations in  $\Delta$  were associated primarily with changes in  $\Phi$  (Meinzer *et al.*, 1994; Saliendra *et al.*, 1996). In a more recent study on *Sorghum bicolor* exposed to free-air  $\text{CO}_2$  enrichment (FACE), both  $c_i/c_a$  and  $\Phi$  contributed to variation in  $\Delta$  under changing water availability (Williams *et al.*, 2001).

In the current study, influences on  $\Delta$  by either  $c_i/c_a$  and  $\Phi$  varied among species (Fig. 2), suggesting that influences on  $\Delta$  may be dependent either on the biochemical subtype or unique species responses. In *B. curtipendula* and in *A. glabrata*, the sensitivity of carbon isotope discrimination to changes in  $c_i/c_a$  was lower than from variations in  $\Phi$ . In fact,  $\Delta$  was substantially mirrored by changes in  $\Phi$  (Fig. 2).

Bundle sheath leakiness remained fairly stable and  $c_i/c_a$  accounted for most of the variations observed in  $\Delta$  in *E. lehmanniana*. This species might better co-ordinate the activities of Rubisco and PEPC, thus maintaining a stable Rubisco to PEPC activity ratio under changing environmental conditions. Because of the stability in  $\Phi$ , photosynthetic performance in *E. lehmanniana* might be more sensitive to stomatal regulation than to biochemical patterns. Decreases in  $g_s$  and the higher internal  $\text{CO}_2$  found under elevated atmospheric  $\text{CO}_2$  may have

**Table 2.** Repeated measures of analysis of variance for water, nitrogen, and carbon dioxide effects on photosynthetic assimilation ( $A$ ), stomatal conductance ( $g_s$ ) and the ratio of intercellular to ambient  $CO_2$  concentration ( $c_i/c_a$ ) in *B. curtipendula* (PCK), *E. lehmanniana*, and *A. glabrata* (NADP-ME)

Parameters were measured weekly through the experiment 'time'.

|                                    | Source of variation <sup>a</sup> |       |           |
|------------------------------------|----------------------------------|-------|-----------|
|                                    | $A$                              | $g_s$ | $c_i/c_a$ |
| <i>B. curtipendula</i> (PCK)       |                                  |       |           |
| Water                              | ***                              | *     | ***       |
| Nitrogen                           | ***                              | ***   | **        |
| CO <sub>2</sub>                    | ***                              | ***   | ***       |
| Water×nitrogen                     | ***                              | *     | *         |
| Water×CO <sub>2</sub>              | ns                               | ns    | ns        |
| CO <sub>2</sub> ×nitrogen          | 0.133                            | ns    | ns        |
| Time                               | ***                              | ***   | **        |
| Time×water                         | ns                               | ns    | ns        |
| Time×nitrogen                      | ns                               | ns    | ns        |
| Time×CO <sub>2</sub>               | ns                               | **    | ns        |
| Time×water×nitrogen                | ns                               | **    | ns        |
| Time×CO <sub>2</sub> ×water        | *                                | ns    | ns        |
| Time×nitrogen×CO <sub>2</sub>      | ns                               | ns    | ns        |
| <i>E. lehmanniana</i> (NAD-ME)     |                                  |       |           |
| Water                              | ns                               | 0.159 | *         |
| Nitrogen                           | ns                               | 0.195 | 0.137     |
| CO <sub>2</sub>                    | 0.14                             | ***   | ***       |
| Water×nitrogen                     | ns                               | ns    | *         |
| Water×CO <sub>2</sub>              | 0.188                            | ns    | ns        |
| CO <sub>2</sub> ×nitrogen          | ns                               | ns    | **        |
| Time                               | ***                              | ***   | ***       |
| Time×water                         | ns                               | *     | ns        |
| Time×nitrogen                      | ns                               | ns    | ns        |
| Time×CO <sub>2</sub>               | ns                               | ***   | ns        |
| Time×water×nitrogen                | ns                               | **    | ns        |
| Time×CO <sub>2</sub> ×water        | ns                               | ***   | ns        |
| Time×nitrogen×CO <sub>2</sub>      | ns                               | 0.136 | 0.174     |
| <i>Aristida glabrata</i> (NADP-ME) |                                  |       |           |
| Water                              | 0.197                            | ***   | ns        |
| Nitrogen                           | ns                               | ns    | ns        |
| CO <sub>2</sub>                    | **                               | ***   | ***       |
| Water×nitrogen                     | ns                               | 0.181 | ns        |
| Water×CO <sub>2</sub>              | ns                               | *     | ns        |
| CO <sub>2</sub> ×nitrogen          | ns                               | ns    | ns        |
| Time                               | **                               | 0.108 | *         |
| Time×water                         | *                                | **    | ns        |
| Time×nitrogen                      | ns                               | ns    | ns        |
| Time×CO <sub>2</sub>               | ns                               | ns    | ns        |
| Time×water×nitrogen                | ns                               | ns    | ns        |
| Time×CO <sub>2</sub> ×water        | ns                               | 0.194 | ns        |
| Time×nitrogen×CO <sub>2</sub>      | ns                               | ns    | ns        |

<sup>a</sup>\*, \*\*, \*\*\*, and ns for  $P \leq 0.1$ ,  $P \leq 0.05$ ,  $P \leq 0.01$  and not significant, respectively. The actual  $P$ -values are reported when  $P > 0.1$  and  $P < 0.2$ .

accounted for changes in  $\Delta$  by increasing the amount of  $CO_2$  present in the bundle sheath, thus maintaining a constant gradient between the bundle sheath and the mesophyll.

In this study carbon isotope discrimination ( $\Delta$ ) and bundle sheath leakiness ( $\Phi$ ) ranged from 4.5‰ to 6.6‰ and 0.38 to 0.63, respectively. Low light conditions in the chambers ( $600 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) may have been respon-

sible for these comparatively high values of  $\Delta$  and  $\Phi$ . In fact, PEP and RuBP regeneration rates tend to decrease under low irradiance limiting  $CO_2$  assimilation rates (Peisker and Henderson, 1992). As a result, the release of  $CO_2$  in the bundle sheath due to dark respiration and photorespiration prevails on the rates of decarboxylation of  $CO_2$  compounds and of RuBP carboxylation increasing  $\Phi$  and  $\Delta$ . Regardless,  $\Phi$  was significantly higher under dry compared to wet conditions in all three grasses. *B. curtipendula* had the highest  $\Phi$  values followed by *A. glabrata* and *E. lehmanniana*. Increases in  $\Phi$  during drought is consistent with previous studies (Saliendra *et al.*, 1996; Williams *et al.*, 2001). The lower bundle sheath leakiness values under dry conditions in *E. lehmanniana* highlights the superior physiological tolerance to drought in this NAD-ME subtype compared to the other two subtypes (Hattersley, 1992; Schulze *et al.*, 1996). Although high water and nutrient supply resulted in only a moderate stimulation of growth of *E. lehmanniana*, high assimilation rates and environmentally stable values of leakiness suggest a greater ability of this species to co-ordinate PEP carboxylase and Rubisco activities under nitrogen and water limitation.

Since the NADP-ME subtype requires fewer enzymes for decarboxylation in the bundle sheath than NAD-ME and PCK subtypes, a higher photosynthetic response in *A. glabrata* under nitrogen limitation is expected. However, consistent with these results, previous studies have reported that the high nitrogen-use efficiency in the NADP-ME subtype tends to diminish under low nitrogen availability (Bowman, 1991). Some species of *Eragrostis* show higher N-use efficiencies and photosynthetic rates under low N availability (Taub and Lerda, 2000). It has been hypothesized that although the NADP-ME subtype has higher nitrogen-use efficiency than the other subtypes, the NAD-ME grasses have greater proportional allocation of nitrogen to photosynthetic organs (Bowman, 1991). This attribute may ensure PEP and Rubisco regeneration and co-ordination under nitrogen stress.

The two species that were most affected by low water supply, *B. curtipendula* and *A. glabrata* also had lower biomass compared to *E. lehmanniana* under these conditions, suggesting that plant size did not affect the intensity of drought stress. The higher above-ground biomass production and photosynthetic rates in *B. curtipendula*, compared to the other two grasses at elevated atmospheric  $CO_2$  concentration and with ample nitrogen and water supply, suggests that when soil resources are available this species can be more responsive to increasing atmospheric  $CO_2$  compared to the other two species. However, recent studies reported opposite trends showing no significant above-ground enhancement of two South African PCK subtype grasses exposed to elevated  $CO_2$  (Wand *et al.*, 2001). The relative responsiveness of different  $C_4$  subtypes to elevated atmospheric  $CO_2$  concentrations is apparently

unresolved at present due to the low number of species that have been investigated.

In conclusion, increases in above-ground biomass and photosynthetic response to elevated CO<sub>2</sub> in different C<sub>4</sub> biochemical subtypes are strongly dependent on water and nitrogen availability. Although responses of C<sub>4</sub> plants to increasing atmospheric CO<sub>2</sub> are smaller than for C<sub>3</sub> plants, their ecological consequences should not be undervalued. Most C<sub>4</sub> grasses occupy dry and nutrient-poor environments where even a small improvement in either water or nutrient-use efficiency can result in a dramatic change in competitive ability and plant community structure along temporal and spatial scale. The greater stability of Φ and higher above-ground biomass accumulation in *E. lemmaniana* (NAD-ME) under limited water and nitrogen availability could, in part, explain the recent proliferation and dominance of this non-native species in semi-arid grasslands throughout the south-western United States. Conversely, if increasing atmospheric CO<sub>2</sub> is accompanied by increases in monsoonal precipitation and/or nitrogen deposition, *B. curtipendula* (PCK) may be favoured. In any event, the response to changing environmental conditions and nitrogen availability can not be explained exclusively by biochemical differences among subtypes. Indeed, in this study the differences in biomass production at elevated CO<sub>2</sub> were not always correlated with Δ and Φ, suggesting that increases in biomass production could only be partly explained by photosynthetic processes at the leaf level. Other factors based on species-specific allocation traits and interaction between different functional types could influence and may in some cases overcome anatomical and biochemical differences related to C<sub>4</sub> subtype.

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