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Carbon isotope composition of C_4 grasses is influenced by light and water supply

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ABSTRACT

The carbon isotope composition of C_4 grasses has the potential to be used as an indicator of changes in the isotopic composition and concentration of atmospheric CO_2, especially for climate reconstruction. The usefulness of C_4 grasses for this purpose hinges on the assumption that their photosynthetic discrimination against ^{13}C remains constant in a wide range of environmental conditions. We tested this assumption by examining the effects of light and water stress on the carbon isotope composition of C_4 grasses using different biochemical subtypes (NADP-ME, NAD-ME, PCK) in glasshouse experiments. We grew 14 different C_4 grass species in four treatments: sun-watered, sun-drought, shade-watered and shade-drought. Carbon isotope discrimination (Δ) rarely remained constant. In general, Δ values were lowest in sun-watered grasses, greater for sun-drought plants and even higher for plants of the shade-watered treatment. The highest Δ values were generally found in the most stressed grasses, the shade-drought plants. Grasses of the NADP-ME subtype were the least influenced by a change in environmental variables, followed by PCK and NAD-ME subtypes. Water availability affected the carbon isotope discrimination less than light limitation in PCK and NAD-ME subtypes, but similarly in NADP-ME subtypes.

In another experiment, we studied the effect of increasing light levels (150 to 1500 μmol photons m^{-2} s^{-1}) on the Δ values of 18 well-watered C_4 grass species. Carbon isotope discrimination remained constant until photon flux density (PFD) was less than 700 μmol photons m^{-2} s^{-1}. Below this light level, Δ values increased with decreasing irradiance for all biochemical subtypes. The change in Δ was less pronounced in NADP-ME and PCK than in NAD-ME grasses. Grasses grown in the field and in the glasshouse showed a similar pattern. Thus, caution should be exercised when using C_4 plants under varying environmental conditions to monitor the concentration or carbon isotopic composition of atmospheric CO_2 in field/glasshouse studies or climate reconstruction.

Key-words: C_4 grasses; carbon isotope; climate reconstruction; discrimination; global change; light; water; $\delta^{13}C$.

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Abbreviations: a, discrimination factor associated with diffusion in air (‰); b_3, discrimination factor associated with photosynthetic fixation by Rubisco (‰); b_4, discrimination factor associated with carbonic anhydrase and PEP (‰); c_i/c_a, ratio of internal CO_2 concentration in the mesophyll airspaces to ambient atmospheric concentration of carbon dioxide (dimensionless); Δ, carbon isotope discrimination (‰); $\delta^{13}C$, carbon isotope ratio (‰); NAD-ME, nicotinamide adenine dinucleotide malic enzyme; NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; PCR, photosynthetic carbon reduction tissue; PDB, Pee Dee Belemnite; PEP, phosphoenolpyruvate; PFD, photon flux density (μmol m^{-2} s^{-1}); ϕ, leakiness (dimensionless); R, $^{13}C/^{12}C$ ratio; Rubisco, ribulose-1,5-biphosphate carboxylase oxygenase.

INTRODUCTION

The isotopic composition of atmospheric carbon dioxide provides valuable information about global carbon cycling and prehistoric climates (Stuiver 1978; Friedli *et al.* 1986; Leavitt & Long 1988; Beerling & Woodward 1993; Lloyd & Farquhar 1994; White *et al.* 1994). Historical patterns of atmospheric CO_2 concentrations have been derived from a number of different sources, such as ice cores, peats, sediments, tree rings and packrat middens. Recently, C_4 grasses and shrubs have been used as a proxy for the carbon isotope composition of atmospheric CO_2 (Marino & McElroy 1991; Marino *et al.* 1992) and changes in atmospheric CO_2 concentrations (Kelly, Yoker & Marino 1993; Cerling, Wang & Quade 1993). It was pointed out by Marino & McElroy (1991) that the use of C_4 grasses might fill the gap between ice core data and recent measurements of the carbon isotopic composition of the atmosphere (Keeling *et al.* 1989). Furthermore, the potential use of C_4 grasses might be favoured over other sources because C_4 plants offer a finer temporal resolution and smaller variability in the isotopic signal, combined with simpler sample preparation (Marino & McElroy 1991).

A fundamental assumption in these C_4 studies is that the carbon isotope composition of C_4 tissues reflects the atmospheric signal without physiological variations. Thus, discrimination against ^{13}C (Δ) is assumed to remain constant during C_4 photosynthesis. Farquhar (1983) developed a model to relate the carbon isotope composition ($\delta^{13}C$) of

C_4 plants to that of atmospheric carbon dioxide:

$$\delta^{13}C_{plant} = \delta^{13}C_{air} - a - (b_4 + b_3\,\phi - a)\,c_i/c_a, \quad (1)$$

which includes discrimination factors associated with diffusion in air (a), photosynthetic fixation by ribulose-1,5-biphosphate carboxylase, Rubisco (b_3), carbonic anhydrase and phosphoenolpyruvate activity, PEP (b_4), and the fraction of CO_2 fixed by PEP carboxylation which subsequently leaks out of bundle sheath cells (ϕ). c_i/c_a represents the ratio of internal CO_2 concentration in the mesophyll airspaces to the ambient atmospheric concentration of carbon dioxide. While the fractionation factors a, b_3 and b_4 are constant (for temperature dependence of b_4, see Mook, Bommerson & Staverman 1974), the c_i/c_a ratio and the leakage of CO_2 depend on physiological and structural constraints (O'Leary 1981; Evans *et al.* 1986). However, Wong, Cowan & Farquhar (1985a–c) showed that the c_i/c_a ratio of C_4 plants remained fairly stable under a wide range of light, water and nutrient conditions. In contrast, various C_4 plant species have been found to differ in the fraction of CO_2 leaking out of bundle sheath cells, either via symplastic or apoplastic pathways (Brown & Byrd 1993; Dai, Ku & Edwards 1993). This leakage (ϕ) varied diurnally and increased with increasing water and salt stress (Bowman *et al.* 1989). Increasing leakage out of the photosynthetic carbon reduction tissue (PCR) results in higher carbon isotope discrimination because b_3 is increasingly expressed. Thus, changes in the isotopic composition of C_4 plant tissues might reflect changes in C_4 photosynthetic discrimination as well as changes in the isotopic signature of atmospheric CO_2.

Before we can use the carbon isotope composition of C_4 grasses to reconstruct historical patterns of atmospheric CO_2, we need to test the hypothesis that Δ by C_4 grass species of different biochemical subtypes remains constant. Hattersley (1982) observed that different biochemical C_4 subtypes had distinctly different $\delta^{13}C$ ratios. His results demonstrated that the carbon isotope discrimination of different photosynthetic C_4 subtypes varies even under controlled glasshouse conditions. The NADP-ME subtype (nicotinamide adenine dinucleotide phosphate malic enzyme) showed the highest $\delta^{13}C$ values, while the NAD-ME subtype had the lowest ratios and the PCK subtype (phosphoenolpyruvate carboxykinase) was intermediate. Bowman *et al.* (1989) showed that salinity stress could induce a change in C_4 carbon isotope discrimination in the short term. However, the effects of light and water stress on Δ of a wide variety of C_4 grass species have yet to be explored. Because these environmental variables seem the most likely to change over time, they are the most critical variables for climate reconstruction using C_4 plants. Thus, understanding how each biochemical subtype will respond to light and water stress is a prerequisite for using carbon isotope composition of C_4 plants to monitor present concentrations and isotopic composition of CO_2, to reconstruct historic atmospheric CO_2 concentrations, and also to integrate ambient CO_2 concentrations in the long term, for example over a growing season in remote areas or in glasshouse studies.

In order to gain more insight into the relationships between changing environmental conditions and carbon isotope discrimination, we studied the effects of light and water on the carbon isotope composition of a wide variety of C_4 grasses in glasshouse experiments. In a first experiment, we examined the effects of light history on the carbon isotope composition of well-watered C_4 grasses. In a second, multi-factorial experiment, we studied the effects of light level and water supply on the carbon isotope discrimination of C_4 grasses of different biochemical subtypes. In a third experiment, we investigated the effects of a light gradient on the Δ values of a variety of C_4 grasses. Finally, we compared these results from controlled environmental conditions to results from a field study.

MATERIALS AND METHODS

Plant material

Eighteen species of C_4 grasses with different biochemical C_4 subtypes were chosen for this study (Table 1). Five of these grasses had the NADP-ME subtype pathway, five species were of the PCK subtype (Hattersley & Watson 1992) and eight species were NAD-ME subtypes (Gutierrez, Gracen & Edwards 1974; Hattersley & Watson 1992). Contrasting biochemical classifications were found in the literature for the species *Eragrostis curvula*. Gutierrez, Gracen & Edwards (1974) described this species as a NAD-ME subtype, whereas Hattersley & Browning (1981) provided evidence that it was PCK subtype. In this study, we adopted the more recent classifica-

Table 1. Selected C_4 grasses and their biochemical subtype of C_4 photosynthesis (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme)

Subtype	Species
NADP-ME	*Andropogon gerardii* Vit.
NADP-ME	*Echinochloa crus-galli* (L.) Beauv.
NADP-ME	*Paspalum dilatum* Poir.
NADP-ME	*Pennisetum setaceum* (Forssk.) Chiov.
NADP-ME	*Setaria lutescens* (Weigel) Hubb.
PCK	*Bouteloua curtipendula* (Michx.) Torr.
PCK	*Chloris gayana* Kunth.
PCK	*Eragrostis curvula* (Schrader) Nees
PCK	*Spartina pectinata* Link
PCK	*Zoysia japonica* Steudel
NAD-ME	*Bouteloua gracilis* (H.B.K.) Lag. ex Steudel
NAD-ME	*Buchloe dactyloides* (Nutt.) Engelm.
NAD-ME	*Cynodon dactylon* (L.) Pers.
NAD-ME	*Distichlis spicata* (L.) Greene
NAD-ME	*Leptochloa dubia* (H.B.K.) Nees
NAD-ME	*Muhlenbergia wrightii* Vasey ex Coult
NAD-ME	*Sporobolus airoides* (Torr.) Torr.
NAD-ME	*Sporobolus cryptandrus* (Torr.) Gray

tion of Hattersley & Browning (1981). No C_4 subtype information was available for *Muhlenbergia wrightii*. Other species of the same genus are known to be either NAD-ME (*Muhlenbergia montana*; Hattersley & Browning 1981) or PCK (*Muhlenbergia schreberi*; Hattersley 1992). The results of this study indicate that *Muhlenbergia wrightii* is a NAD-ME subtype.

Experiments were conducted in a glasshouse with supplementary high-intensity discharge lighting at the University of Utah. Seeds of all species (obtained commercially) were germinated and then transferred into pots (12·5 cm diameter) filled with a 1:1 mixture of vermiculite to sand. During a 4 d pre-treatment period, plants were located in full sunlight and watered to field capacity. After this time period, plants were randomly assigned to the different treatments (described below). Grasses were fertilized twice a week with standard nutrient solution and rotated once a week within their treatment bay to reduce edge effects.

Glasshouse conditions

The air temperature in the glasshouse was 25–32 °C during the day and above 20 °C at night. Full sun conditions at midday averaged 860 ± 30 μmol photons m^{-2} s^{-1} between January and April 1994. The total amount of photons received per day was about 34·0 ± 1·7 mol m^{-2} (L. Sperry, University of Utah, personal communication).

Carbon dioxide concentrations were monitored continuously during 1 week in May 1994 (using a LI-COR 6200, LI-COR, NE, USA). CO_2 concentrations did not differ between sun and shade treatments (analysis of variance, $P > 0{\cdot}05$), indicating adequate ventilation within the glasshouse. The carbon dioxide concentrations remained near constant at 392 ± 0·4 cm^3 m^{-3} over a 24 h period (mean ± standard error, $n = 288$). The carbon isotope ratio of carbon dioxide in the glasshouse air ($\delta^{13}C_{air}$) was measured over a 2 d period. Glasshouse air was collected in 2 dm^3 glass flasks. Carbon dioxide was cryogenically extracted using a stainless-steel vacuum line, purified and analysed for $^{13}C/^{12}C$ composition using an isotope ratio mass spectrometer (delta S, Finnigan MAT, San Jose, CA). The $\delta^{13}C_{air}$ value averaged –9·1 ± 0·2‰ ($n = 10$) and was used to calculate carbon isotope discrimination values.

Treatments

Two NAD-ME subtype grass species were used in the first experiment: *Sporobolus airoides* and *Muhlenbergia wrightii*. Five replicate plants of each species were used for sun and shade treatments, starting in May. Light in the shade treatment was reduced to about 10% of that in the sun treatment by multiple layers of shade cloth. All plants were fertilized twice a week and watered daily to field capacity. Prior to the treatments, leaves of all grasses were cut close to the base to avoid sampling of leaf tissue that was laid down before treatments started. After two harvests, in July and August, three plants of each treatment (sun or shade) were subjected to a different light environment (sun→shade, shade→sun). Two plants of each treatment remained in the old environment (sun→sun, shade→shade) and served as controls. After an additional 2 months, leaves were sampled in October. All plants were then transferred back to their original environment (sun→shade→sun, shade→sun→shade) or kept as control plants (sun→sun→sun, shade→shade→shade). Five weeks after the switch, a final harvest of all plants was completed in December.

In a second experiment, five replicate plants of the C_4 grass species listed in Table 1 were assigned to one of four different treatments: sun-watered, sun-drought, shade-watered and shade-drought. *Paspalum dilatum*, *Sporobolus* sp. and *Spartina pectinata* were not included in the second experiment. Six of the species were not used in the water stress treatments. Plants in the well-watered treatments were watered daily to field capacity. Plants of the drought treatments received 50 cm^3 of water every other day, except for *Chloris gayana* and *Setaria lutescens* in the sun-drought treatment, which received 100 cm^3 of water every other day. Light levels in the shade treatments were reduced to about 10% of those in the sun treatment by multiple layers of shade cloth. All treatments started at the beginning of February. Results of the first harvest 4 weeks later are presented; data from a second harvest showed a similar pattern and are not presented. After the second experiment was completed, all grasses were kept in full sunlight, well-watered conditions for an additional 3 weeks and subsequently used in a third experiment.

Eighteen C_4 grass species were used in the third experiment (see Table 1). Plants that were used in the second experiment were blocked to account for the variance due to the previous treatments and then randomly assigned to the different new light treatments so that each light treatment included at least one plant from each treatment of the second experiment, with a maximum of six replicate plants per light treatment. Starting at the beginning of April, plants were exposed to five different light environments, ranging from 9 to 100% light using multiple layers of shade cloth (100% light = 1475 μmol photons m^{-2} s^{-1}, 55% light = 815 μmol photons m^{-2} s^{-1}, 30% light = 440 μmol photons m^{-2} s^{-1}, 20% light = 300 μmol photons m^{-2} s^{-1}, 9% light = 135 μmol photons m^{-2} s^{-1}). PFD at noon was measured with a quantum sensor (LI-185A, LI-COR, NE, USA) and averaged for each treatment. All plants were watered to field capacity every other day and fertilized twice a week. After 4 weeks, plants were sampled for carbon isotope analyses.

Sampling and $\delta^{13}C$ analysis

Samples for carbon isotope discrimination (Δ) were taken from leaf tissue grown during the experiment only. Plant material was dried for 48 h at 70 °C and then ground with a mortar and pestle to a fine powder. A 2 mg subsample was combusted and analysed for $^{13}C/^{12}C$ composition using an isotope ratio mass spectrometer (delta S, Finnigan MAT,

San Jose, CA). The carbon isotope ratio ($\delta^{13}C$) was calculated as

$$\delta^{13}C = (R_{sample} / R_{standard} - 1) * 1000\ ‰, \quad (2)$$

and then carbon isotope discrimination (Δ) was calculated as

$$\Delta = (\delta^{13}C_{air} - \delta^{13}C_{plant}) / (1 + \delta^{13}C_{plant}), \quad (3)$$

where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios of the plant sample and standard (PDB) (Farquhar, Ehleringer & Hubick 1989). For $\delta^{13}C_{air}$ the average value of glasshouse air (–9·1 ‰) was used (see description of glasshouse conditions). The overall precision of the measurements of plant materials was ± 0·11 ‰.

Statistics

Analyses of variance were used to analyse the effects of treatment, subtype and environmental history (Norusis 1990). When the interaction term was not significant at the 0·05 level, the data were combined for further analysis. The least-significant-difference test (multiple range test, $LSD_{0.05}$) was used to separate means. Although we accounted for the four different environmental histories of grasses used in the second experiment by assigning these plants equally to the five light treatments in the third experiment, we tested for possible significant history effects before data analyses for experiment 3 (Table 2). No history effect was detected for NADP-ME and NAD-ME grass species except for *Bouteloua gracilis* (NAD-ME). However, most of the grasses with the PCK subtype showed significant history effects (except *Bouteloua curtipendula*), and were therefore treated separately in the later analyses. Linear regression analyses of carbon isotope discrimination values were performed with $\log_{10}$-transformed PFD data as the independent variable.

Table 2. Test for significant interactions between treatments of experiment 2 (history) and light levels of experiment 3 (light). Results of one-way analysis of variance for $\delta^{13}C$ ratios are presented. NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme

Species	*n*	$P_{Interactions}$ history × light
NADP-ME		
Andropogon gerardii	21	0·352
Echinochloa crus-galli	11	0·537
Paspalum dilatum	11	0·061
Pennisetum setaceum	10	0·471
Setaria lutescens	9	0·128
PCK		
Bouteloua curtipendula	10	0·453
Chloris gayana	22	0·054
Eragrostis curvula	11	0·035
Spartina pectinata	5	0·038
Zoysia japonica	22	0·004
NAD-ME		
Bouteloua gracilis	9	0·008
Buchloe dactyloides	21	0·366
Cynodon dactylon	22	0·562
Distichlis spicata	24	0·208
Leptochloa dubia	10	0·419
Muhlenbergia wrightii	22	0·142
Sporobolus airoides	22	0·189
Sporobolus cryptandrus	9	0·096

RESULTS

Past and current light conditions affect carbon isotope discrimination of C_4 grasses grown under well-watered conditions

In the first experiment, we studied the carbon isotope composition of *Sporobolus airoides* and *Muhlenbergia wrightii* growing under different light conditions in the glasshouse (Table 3). The Δ values of grasses growing in full sun light were about 2‰ lower than those of plants growing in the shade ($P < 0·001$; July and August 1993). Changing the environment for a subset of plants demonstrated the importance of the current environment. The discrimination against ^{13}C of plants transferred from the sun into the shade treatment (sun→shade) increased significantly, whereas the Δ values at plants transferred from the shade into full sun light (shade→sun) decreased. After 2 months (October), transferred plants had significantly different Δ values from their non-transferred controls (sun→sun, sun→shade or shade→shade, shade→sun; $P < 0·005$). Within a species, plants growing under the same current light level (sun→sun, shade→sun or shade→shade, sun→shade) did show similar Δ values independently of their past light level treatment. However, the light history effect began to influence the Δ values after grasses were transferred back and grown for 5 more weeks under their original light treatment (sun→shade→sun and shade→sun→shade). The Δ values of plants transferred back into full sun (sun→shade→sun) did not decrease to the levels of the sun control grasses (sun→sun→sun, $P < 0·05$). Yet, plants transferred back into the shade (shade→sun→shade) did show similar Δ values to those of the shade control plants (shade→shade→shade).

Light limitation influences the carbon isotope discrimination of C_4 grasses with different biochemical pathways more than water availability

In our second experiment, we studied the effect of light levels and water supply on the Δ values of C_4 grasses of different biochemical subtypes. In this multi-factorial experiment, six C_4 grass species were grown under two different light regimes: sun-watered and shade-watered, and eight species were grown under four different

Species	Harvest	*P*	Treatment	Δ (‰)
Sporobolus airoides	7/2/93	<0·001	sun	5·13 ± 0·02[a] (5)
			shade	8·04 ± 0·15[b] (5)
	8/19/93	<0·001	sun	5·13 ± 0·07[a] (5)
			shade	6·87 ± 0·13[b] (5)
	10/28/93	0·004	sun→sun	4·98 ± 0·11[a] (2)
			shade→sun	5·47 ± 0·19[a] (3)
			sun→shade	7·29 ± 0·42[b] (3)
			shade→shade	7·08 ± 0·16[b] (2)
	12/1/93	0·002	sun→sun→sun	5·42 ± 0·07[a] (2)
			sun→shade→sun	6·41 ± 0·16[b] (3)
			shade→sun→shade	7·39 ± 0·21[c] (3)
			shade→shade→shade	7·45 ± 0·32[c] (2)
Muhlenbergia wrightii	7/2/93	<0·001	sun	4·91 ± 0·04[a] (5)
			shade	6·53 ± 0·11[b] (5)
	8/19/93	<0·001	sun	4·96 ± 0·07[a] (5)
			shade	7·45 ± 0·39[b] (5)
	10/28/93	<0·001	sun→sun	5·09 ± 0·03[a] (2)
			shade→sun	5·30 ± 0·04[a] (3)
			sun→shade	7·02 ± 0·08[b] (3)
			shade→shade	7·01 ± 0·04[b] (2)
	12/1/93	0·023	sun→sun→sun	4·70 ± 0·31[a] (2)
			sun→shade→sun	5·24 ± 0·13[ac] (3)
			shade→sun→shade	5·80 ± 0·17[bc] (3)
			shade→shade→shade	6·41 ± 0·52[b] (2)

Table 3. Carbon isotope discrimination (Δ) of *Sporobolus airoides* (NAD-ME type, nicotinamide adenine dinucleotide malic enzyme) and *Muhlenbergia wrightii* (NAD-ME type) under changing light environments. The probability level *P* (analysis of variance with treatment as main factor) and the means ± standard errors (*n*) are given. Different letters following the means represent significantly different treatments (LSD test)

Table 4. Carbon isotope discrimination (in ‰) of C_4 grass species of different biochemical subtypes (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme) in different environmental conditions (different light levels and water supply). Means and standard errors ($n = 5$) are given. Letters following the means represent significantly different treatments (LSD test)

		Treatments			
Species	Subtype	sun-watered	sun-drought	shade-watered	shade-drought
Andropogon gerardii	NADP-ME	3·44±0·10[c]	4·09±0·04[d]	2·97±0·08[a]	3·34±0·08[b]
Echinochloa crus-galli	NADP-ME	3·76±0·04[a]	4·41±0·05[b]	4·57±0·04[c]	5·07±0·04[d]
Pennisetum setaceum	NADP-ME	2·83±0·14[a]		3·38±0·06[b]	
Setaria lutescens	NADP-ME	2·89±0·03[b]	3·55±0·03[d]	2·47±0·03[a]	3·22±0·03[c]
Bouteloua curtipendula	PCK	4·71±0·05[a]		6·52±0·02[b]	
Chloris gayana	PCK	4·17±0·06[a]	4·84±0·06[b]	5·28±0·03[c]	6·21±0·03[d]
Eragrostis curvula	PCK	2·71±0·05[a]		4·44±0·04[b]	
Zoysia japonica	PCK	4·62±0·06[a]	5·22±0·14[b]	6·06±0·08[c]	6·38±0·07[d]
Bouteloua gracilis	NAD-ME	5·11±0·04[a]		8·99±0·04[b]	
Buchloe dactyloides	NAD-ME	4·96±0·04[a]		6·85±0·06[b]	
Cynodon dactylon	NAD-ME	4·11±0·05[a]	5·49±0·06[b]	8·06±0·06[c]	8·63±0·09[d]
Distichlis spicata	NAD-ME	4·90±0·07[a]	5·25±0·08[a]	7·14±0·15[b]	7·48±0·26[b]
Leptochloa dubia	NAD-ME	4·30±0·04[a]		6·66±0·05[b]	
Muhlenbergia wrightii	NAD-ME	4·02±0·03[a]	5·03±0·07[b]	8·08±0·15[c]	9·19±0·16[d]

treatments: sun-watered, sun-drought, shade-watered and shade-drought. Carbon isotope discrimination differed significantly amongst all four treatments for all species of all pathways (Table 4, $P < 0{\cdot}001$; except for *Distichlis spicata*, where differences existed only between light treatments). In general, carbon isotope discrimination was lowest for grasses of the sun-watered treatment, greater for sun-droughted plants and even higher in plants under shade-watered conditions. The highest Δ values were found in the most stressed grasses, the shade-drought plants. Two exceptions to this trend were the NADP-ME species *Andropogon gerardii* and *Setaria lutescens*. Both species showed lower carbon isotope discrimination in the shade than in the corresponding sun treatments. *A. gerardii* had even lower Δ values under shade-droughted than under sun-watered conditions.

NADP-ME species were the least influenced by a change in environmental variables (–0·75 to 0·81‰), followed by PCK subtypes (0·32 to 1·8‰). The largest changes occurred in NAD-ME subtype grasses (0·34 to 3·95‰). This pattern suggests that *Muhlenbergia wrightii* might belong to the NAD-ME rather than to the PCK subtype. Changes in the Δ values of *M. wrightii* caused by both environmental variables were between 1·01 and 4·16‰, in the range of all the other NAD-ME grasses and far greater than such changes in PCK species. Comparison of the change in Δ values caused by changes in water or light level showed that, for PCK and NAD-ME subtypes, a change in water availability affected the Δ values less than did light limitation (PCK water: 0·32 to 0·93‰; PCK light: 1·16 to 1·81‰; NAD water: 0·34 to 1·38‰; NAD light: 1·89 to 3·95‰). This trend was not observed for the NADP-ME subtypes, where the two variables had similar influences (NADP water: 0·37 to 0·75‰; NADP light: –0·75 to 0·81‰).

Carbon isotope discrimination of all well-watered C_4 grasses increased with decreasing light levels

In the third experiment, we studied the effect of decreasing light levels (1500 to 150 μmol photons m^{-2} s^{-1}) under well-watered conditions on carbon isotope discrimination. Independently of the biochemical subtype, Δ values of all grasses remained constant until PFD decreased below 50% (700 μmol photons m^{-2} s^{-1}; Fig. 1). Below this critical light level, carbon isotope discrimination increased with decreasing light for all grasses. The increase in carbon isotope discrimination with light was as low as 0·1‰ in *A. gerardii* or as high as 3·7‰ in *M. wrightii*. While the rates of change were variable among species of the same biochemical subtype (e.g. *A. gerardii* versus *P. dilatum* or *C. gayana* versus *E. curvula*), all relationships were highly significant (Table 5) except for *S. lutescens* and *S. pectinata*.

In spite of the variability within a biochemical subtype, the differences in response between subtypes were quite pronounced (Table 6). The slope of the Δ–light regression for NADP-ME grasses (–1·08 ± 0·33) did not differ from that for PCK (–1·44 ± 0·27), but both slopes were significantly shallower than that of the NAD-ME subtype (–2·64 ± 0·21). Differences in the past environmental conditions (the history effect from experiment 2) did not change this overall pattern (Table 6). Within any given light treatment, carbon isotope discrimination of former shade-droughted grasses was slightly higher than discrimination of less stressed plants, either shade-watered or sun-droughted plants. Former sun-watered grasses showed the lowest Δ values (Fig. 2), confirming the results of the second experiment. Regardless of light level, carbon isotope discrimination was lowest in NADP-ME grasses, highest in NAD-ME and intermediate in PCK subtypes. Differences in discrimination between the biochemical subtypes were highly significant in all light treatments (analysis of variance with subtype as main factor, $P < 0{\cdot}001$).

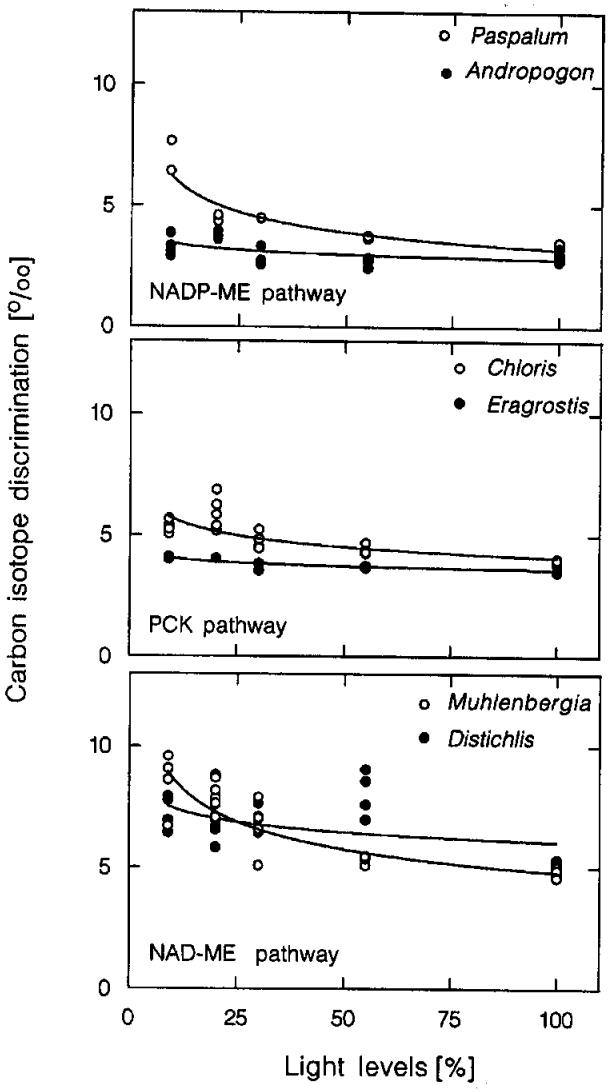

Figure 1. Carbon isotope discrimination as a function of light levels for C_4 grasses of different biochemical subtypes (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme). Two species are plotted, representing the extremes within one subtype (for regression equations, see Table 5).

Subtype	Species	*n*	Regression	*r*	*P*
NADP-ME	*Andropogon gerardii*	22	$y=-0.682 \log(x)+4.936$	0·31	0·008
NADP-ME	*Echinochloa crus-galli*	11	$y=-2.320 \log(x)+11.139$	0·74	< 0·001
NADP-ME	*Paspalum dilatum*	12	$y=-2.796 \log(x)+11.930$	0·69	< 0·001
NADP-ME	*Pennisetum setaceum*	11	$y=-1.542 \log(x)+7.377$	0·87	< 0·001
NADP-ME	*Setaria lutescens*	10	$y=-1.208 \log(x)+7.212$	0·05	0·555
PCK	*Bouteloua curtipendula*	11	$y=-1.193 \log(x)+9.001$	0·49	0·016
PCK	*Chloris gayana*	23	$y=-1.661 \log(x)+9.254$	0·64	< 0·001
PCK	*Eragrostis curvula*	11	$y=-0.440 \log(x)+4.983$	0·64	0·003
PCK	*Spartina pectinata*	6	$y=-1.686 \log(x)+9.803$	0·63	0·058
PCK	*Zoysia japonica*	22	$y=-1.728 \log(x)+10.216$	0·42	0·001
NAD-ME	*Bouteloua gracilis*	9	$y=-2.435 \log(x)+13.177$	0·79	0·001
NAD-ME	*Buchloe dactyloides*	22	$y=-1.911 \log(x)+11.220$	0·53	< 0·001
NAD-ME	*Cynodon dactylon*	23	$y=-3.257 \log(x)+14.994$	0·59	< 0·001
NAD-ME	*Distichlis spicata*	25	$y=-1.276 \log(x)+10.177$	0·18	0·037
NAD-ME	*Leptochloa dubia*	11	$y=-2.418 \log(x)+12.264$	0·94	< 0·001
NAD-ME	*Muhlenbergia wrightii*	23	$y=-3.868 \log(x)+16.853$	0·79	< 0·001
NAD-ME	*Sporobolus airoides*	23	$y=-3.518 \log(x)+16.493$	0·60	< 0·001
NAD-ME	*Sporobolus cryptandrus*	10	$y=-1.862 \log(x)+11.574$	0·50	0·022

Table 5. Carbon isotope discrimination as a function of light level calculated for 18 C_4 grasses of different biochemical C_4 subtypes (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme). Photon flux density data were log-transformed (base 10) for regression analyses

Carbon isotope discrimination of C_4 grasses grown under field conditions followed the same patterns as seen in glasshouse experiments

Results presented so far have focused on C_4 grasses grown under glasshouse conditions. Are similar patterns seen in plants growing in the field? Kephart, Buxton & Taylor (1992) studied the effect of light on growth of C_3 and C_4 perennial grasses under field conditions during 1984 and 1985. We were able to analyse the carbon isotope discrimination of leaf samples of *A. gerardii* and *Panicum clandestinum* from Kephart's study grown under three light regimes, from 690 to 1993 μmol photons m^{-2} s^{-1}. The NADP-ME grass, *A. gerardii*, showed more enriched $\delta^{13}C$ values than the NAD-ME grass, *P. clandestinum* (Fig. 3), which corresponds with higher carbon isotope discrimination we found in NAD-ME subtypes in glasshouse experiments 2 and 3. The $\delta^{13}C$ values of both species, field- and glasshouse-grown grasses, showed no light effect at high light environments. Light levels for grasses grown in the field never reached the critical level of below 700 μmol m^{-2} s^{-1} at which carbon isotope discrimination begins to increase (glasshouse experiment 3). Both field-grown C_4

Subtype	*n*	regression	*r*	*P*	SE slope	SE intercept
NADP-ME	66	$y=-1.08 \log(x)+6.652$	0·14	0·002	0·33	0·910
sun-watered	30	$y=-1.25 \log(x)+7.112$	0·22	0·008	0·44	1·215
sun-drought	11	$y=0.07 \log(x)+3.435$	0·0	0·948	1·05	2·916
shade-watered	17	$y=-1.57 \log(x)+8.063$	0·22	0·060	0·77	2·103
shade-drought	8	$y=-0.50 \log(x)+4.939$	0·08	0·490	0·69	1·931
PCK	73	$y=-1.44 \log(x)+8.936$	0·28	<0·001	0·27	0·731
sun-watered	31	$y=-1.19 \log(x)+8.008$	0·27	0·003	0·37	1·003
sun-drought	11	$y=-1.46 \log(x)+9.247$	0·21	0·162	0·96	2·517
shade-watered	21	$y=-1.30 \log(x)+8.744$	0·23	0·026	0·54	1·459
shade-drought	10	$y=-2.21 \log(x)+11.161$	0·69	0·003	0·53	1·394
NAD-ME[1]	158	$y=-2.64 \log(x)+13.515$	0·51	0·001	0·21	0·552
sun-watered	65	$y=-2.28 \log(x)+12.229$	0·54	<0·001	0·26	0·709
sun-drought	23	$y=-2.82 \log(x)+13.994$	0·59	<0·001	0·52	1·356
shade-watered	48	$y=-2.94 \log(x)+14.505$	0·61	<0·001	0·35	0·917
shade-drought	22	$y=-3.01 \log(x)+15.152$	0·46	<0·001	0·74	1·953

[1] Including *Muhlenbergia*.

Table 6. Carbon isotope discrimination as a function of light for three biochemical C_4 subtypes with different environmental histories (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme). Photon flux density data were log-transformed (base 10) prior to regression analyses

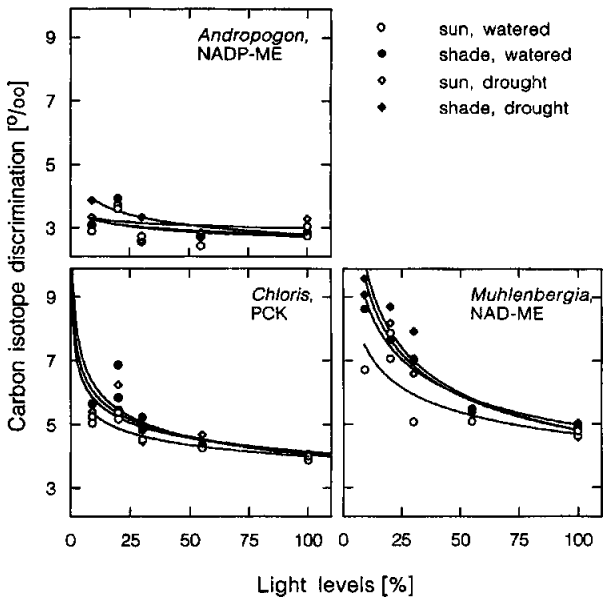

Figure 2. Response of carbon isotope discrimination to light is unrelated to past environmental growing conditions of C_4 grasses of different biochemical subtypes: *Andropogon gerardii* (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme), *Chloris gayana* (PCK, phosphoenolpyruvate carboxykinase), *Muhlenbergia wrightii* (NAD-ME, nicotinamide adenine dinucleotide malic enzyme). Regression analyses were performed with $\log_{10}$-transformed PFD data (*A. gerardii* sun-watered: $y=-0{\cdot}566 \log(x) + 4{\cdot}539$, $n=7$, $r=0{\cdot}24$, $P=0{\cdot}264$; sun-drought: $y=-0{\cdot}378 \log(x) + 4{\cdot}168$, $n=5$, $r=0{\cdot}12$, $P=0{\cdot}568$; shade-watered: $y=-0{\cdot}695 \log(x) + 4{\cdot}862$, $n=5$, $r=0{\cdot}29$, $P=0{\cdot}371$; shade-drought: $y=-1{\cdot}108 \log(x) + 6{\cdot}254$, $n=5$, $r=0{\cdot}91$, $P=0{\cdot}011$; *C. gayana* sun-watered: $y=-1{\cdot}353 \log(x) + 8{\cdot}214$, $n=8$, $r=0{\cdot}90$, $P<0{\cdot}001$; sun-drought: $y=-1{\cdot}756 \log(x) + 9{\cdot}583$, $n=5$, $r=0{\cdot}65$, $P=0{\cdot}101$; shade-watered: $y=-1{\cdot}535 \log(x) + 8{\cdot}986$, $n=5$, $r=0{\cdot}74$, $P=0{\cdot}063$; shade-drought: $y=-2{\cdot}346 \log(x) + 11{\cdot}310$, $n=5$, $r=0{\cdot}67$, $P=0{\cdot}090$; *M. wrightii* sun-watered: $y=-2{\cdot}293 \log(x) + 13{\cdot}077$, $n=7$, $r=0{\cdot}73$, $P=0{\cdot}015$; sun-drought: $y=-4{\cdot}609 \log(x) + 19{\cdot}087$, $n=5$, $r=0{\cdot}94$, $P=0{\cdot}006$; shade-watered: $y=-3{\cdot}740 \log(x) + 16{\cdot}623$, $n=6$, $r=0{\cdot}97$, $p<0{\cdot}001$; shade-drought: $y=-4{\cdot}801 \log(x) + 19{\cdot}787$, $n=5$, $r=0{\cdot}94$, $P=0{\cdot}007$).

grass species showed different carbon isotope ratios between the two years. The $\delta^{13}C$ ratios of field-grown *A. gerardii* were significantly higher than for the glasshouse plants.

DISCUSSION

The carbon isotope discrimination of a wide variety of C_4 grasses changed substantially in response to decreased light levels and water supply. The more the grasses were stressed by low light or limited water supply, the lower their $\delta^{13}C$ ratio and the higher their carbon isotope discrimination. This response occurred for all biochemical subtypes and species tested.

Carbon isotope discrimination exhibited a threshold response when light levels were decreasing (Figs 1 & 2). Discrimination remained constant in high-light environments, and then increased rapidly below 700 μmol photons m^{-2} s^{-1}. This pattern held for all three biochemical subtypes. Grasses of the NAD-ME subtype showed the largest responses to changes in environmental conditions, followed by the PCK subtype. The smallest changes in Δ were observed in NADP-ME subtype grasses. Peisker & Henderson (1992) presented a model to predict Δ values based on anatomical, biochemical and physiological characteristics of C_4 leaves. Their model predicted slightly decreased Δ and ϕ values with decreasing light for *Sorghum bicolor* (NADP-ME) until a PFD of about 500 μmol photons m^{-2} s^{-1} was reached. Below 500 μmol m^{-2} s^{-1}, an irradiance similar to our threshold light level, the model predicted steep increases of Δ and ϕ values. Our results broaden the spectrum of C_4 grasses for which this pattern was observed. Earlier studies have also noted more negative $\delta^{13}C$ values (higher discrimination) with decreased irradiances for *Panicum maximum* (NADP-ME) and *Echinochloa frumentacaea* (PCK) (Smith, Oliver & McMillan 1976; Osmond 1987; Wong & Osmond 1991). Henderson, von Caemmerer & Farquhar (1992) found increased short-term discrimination in very low light environments (below 240 μmol photons m^{-2} s^{-1}) for *Zea mays* (NADP-ME) and *Amaranthus edulis* (NAD-ME) but no changes in Δ for *S. bicolor*. Higher ϕ and Δ values under low-light conditions are thought to be caused by reduced Rubisco activity. This reduction would lead to a stronger expression of b_3, the photosynthetic fixation of CO_2 during the C_3 cycle (see Eqn 1).

Changing the water supply for C_4 grasses also resulted in increased Δ values (Table 4). This change in discrimination might be caused by decreased stomatal conductance and higher internal CO_2 concentrations at low water status. Similar to our results, Tieszen & Boutton (1989) presented a positive correlation of $\delta^{13}C$ values of African C_4 grasses with rainfall and leaf conductances. Of the two environmental variables examined, light levels influenced the $\delta^{13}C$ ratios more than water availability for the C_4 subtypes NAD-ME and PCK (Table 4). Δ values increased by up to 3·95‰ in response to light limitation while only increasing by 1·38‰ in response to water stress. Changes were less pronounced in NADP-ME grasses, ranging between –0·75 and +0·81‰. In any case, discrimination against ^{13}C did not stay constant during C_4 photosynthesis, a major assumption of studies using C_4 grasses as a proxy for changes in the isotopic composition of atmospheric CO_2.

Assuming constant c_i/c_a ratios (Wong, Cowan & Farquhar 1985a–c), the observed changes in carbon isotope discrimination could be explained by changes in leakiness. Bowman *et al.* (1989) showed for two NADP-ME grasses, *Zea mays* and *Andropogon glomeratus*, that the leakage (ϕ) increased with increasing water and salt stress. They calculated that up to 55% of the CO_2 which was fixed by PEP and transported into PCR tissues leaked out again. Hattersley (1982) attributed differences in $\delta^{13}C$ ratios between biochemical subtypes of C_4 grasses grown under well-watered and full-light conditions to an increase in leakiness associated with differences in anatomical

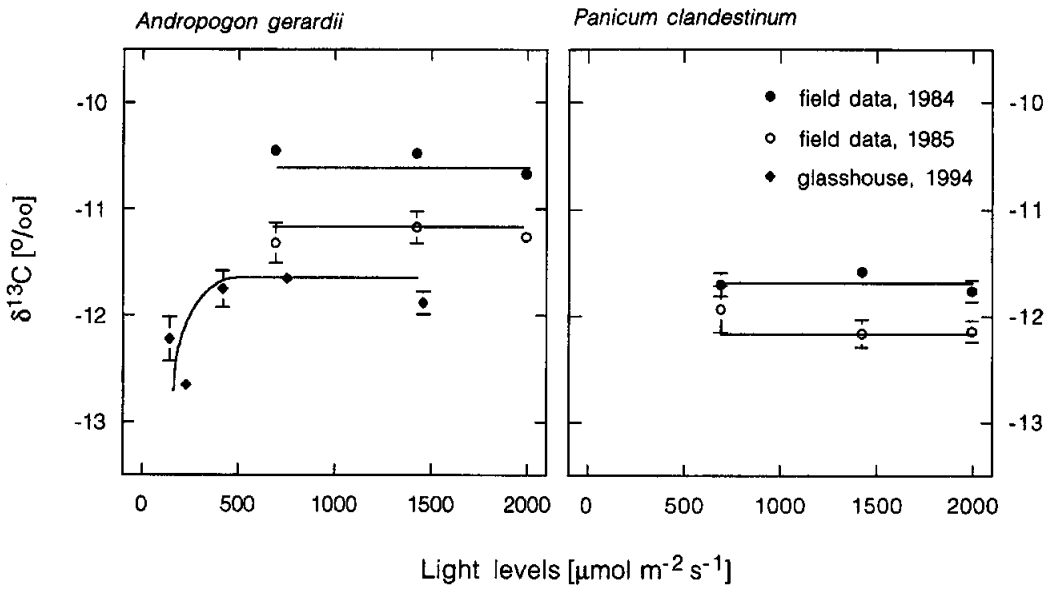

Figure 3. $\delta^{13}C$ ratios as a function of light for field-grown *Andropogon gerardii* and *Panicum clandestinum* (1984 and 1985 samples collected by Kephart and co-workers) and *A. gerardii* grown under glasshouse conditions (1994). Means and standard errors are presented ($n=4$ per species and light treatment in 1984 and 1985; $n=4$ or 5 for each light level in 1994). If error bars are absent, bars are smaller than symbol size.

features. He estimated ϕ to be 0·27 in NADP-ME, 0·34 in PCK and 0·43 in NAD-ME grasses (cited in Farquhar 1983). Ehleringer & Pearcy (1983) predicted leakage to be higher in NAD-ME than in NADP-ME or PCK species because of differences in quantum yield between subtypes. Using the model of Farquhar (1983) to estimate leakage for plants of experiment 2 (Eqn 1; with $a=4{\cdot}4‰$, $b_4=-5{\cdot}7‰$, $b_3=27‰$ and $c_i/c_a=0{\cdot}4$), we calculated increasing ϕ values ranging from 0·22 to 0·75 with increasing stress (Table 7). Leakage estimates for plants of experiments 2 and 3 were very similar and showed the same trend for the different biochemical subtypes. The highest leakage was calculated for NAD-ME plants, and the lowest for NADP-ME, with intermediate values for PCK grasses. These results suggest that the NADP-ME plants are most successful in reducing CO_2 leakage under a wide range of environmental conditions. Both anatomy and biochemistry could play a role in explaining the differences in discrimination we found between the subtypes. Ohsugi *et al.* (1988) observed more positive $\delta^{13}C$ ratios in *Panicum* species which possessed a suberized middle lamella than in *Panicum* species which lacked this cell wall property. Both NADP-ME and PCK subtypes possess a suberized middle lamella whereas NAD-ME does not. This might partially explain why NADP-ME and PCK have more positive $\delta^{13}C$ ratios than NAD-ME. On the other hand, leakiness in the PCK subtypes was greater than in NADP-ME species, in spite of similar anatomical features. The lack of a suberized middle

Table 7. Estimate of leakage (ϕ) of C_4 grass species of different biochemical subtypes (NADP-ME, nicotinamide adenine dinucleotide phosphate malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, nicotinamide adenine dinucleotide malic enzyme) in different environmental conditions (different light levels and water supply). Means ± standard errors (n) are given. The following factors were used for calculation of ϕ: $a=4{\cdot}4$ ‰, $b_3=27$ ‰, $b_4=-5{\cdot}7$ ‰, $c_i/c_a=0{\cdot}4$

		Treatments			
Subtype	Experiment	sun-watered	sun-drought	shade-watered	shade-drought
NADP-ME	2	0·22±0·009 (20)	0·34±0·008 (16)	0·28±0·017 (20)	0·33±0·021 (15)
	3	0·26±0·010 (19)		0·38±0·056 (8)	
PCK	2	0·34±0·017 (20)	0·43±0·009 (10)	0·48±0·017 (20)	0·55±0·004 (10)
	3	0·37±0·011 (19)		0·48±0·022 (13)	
NAD-ME	2	0·38±0·007 (35)	0·45±0·006 (13)	0·69±0·014 (35)	0·75±0·020 (15)
	3	0·42±0·005 (35)		0·65±0·016 (28)	

lamella in NAD-ME species was not compensated for by other anatomical characteristics (Hattersley & Browning 1981). Bowman *et al.* (1989) suggested that the extent of leakage was not dependent on cell wall properties as soon as the conductance to diffusion out of PCR tissues was sufficiently low. Their results showed that diurnal fluctuations in ϕ values were probably due to biochemical changes such as changes in the ratio of PEP carboxylase to Rubisco activity. However, contrasting results were presented by Henderson, von Caemmerer & Farquhar (1992), who found constant values for ϕ over a wide range of irradiances.

While carbon isotope discrimination of C_4 grasses was highly dependent on present environmental conditions, past light levels and water supply also had an effect. The time-dependent history effect seen in the first experiment will affect the use of C_4 grasses as a tool to monitor ambient CO_2 concentrations. If environmental conditions change substantially and grasses are grown over longer time periods (more than 2 months), the isotopic signal of C_4 plants will not only represent the present atmospheric conditions but also reflect water or light conditions during the entire time interval. Although this history effect would limit the use of C_4 plants as indicators in the field or in glasshouse studies, one can easily overcome the problem by frequently exchanging C_4 plant individuals.

Application of results from glasshouse studies to a field experiment revealed amazingly congruent patterns. C_4 grasses grown during two succeeding years in the field showed significantly different carbon isotope ratios, even though light treatments were similar (Fig. 3). On the basis of our water stress results, we would predict that 1984 was a wetter year than 1985. This prediction was confirmed by precipitation data given in Kephart, Buxton & Taylor (1992): 584 mm during March to July 1984 compared to 243 mm in 1985. This consistency shows that our glasshouse observations are applicable to non-controlled field conditions where the carbon isotope composition of C_4 grasses shows variations of a similar magnitude to those we measured in the glasshouse.

Furthermore, our results demonstrate a more limited application of C_4 plants as high-precision tools for climate reconstruction than implied by earlier studies with either cultivated, irrigated corn or *Atriplex* from packrat middens (Marino & McElroy 1991; Marino *et al.* 1992). We could not confirm the basic assumption that carbon discrimination of C_4 plants grown under a wide range of environmental conditions does not change. Instead, we observed substantial changes in Δ in response to water stress and light limitation, which reached about 60% of the variability of C_3 plants grown under the same environmental conditions (N. Buchmann, unpublished results). This variability in carbon isotope discrimination was most pronounced in NAD-ME subtypes and least obvious in grasses of the NADP-ME subtype. However, use of C_4 plants in climate reconstruction studies still has the potential to fill the gap between ice core measurements and recent measurements of carbon isotope ratios in atmospheric CO_2. Modelling techniques, such as those used with tree-ring analyses, could be used to account for the physiological variation in Δ of C_4 plants. The most promising scenario would involve C_4 plants using the NADP-ME subtype and situations where information about other environmental variables such as light or water is available.

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