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Differential ^{18}O enrichment of leaf cellulose in C_3 *versus* C_4 grasses

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Abstract. We show that differences in the oxygen isotope ratio of leaf water between C_3 and C_4 grasses (five species of each photosynthetic type) become less distinct as relative humidity increases, and that ^{18}O leaf water differences translate directly to the oxygen isotope ratio of leaf cellulose. A conceptual model is presented that is based on grass blade growth characteristics and observed patterns of progressive enrichment in grasses. The Barbour and Farquhar (2000) model was capable of explaining the oxygen isotope ratio of bulk leaf cellulose of C_3 and C_4 grasses grown under a variety of growth conditions.

Keywords: C_3 and C_4 photosynthesis, cellulose, grasses, oxygen isotopes, $\delta^{18}O$.

Introduction

Recently, Helliker and Ehleringer (2000) observed that oxygen isotope ratios ($\delta^{18}O$) of bulk leaf water (the whole grass blade, from ligule to tip) in C_3 and C_4 grasses grown in the same, controlled, low relative humidity environment, were significantly different from each other. Leaf water of C_4 grasses was consistently more ^{18}O enriched than in C_3 grasses, with the extent of this ^{18}O enrichment a linear function of both leaf length and interveinal distance. Our hypothesis was that differences in interveinal distance in grasses, in concert with progressive ^{18}O enrichment of leaf water along the leaf, account for the unexpected C_3/C_4 differences in bulk leaf water. This was largely a Péclet effect (Farquhar and Lloyd 1993; Barbour *et al.* 2000) where the smaller interveinal distances of C_4 grasses (smaller effective pathlength for back-diffusion) allowed for greater mixing of ^{18}O-enriched water in stomatal complexes and '^{18}O-unenriched' vein water. The greater mixing of enriched and unenriched water pools results in an overall greater ^{18}O enrichment moving up the leaf. This results in slightly more ^{18}O enriched bulk leaf water in C_4 grasses.

Previous survey studies on organic oxygen isotope ratios have identified a distinct 5–8‰ difference in the oxygen isotope ratio of leaf cellulose ($\delta^{18}O_{cl}$) values of C_3 and C_4 plants grown in the same environment (Sternberg and DeNiro 1983; Sternberg *et al.* 1984*a*, *b*). Because the previously observed $\delta^{18}O_{cl}$ differences between C_3 and C_4 plants were similar in magnitude to the leaf water differences observed by Helliker and Ehleringer (2000), we hypothesized that the C_3 and C_4 leaf water differences in grasses would be recorded directly as cellulose ^{18}O differences. However, previous cellulose studies had concentrated primarily on C_3 dicots and C_4 grasses, making it unclear whether those observed $\delta^{18}O_{cl}$ differences were the result of differences in photosynthetic pathway, plant microclimate associated with height differences, plant morphology, or combinations of these parameters. Additionally, Yakir *et al.* (1990*a*) showed no significant difference between the $\delta^{18}O_{cl}$ of well watered *versus* drought-stressed plants, even though there were substantial differences in the level of leaf water ^{18}O enrichment between the two treatments.

In aquatic leaves, where evaporative enrichment of leaf water is not a factor,

$$\delta^{18}O_{cl} = \delta^{18}O_{wl} + \varepsilon_O \quad (1)$$

where $\delta^{18}O_{cl}$ and $\delta^{18}O_{wl}$ represent the oxygen isotope ratio in leaf cellulose and bulk leaf water, respectively, and ε_O was approximately 27‰ (Yakir and DeNiro 1990). The fractionation factor, ε_O, is an equilibrium fractionation resulting from the exchange of the oxygen in a carbonyl group with the surrounding oxygen in medium water (DeNiro and Epstein 1981; Sternberg 1989). The oxygen isotope ratio of leaf water in aerial leaves is a function of known kinetic and equilibrium fractionation factors and the physical environment (Craig and Gordon 1965; Farris and Strain 1978; Farquhar *et al.* 1989; Yakir *et al.* 1990*b*; Flanagan *et al.* 1991). Additionally, considerable heterogeneity can develop

Abbreviations used: HH, MH, LH, high, medium and low relative humidity, respectively; p_{ex}, proportion of exchangeable oxygen atoms during cellulose synthesis; p_x, proportion of xylem and/or phloem water within the cells where cellulose is being synthesized; VPd, vapour pressure deficit; ε_O, fractionation factor; $\delta^{18}O_{cl}$, $\delta^{18}O_{wl}$, $\delta^{18}O_{wx}$, oxygen isotope ratio of leaf cellulose, bulk leaf water and source water, respectively.

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across the aerial leaves (Luo and Sternberg 1992; Yakir 1992; Wang and Yakir 1995). In this case, the final $\delta^{18}O_{cl}$ need not follow Eqn 1 if the $\delta^{18}O$ of bulk leaf water is not representative of the isotopic signature of water at the site of cellulose synthesis. For example, Yakir (1992) showed that leaf cellulose $\delta^{18}O$ of a maize leaf blade was 27.6‰, while leaf water $\delta^{18}O$ was 5.4‰, which is inconsistent with ε_O of 27‰.

To explain the final $\delta^{18}O$ signature of grass blade bulk leaf cellulose we must take into account both the developmental aspects of grass blade expansion and the dynamics of leaf water ^{18}O enrichment that are unique to grass blades (Fig. 1). With the exception of the absolute tip of the leaf, new growth in a grass blade occurs at the intercalary meristem located at the base of the leaf (Fahn 1990), so that the oldest portion of the leaf is the tip, and new cellulose is added at the base. Superimposed on this growth pattern is the large and distinct isotope enrichment that has been observed along the length of the leaf, where the water at the base of the leaf can be as much as 50‰ less enriched in ^{18}O than water at the tip (Helliker and Ehleringer 2000). Any model that explains the $\delta^{18}O$ of bulk leaf cellulose of grasses must take into account the production of sucrose across the leaf in an ^{18}O-enriched water environment, and the use of that sucrose to produce cellulose at the intercalary meristem, a relatively unenriched water environment.

Recently, Barbour and Farquhar (2000) developed a general biochemical model to explain $\delta^{18}O_{cl}$ in plants:

$$\delta^{18}O_{cl} = \delta^{18}O_{wx} + (\delta^{18}O_{wl} - \delta^{18}O_{wx})(1 - p_{ex} \times p_x) + \varepsilon_O \quad (2)$$

where $\delta^{18}O_{wx}$ represents the isotope ratio of source water, p_{ex} is the proportion of exchangeable oxygen atoms during

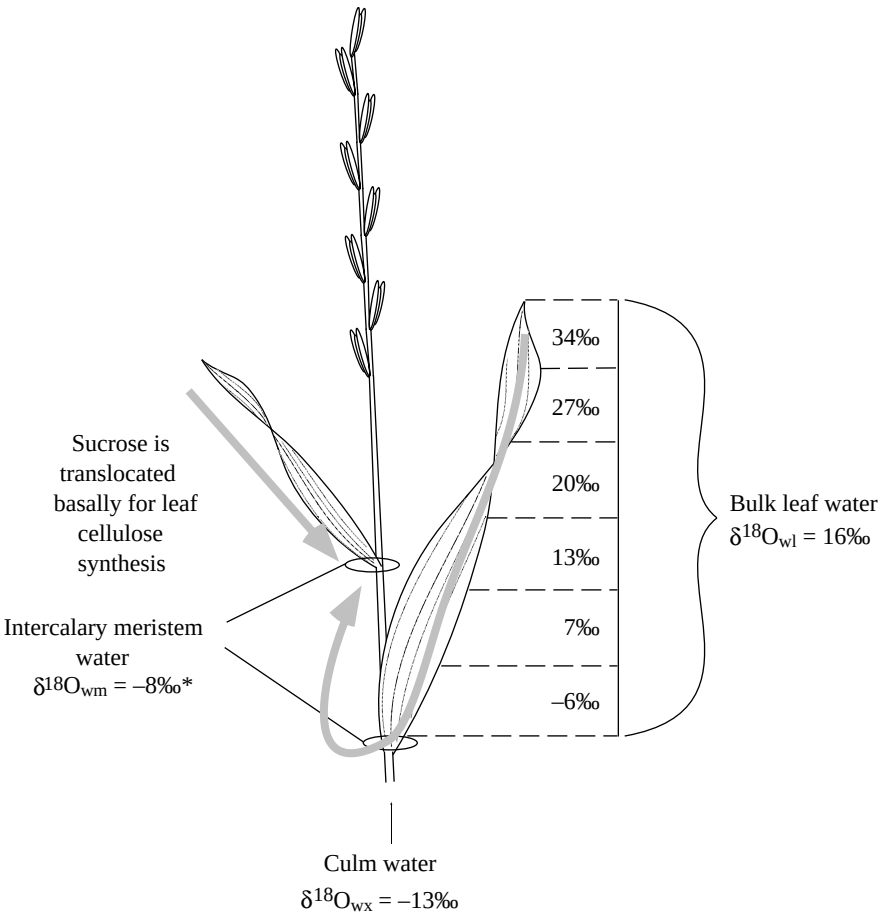

Fig. 1. Mechanistic model of cellulose formation in grass leaves. There is a high degree of progressive enrichment where the leaf tip may be 40‰ more enriched than the leaf base. Sucrose is formed in the highly enriched environment of the leaf and translocated to the intercalary meristem for cellulose synthesis. The intercalary meristem is much less enriched than bulk leaf water and the translocated sucrose. Hence, the cellulose formed is comprised of the oxygen isotope ratio of the enriched leaf water and the considerably less enriched meristem water. $\delta^{18}O$ water values are from *Zea mexicana* (teosinte). *, meristem water value was assumed, not measured.

cellulose synthesis, and p_x is the proportion of xylem and/or phloem water within the cells where cellulose is being synthesized. In a best-fit analysis, Barbour and Farquhar (2000) found $p_{ex} \times p_x$ to be 0.38 for cotton leaves. By applying the Barbour and Farquhar model to five C_3 and five C_4 grass species, we can access the ability of the model to explain the bulk leaf cellulose $\delta^{18}O$ in monocotyledonous leaves, which have a distinctly different leaf expansion pattern than dicotyledonous leaves.

In this study, the following questions were examined; (i) how are C_3/C_4 ^{18}O leaf-water differences influenced by relative humidity, (ii) are leaf water ^{18}O differences recorded as organic ^{18}O differences and, (iii) can we successfully model the $\delta^{18}O$ signature of bulk leaf cellulose (defined as the whole leaf from ligule to tip) of grass leaves with Eqn 2? It was expected that as the evaporative gradient for water loss decreased, overall ^{18}O leaf-water enrichment would also decrease and consequently, leaf water differences between the two photosynthetic types would diminish. Furthermore, it was expected that the leaf water $\delta^{18}O$ signature would be recorded in the organic $\delta^{18}O$ signature of bulk leaf cellulose, and that the $\delta^{18}O$ of bulk leaf cellulose would follow Eqn 2.

Materials and methods

Plant material and growth conditions

In our experiments, we used five C_3 grasses and five C_4 grasses (species listed in Table 1). For each species, $n = 3$ at each of three growth environments, which will be referred to as high (HH), medium (MH), and low (LH) relative humidity. The growth environment characteristics were; (i) HH with a 24-h mean relative humidity of 99% and temperature of 25.9°C, with a midday mean relative humidity of 96% and temperature of 31.8°C with a leaf-to-air vapour pressure deficit (VPd) of 0.2 kPa, (ii) MH environment with a 24-h mean relative humidity of 87% and temperature of 24.7°C, with a midday mean relative humidity of 81% and temperature of 30.1°C with a VPd of 0.8 kPa, and (iii) LH environment with a 24-h mean relative humidity of 37% and temperature of 22.1°C, with a midday mean relative humidity of 34% and temperature of 23.1°C and a VPd of 2.6 kPa. Midday means represent the 2 h of the photoperiod where maximum temperatures and minimum relative humidities were achieved. Mean source water and atmospheric water vapour $\delta^{18}O$ did not differ significantly between chambers and were –15.8 and –27.4‰, respectively.

Table 1. Leaf water $\delta^{18}O$ of C_3 and C_4 grasses

The $\delta^{18}O$ of bulk leaf water (from ligule to tip) for all grass species examined, sampled mid-way through the experiment. Each value represents a mean of three plants. C_3 and C_4 means are significantly different at a growth humidity if followed by * ($P<0.01$). Source water was –15.8‰

Growth conditions	$\delta^{18}O_{wl}$ (‰) HH	MH	LH
C_3 grasses			
Agropyron desertorum	–9.4 ± 1.6	–9.0 ± 0.9	7.7 ± 1.6
Agrostis stolonifera	–9.7 ± 1.1	–7.3 ± 1.3	9.2 ± 2.8
Alopecurus pratensis	–9.2 ± 1.7	–8.1 ± 1.3	8.3 ± 3.6
Bromus inermis	–9.0 ± 0.5	–7.2 ± 0.3	8.2 ± 1.7
Lolium multiflorum	–9.2 ± 0.8	–8.8 ± 0.6	8.0 ± 1.2
C_4 grasses			
Andropogon gerardii	–8.7 ± 0.8	–7.6 ± 0.3	15.1 ± 3.7
Bouteloua curtipendula	–6.7 ± 0.9	–7.7 ± 1.6	12.7 ± 0.3
Eragrostis curvula	–9.4 ±1.8	–8.2 ± 0.7	12.4 ± 4.2
Leptochloa dubia	–8.4 ± 1.2	–7.7 ± 1.2	14.2 ± 2.8
Muhlenbergia wrightii	–9.0 ± 1.1	–8.0 ± 1.7	9.0 ± 2.6
C_3 mean	**–9.3 ± 1.0**	**–8.1 ± 1.1**	**8.3 ± 0.5**
C_4 mean	**–8.4 ± 1.4**	**–7.8 ± 1.1**	**12.6 ± 3.2***

To maintain an isotopically-constant water source for all plants, plants were grown from germination in 1-L pots with a 1 : 1 : 1 sterilized soil/perlite/vermiculite mixture contained within 190-L open-top plastic chambers (stock tanks; Rubbermaid Inc., Wooster, OH, USA). The chambers had source-water entry and exit ports on either end, and tap water was fed to each chamber at a rate of approximately 8 mL s^{-1}. The water level in the chambers was kept constant at a level that submerged all but the upper 1.5 cm of the 1-L pots. Aquarium pumps (2–4) oxygenated the source water in each chamber, with the airflow separated into ten diffusers distributed evenly throughout the bottom of the chambers. Thermocouples (wet and dry bulb) were placed at plant height within each chamber to measure air temperature and relative humidity throughout the experiments. Photon flux density at plant height averaged approximately 1000 μmol m^{-2} s^{-1} (Li-Cor light sensor; Lincoln, NE, USA) throughout a 16-h photoperiod. Thermocouples and light sensor were averaged at 15-min intervals with a data logger (Campbell Scientific model 21X; Logan, UT, USA).

For high HH and MH growth environments, Plexiglas tops were sealed to the upper lips of the chambers. Relative humidity differences between the HH and MH treatments were achieved simply by varying the tightness of the seal between Plexiglas and the upper lip of the chambers. In both the HH and MH environments, the aquarium pumps were the sole source of O_2 and CO_2 for the plants. The $[CO_2]$ within these chambers was monitored (Li-Cor 6252) to assure that CO_2 remained near ambient concentrations. The Plexiglas chamber tops had two 20-cm^2 removable Plexiglas insertions. These insertions were replaced by soft plastic (Tygon; Saint-Gobain Performance Plastics, Akron, OH, USA) sampling ports during leaf water sampling. By sampling leaf water in this manner, any change in relative humidity or chamber temperature during the sampling period was prevented (data not shown). There was no Plexiglas top on the LH chamber; thus this chamber experienced Salt Lake City (UT, USA) ambient relative humidity. Closed-cell foam was placed at water level around each of the plants in the LH chamber to mitigate evaporative enrichment of source water.

Sample collection and isotope analysis

Leaf samples for water analysis were immediately sealed in glass vials after excision, and later extracted by cryogenic distillation (Ehleringer and Osmond 1989). With the exception of Figs 1 and 2, all leaf water and leaf cellulose $\delta^{18}O$ values represent the whole leaf blade from ligule to tip, and are referred to as 'bulk leaf water' ($\delta^{18}O_{wl}$) and 'bulk leaf cellulose' ($\delta^{18}O_{cl}$). Leaf water samples were taken at midday in the middle of the growth experiment (week 3 of 6). Leaf cellulose samples were taken at the end of week 6. Preliminary studies using these growth chambers showed that conditions were constant enough to result in no significant change in midday $\delta^{18}O_{wl}$ from week to week (Helliker and Ehleringer 2000). Samples of chamber inlet and outlet water were taken prior to sampling leaf material. All water samples were analysed by the CO_2:H_2O equilibration technique of Socki *et al.* (1992), and the isotope ratio was determined by on-column injection into a gas chromatograph (GC)–isotope ratio mass spectrometer

(IRMS) system in continuous flow mode (GC – Varian model 3300; Walnut Creek, CA, USA; MS – model 252; Thermo Finnigan, San Jose, CA, USA). The equilibration technique and GC–IRMS set-up are described in Helliker and Ehleringer (2000). Leaf materials for cellulose extraction were placed in coin envelopes and dried at 70°C. α-Cellulose was extracted from whole leaf material using methods of Leavitt and Danzer (1992) and Roden and Ehleringer (1999). The $\delta^{18}O$ of leaf α-cellulose was determined on a Delta S isotope ratio mass spectrometer (Thermo Finnigan) by the pyrolysis method described by Saurer *et al.* (1998) and Roden and Ehleringer (1999).

The Gat–Bowser model

Direct samples of progressive enrichment along the leaf were taken under LH conditions only. The Gat–Bowser model was used to predict progressive enrichment under HH and MH conditions. The 'string of lakes' equation developed by Gat and Bowser (1991) has been previously shown to adequately explain the progressive leaf water enrichment in grasses (Helliker and Ehleringer 2000):

$$\delta_n = \delta_{n-1} + \frac{\left(\delta_a + \frac{\varepsilon}{h}\right) - \delta_{n-1}}{1 + \frac{F \times (1-h)}{Eh}} \quad (3)$$

where the subscript n represents the nth element in a series, δ_a and δ_{n-1} refer to the $\delta^{18}O$ of atmospheric water vapour (measured as –27.4‰) and water entering a leaf section (for the first element at the base of a leaf we assumed δ_{n-1} was equal to source water, –15.8‰), respectively. Relative humidity is represented by h and $\varepsilon = \varepsilon_{eq} + (1 - h)\,\varepsilon_k$, where ε_{eq} (temperature dependent, 9.4‰ at 25°C) and ε_k (28.5‰) represent the equilibrium and kinetic fractionation factors, respectively. F and E represent the flux into, and the evaporative flux out of, an element. The assumptions to apply this model are isotopic steady-state conditions and well-mixed pools of water.

The evaporative flux (E) was assumed to be the same in each string element. There was no assumed capacitance and we assumed steady-state flux conditions where $F_1 = E_{total}$, $F_2 = E_{total} - E_1$, $F_3 = E_{total} - (E_1 + E_2)$, and so on. The value or units of F and E are unimportant under these assumptions as F is divided by E in the Gat–Bowser model so that units and order of magnitude cancel. The absolute number of elements in the series is important, because as segment numbers increase the level of ^{18}O enrichment within each segment decreases and the enrichment in the terminal segment increases, while not changing the mean $\delta^{18}O$ of all the segments combined (only when assuming E is constant along all segments; see Helliker and Ehleringer 2000). For predictions in Fig. 3, six series elements ($|n| = 6$) were used.

Results

δ18O leaf water

When grass blades were sectioned and the $\delta^{18}O$ of leaf water of each section was determined, all grass species showed large progressive enrichment along the leaf blade (Fig. 2). Some base-to-tip $\delta^{18}O$ differences exceeded 40‰ with an all species mean base-to-tip difference of 32‰. For a given species, the number of leaf segments (from 2–4 segments) was determined by overall leaf length, which varied substantially between species. Because of this, direct comparison of progressive ^{18}O leaf water enrichment across species was not possible. Leaf section samples were taken only at the LH conditions. For comparison at the MH and HH conditions, Gat–Bowser model predictions are also presented in Fig. 2.

The $\delta^{18}O$ of bulk leaf water (whole leaves, sampled from the ligule to the blade tip) of all species became more enriched as relative humidity decreased, as would be expected. In all growth conditions the mean C_4 value was more enriched than mean C_3 values. However only at the LH growth conditions were the differences significant ($P<0.01$; Table 1), with a mean C_3/C_4 difference of 4.3‰. Leaf temperatures were not measured in the HH and MH chambers. However, leaf temperatures measured in the LH chamber showed no significant differences across species (data not shown). Therefore, we assumed that any difference in transpiration that may have existed between species was not affecting leaf temperature and hence evaporative enrichment of ^{18}O.

δ18O leaf cellulose

The mean values of $\delta^{18}O$ of bulk leaf cellulose of C_3 and C_4 grasses at the HH growth environment were 16.2 and 17.1‰, respectively (Table 2). The same pattern and degree of separation held in the MH growth environment, where the C_3 and C_4 means were 15.1 and 16.4‰, respectively. In the LH experiment, leaf cellulose of C_3 grasses was 3–8‰ less ^{18}O enriched than C_4 grasses, with a mean difference of about 5‰, which is very similar to previously observed differences (Sternberg and DeNiro 1983; Sternberg *et al.* 1984*a*, *b*). In Fig. 3, we plotted the observed $\delta^{18}O_{cl}$ *versus* $\delta^{18}O_{wl}$ for all species at all growth conditions. The highly significant correlation ($P<0.0001$; $^{18}O_{cl} = 0.72\delta^{18}O_{wl} + 22.23$; $r^2=0.97$) implied that $\delta^{18}O_{wl}$ was the primary control of

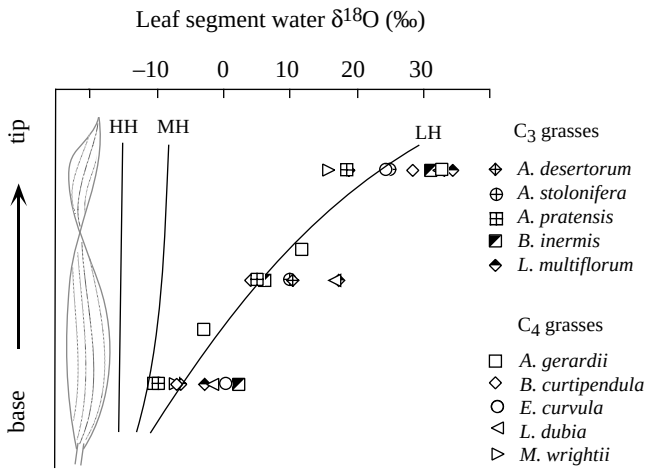

Fig. 2. $\delta^{18}O$ of leaf water for leaf segments of all grass species. Samples were taken in the LH experiment only. Segments do not correspond to any uniform length or water/mass quantity. For a given species, the number of leaf segments (2–4) was determined by overall leaf length, which varied substantially between species. Because of this, direct comparison of progressive enrichment across species was not possible. The lines indicate predicted evaporative enrichment of segments using the Gat–Bowser model (Eqn 3) at each growth relative humidity. Parameters for the Gat–Bowser model are listed in the text.

$\delta^{18}O_{cl}$. Predicted values of $\delta^{18}O_{cl}$ from Eqns 1 and 2 are plotted in Fig. 4. For predictions of $\delta^{18}O_{cl}$, we used observed $\delta^{18}O_{wl}$ from Table 1, ε_O = 27‰. $p_{ex} \times p_x$ was obtained by best fit and was 0.25.

Table 2. Leaf cellulose $\delta^{18}O$ of C_3 and C_4 grasses
The $\delta^{18}O$ of bulk leaf cellulose (from ligule to tip) for all grass species examined. Each value represents a mean of three plants. C_3 and C_4 means are significantly different at a growth humidity if followed by * (P<0.01)

Growth conditions	$\delta^{18}O_{cl}$ (‰) HH	MH	LH
C_3 grasses			
Agropyron desertorum	16.3 ± 1.4	15.1 ± 1.1	27.1 ± 0.3
Agrostis stolonifera	16.1 ± 0.1	15.5 ± 0.7	26.5 ± 1.0
Alopecurus pratensis	16.0 ± 0.5	13.9 ± 0.6	26.1 ± 0.4
Bromus inermis	16.9 ± 0.6	16.0 ± 0.3	27.4 ± 0.2
Lolium multiflorum	15.8 ± 0.7	15.0 ± 1.2	27.7 ± 0.7
C_4 grasses			
Andropogon gerardii	17.9 ± 1.3	17.0 ± 1.8	34.0 ± 0.1
Bouteloua curtipendula	16.9 ± 0.3	16.3 ± 0.8	32.5 ± 2.0
Eragrostis curvula	16.6 ± 0.2	15.5 ± 0.2	30.8 ± 1.1
Leptochloa dubia	17.3 ± 1.4	17.3 ± 0.5	34.1 ± 0.3
Muhlenbergia wrightii	16.9 ± 1.2	15.8 ± 0.8	30.9 ± 0.4
C_3 mean	**16.2 ± 0.4**	**15.1 ± 0.8**	**27.0 ± 0.7**
C_4 mean	**17.1 ± 0.5***	**16.4 ± 0.8***	**32.5 ± 1.6***

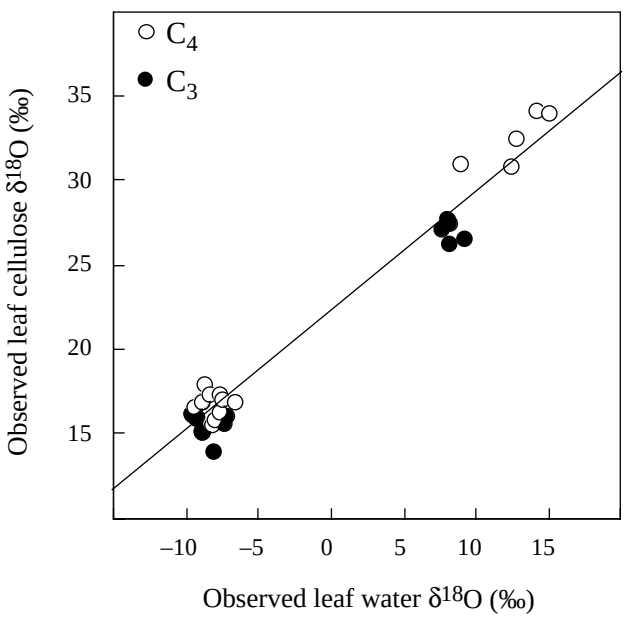

Fig. 3. Oxygen isotope ratios of observed bulk leaf cellulose ($\delta^{18}O_{cl}$) *versus* observed bulk leaf water ($\delta^{18}O_{wl}$) for all grasses at all growth RH. C_4 grasses are represented by open circles, C_3 grasses by closed circles. The least squares line is $\delta^{18}O_{cl} = 0.72^{18}O_{wl} + 22.23$; r^2=0.97. The relationship is significant at P<0.0001.

Discussion

Several studies have shown a distinct difference in $\delta^{18}O$ of leaf cellulose of C_3 and C_4 plants (Sternberg and DeNiro 1983; Sternberg *et al.* 1984*a*, *b*). Yet, a leaf water study by Flanagan *et al.* (1991) showed that there was no effect of photosynthetic pathway on the $\delta^{18}O$ of leaf water. It is suspected that the basis of this observation is that published ^{18}O cellulose studies had been conducted on C_3 dicots and C_4 grasses, whereas the ^{18}O leaf water study was a comparison of C_3 and C_4 dicots. One might conclude from these studies that the observed differences in leaf cellulose $\delta^{18}O$ between C_3 and C_4 plants were a result of differences in leaf water or source water between dicots and grasses, and/or differences in timing of metabolic activity (Sternberg 1989). In these studies, comparisons were made among plants that have different physiology, morphology and, potentially, phenology. Those concerns were avoided by working solely with C_3 and C_4 grasses grown in identical environments to elucidate leaf water and cellulose patterns of $\delta^{18}O$.

One of the initial predictions was that the ^{18}O leaf water differences between C_3 and C_4 grasses would diminish as relative humidity increased. The hypothesis was supported, because leaf water differences between C_3 and C_4 grasses were significant under the LH growth conditions.

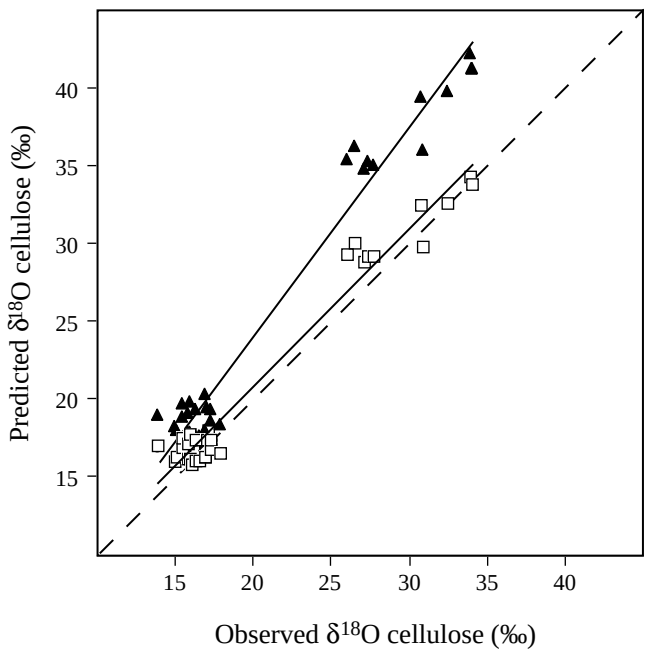

Fig. 4. Predicted leaf cellulose $\delta^{18}O$ *versus* observed leaf cellulose $\delta^{18}O$ for all grasses at all growth relative humidities. Open squares represent predictions from the model of Barbour and Farquhar (2000; $p_{ex} \times p_x$ = 0.25). Closed triangles represent predictions from Eqn 1 ($\delta^{18}O_{wl}$ + 27‰). Predictions of $\delta^{18}O_{cl}$ were obtained by using observed $\delta^{18}O_{wl}$ from Table 1 and ε_O = 27‰ for all calculations. The dashed line represents a 1:1 correlation.

Additionally, there was a definite trend for leaf water of C_4 grasses to be more ^{18}O-enriched than the leaf water of C_3 grasses at all growth conditions. Leaf water measurements were made at one point in time and hence, did not allow for an integrated picture of leaf water throughout the growth period. If it is assumed that $\delta^{18}O_{wl}$ was a primary factor in determining $\delta^{18}O_{cl}$, as Fig. 3 suggests, then cellulose measurements can be viewed as an integration of leaf water over the whole growth period. The $\delta^{18}O_{cl}$ values of C_4 grasses were significantly more enriched than those of C_3 grasses at all growth conditions, and ε_O was not different between photosynthetic types. Considering this, it was concluded that the leaf water of C_4 grasses was always more ^{18}O enriched than in C_3 grasses, even under growth conditions where the minimum relative humidity was as high as 96%.

In the HH growth condition, the leaf water differences between C_3 and C_4 grasses were small, and the $\delta^{18}O$ of water at the site of cellulose synthesis was very close to bulk leaf water, as indicated by the close fit of $\delta^{18}O_{wl}$ + 27‰ in the HH conditions (triangles in Fig. 4). These two results are important, as they show that ε_O was the same across a variety of C_3 and C_4 species and similar to a previously published value of 27‰. At low relative humidity in the LH growth conditions, the C_3/C_4 differences in $\delta^{18}O_{wl}$ became apparent, and this difference was manifested in distinctly different $\delta^{18}O_{cl}$ between C_3 and C_4 grasses.

Across all growth conditions and species, $\delta^{18}O_{wl}$ explained about 97% of the variation in $\delta^{18}O_{cl}$ (Fig. 3). However, the offset of $\delta^{18}O_{cl}$ from $\delta^{18}O_{wl}$ by 22‰ requires explanation. There is substantial evidence in the literature for a constant ε_O value of 27‰ across a range of plant types (Sternberg 1989; Yakir and DeNiro 1990) and chemical experimental systems (the labelling of acetone in water; Sternberg and DeNiro 1983; Sternberg 1989). When we subtracted mean $\delta^{18}O_{wl}$ from mean $\delta^{18}O_{cl}$ we obtained a value of 25.5‰ in the HH treatment, which was very near the previously observed value of 27‰. This value decreased to 23.7 and 19.3‰ in the MH and LH treatments, respectively. Additionally, ^{18}O data for both C_3 and C_4 grasses fell on the same line in Fig. 3, which suggests a common underlying pattern of cellulose synthesis in grasses that is independent of photosynthetic pathway. It is unlikely that the oxygen isotope fractionation factor (ε_O) from leaf water to cellulose decreased as relative humidity decreased. It is more likely that the heterogeneity of leaf water associated with the location of sucrose and cellulose synthesis tended to mask the full expression of ε_O. Or more succinctly, $\delta^{18}O_{wl}$ was an increasingly weak indicator of the $\delta^{18}O$ of water at the site of cellulose synthesis as relative humidity decreased.

During grass blade development, either from the apical meristem or from elongation at the intercalary meristem, cellulose precursors are imported from more mature leaves and/or from the metabolically active portion of the elongating leaf (Fig. 1). The $\delta^{18}O$ of sucrose formed in the leaf should be equal to $\delta^{18}O_{wl}$ + 27‰. During cellulose synthesis, some of the oxygen in sucrose will be exchanged with water at the meristem and will obtain the oxygen isotope signature of meristem water + 27‰. As relative humidity decreases, the $\delta^{18}O$ of meristem water and bulk leaf water should become less similar (Fig. 2). This would explain why, as relative humidity decreased, $\delta^{18}O_{wl}$ + 27‰ became a poorer predictor of $\delta^{18}O_{cl}$. The changing relationship between meristem water and bulk leaf water $\delta^{18}O$ could also explain that while the $\delta^{18}O_{wl}$ values of the MH treatment were intermediate of the HH and LH treatments, the $\delta^{18}O_{cl}$ values were not.

Barbour and Farquhar (2000) developed a general biochemical model to explain the $\delta^{18}O_{cl}$ of plant leaves, and successfully applied the model to cotton leaves. We applied this model to our results and found that it performed as well when applied to grass leaves (Fig. 4). When using a $p_{ex} \times p_x = 0.25$, we obtained a slope of 1 ($y = 1.007x + 0.6$) for predicted *versus* observed $\delta^{18}O_{cl}$ for grass leaves. For cotton leaves, Barbour and Farquhar found that $p_{ex} \times p_x = 0.38$. The different $p_{ex} \times p_x$ values between cotton leaves and grasses was expected, considering the different manner of leaf expansion between cotton and grass leaves. If p_{ex} values are bounded between 0.4 and 0.5, then the p_x values would range between 0.95 and 0.76 for cotton leaves. This estimation is well suited for dicot leaves, where all portions of dicot leaves expand more or less simultaneously, and where the leaves are fully expanded (or near full expansion) before becoming photosynthetically active. Hence, all of the water within the expanding dicot leaf could be xylem or phloem water. This is not the case for grass blade expansion. If the same boundaries are applied for grass leaves, then p_x would range between 0.62 and 0.5. This is reasonable considering that cell elongation occurs at the base of a physiologically active leaf where xylem and phloem water could readily mix with enriched leaf water.

There are several estimates in the literature for the proportion of oxygen atoms that are exchanged with water during cellulose synthesis (p_{ex}). Barbour and Farquhar (2000) showed theoretically that there need be no *a priori* assumption that p_{ex} is a fixed value, even within one plant. However, numerous studies show close agreement between estimates of p_{ex}, even across study systems that are as contrasting as cultured carrot cells and greenhouse-grown trees. The most commonly observed, empirically tested, values of p_{ex} range between 0.4 and 0.5 (Sternberg *et al.* 1986; Yakir 1992; Roden and Ehleringer 1999*a*, *b*; Barbour and Farquhar 2000). Roden *et al.* (2000) determined p_{ex} to be 0.42 in several tree species grown in controlled greenhouse conditions and in wide-ranging field conditions, thus representing the most robust estimation of p_{ex} in higher plants.

By using the Barbour and Farquhar model (Eqn 2) the $\delta^{18}O_{cl}$ of grass blade cellulose was successfully explained.

In the Barbour and Farquhar model, a single value for $p_{ex} \times p_x$ was found by the best-fit method that worked well for ten different grass species at three different growth conditions. This suggests that similar growth patterns are present in all the grasses tested, irrespective of photosynthetic pathway or growth conditions, and that both p_{ex} and p_x remain relatively constant across grass species. The leaf expansion patterns of grasses offer a unique method to directly test for the value of p_{ex}. If the $\delta^{18}O$ of water at the site of cellulose expansion is quantified over a range of growth conditions, hence obtaining an isotopic signature for p_x, then a value of p_{ex} can be constrained.

Based on the pattern of grass blade expansion, it is hypothesized that the length of the leaf blade may represent a historical 'isotopic record' of environmental characteristics. That is, longitudinally, ^{18}O of a grass blade may be directly analogous to ^{18}O variation across a tree ring. As such, there should be an isotopic record of seasonal environmental change along a grass blade. The testing of this hypothesis, as well as quantitatively sampling for the $\delta^{18}O$ of water in the expanding region of the grass blade will help refine the values of p_{ex} and p_x and further our understanding of oxygen isotope processes in grass organics.

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