RESEARCH PAPER

Relationship of leaf oxygen and carbon isotopic composition with transpiration efficiency in the C4 grasses *Setaria viridis* and *Setaria italica*

Patrick Z. Ellsworth, Patricia V. Ellsworth and Asaph B. Cousins*

School of Biological Sciences, Washington State University, PO Box 644236, Pullman, WA 99164-4236, USA

* Correspondence: acousins@wsu.edu

Received 21 April 2017; Editorial decision 11 May 2017; Accepted 26 May 2017

Editor: Howard Griffiths, University of Cambridge

Abstract

Leaf carbon and oxygen isotope ratios can potentially provide a time-integrated proxy for stomatal conductance (*g*<sub>s</sub>) and transpiration rate (*E*), and can be used to estimate transpiration efficiency (TE). In this study, we found significant relationships of bulk leaf carbon isotopic signature (δ<sup>13</sup>C<sub>BL</sub>) and bulk leaf oxygen enrichment above source water (Δ<sup>18</sup>O<sub>BL</sub>) with gas exchange and TE in the model C4 grasses *Setaria viridis* and *S. italica*. Leaf δ<sup>13</sup>C had strong relationships with *E*, *g*<sub>s</sub>, water use, biomass, and TE. Additionally, the consistent difference in δ<sup>13</sup>C<sub>BL</sub> between well-watered and water-limited plants suggests that δ<sup>13</sup>C<sub>BL</sub> is effective in separating C4 plants with different availability of water. Alternatively, the use of Δ<sup>18</sup>O<sub>BL</sub> as a proxy for *E* and TE in *S. viridis* and *S. italica* was problematic. First, the oxygen isotopic composition of source water, used to calculate leaf water enrichment (Δ<sup>18</sup>O<sub>LW</sub>), was variable with time and differed across water treatments. Second, water limitations changed leaf size and masked the relationship of Δ<sup>18</sup>O<sub>LW</sub> and Δ<sup>18</sup>O<sub>BL</sub> with *E*. Therefore, the data collected here suggest that δ<sup>13</sup>C<sub>BL</sub> but not Δ<sup>18</sup>O<sub>BL</sub> may be an effective proxy for TE in C4 grasses.

Key words: C4 photosynthesis, carbon isotopic composition, drought, gas exchange, oxygen isotopic composition, *Setaria italica*, *Setaria viridis*, stable isotopes, transpiration efficiency, water limitation.

Introduction

The bulk leaf carbon isotopic signature (δ<sup>13</sup>C<sub>BL</sub>) can potentially provide time-integrated proxies of stomatal conductance and transpiration efficiency (TE), where TE is defined as the quantity of carbon fixed per unit water lost through transpiration (for a glossary of terms, see Table 1). For example, δ<sup>13</sup>C<sub>BL</sub> has been successfully used in wheat breeding programs to screen for TE (Farquhar and Richards, 1984; Cabrera-Bosquet *et al.* 2009a, 2011; Yousfi *et al.*, 2012; Araus *et al.*, 2013) and has been studied in C4 species such as maize (Monneveux *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009b,c), sorghum (Henderson *et al.*, 1998), sugarcane (Saliendra *et al.*, 1996), and pearl millet (Brück *et al.*, 2000). Additionally, bulk leaf oxygen enrichment above source water (S) (Δ<sup>18</sup>O<sub>BL</sub>=δ<sup>18</sup>O<sub>BL</sub>−δ<sup>18</sup>O<sub>3</sub>) has been proposed as a proxy for transpiration rate (*E*) when comparing plants grown together under the same atmospheric and climatic conditions (Barbour *et al.*, 2000a; Condon *et al.*, 2004; Barbour, 2007). For example, Δ<sup>18</sup>O<sub>BL</sub> has been shown to vary with *E* in several crop species such as tea (Sheshshayee *et al.*, 2010), sunflower (Sheshshayee *et al.*, 2005), cowpea (Bindu Madhava *et al.*, 1999), and sunflower (Barbour, 2007)
and wheat (Cabrera-Bosquet et al., 2009a). However, to date C₄ plant-breeding programs have not generally used stable isotopes to phenotype or select for TE.

Variation in δ¹³Cᵦ in plants grown under the same climatic conditions is primarily determined by leaf photosynthetic CO₂ isotope discrimination:

\[
\Delta^{13}C_{BL} = \frac{\delta^{13}C_{ambient} - \delta^{13}C_{BL}}{1 + \delta^{13}C_{BL}/1000} \tag{1}
\]

where δ values are in ‰ notation and \(\delta^{13}C_{ambient}\) is the signature of available atmospheric CO₂. In C₄ plants, \(\Delta^{13}C\) is influenced by fractionations associated with diffusion of CO₂, carboxylation reactions, and the ratio of bundle sheath CO₂ leak rate to PEP carboxylase rate (leakiness, \(\phi\)), and it is proportional to the partial pressure of intercellular to ambient CO₂ \((C/\text{Ca})\). \(C/\text{Ca}\) is a measure of the supply of CO₂ to photosynthesis, and as \(C/\text{Ca}\) increases, discrimination decreases (for the simplified model; von Caemmerer et al., 2014):

\[
\Delta^{13}C_{BL} = a + (b_{4} + \phi(b_{3} - s) - a)\frac{C_{1}}{C_{a}} \tag{2}
\]

where \(a\) is the fractionation during diffusion of CO₂ in air through stomata (4.4‰), \(b_{4}\) is the combined fractionation of PEP carboxylation and the preceding isotopic equilibrium during dissolution and hydration of CO₂ (~5.2‰ at a leaf temperature of 30 °C) as described in Henderson et al. (1992), \(b_{3}\) is the fractionation by Rubisco (30‰), \(s\) is the fractionation during the leakage of CO₂ out of the bundle sheath cells (1.8‰), and \(\phi\) is the leakiness of CO₂ from the bundle sheath (Henderson et al., 1992, 1998).

The CO₂ concentrating mechanism in C₄ plants minimizes Rubisco fractionation, so the relationship between \(\Delta^{13}C\) and \(C/\text{Ca}\) in C₄ plants is dampened compared with C₃ plants and is less variable across growth conditions and genotypes (Henderson et al., 1998). Leakiness (\(\phi\)) determines the slope of the relationship between \(\Delta^{13}C\) and \(C/\text{Ca}\), controlling the directionality of this relationship from positive to negative. However, \(\phi\) has been shown to be relatively constant in many C₄ species, varying little across light intensities, temperatures, and CO₂ partial pressures (Ubierna et al., 2011; Sun et al., 2012; Kromdijk et al., 2014; Sage, 2014). Therefore, if \(\phi\) is relatively robust and constant across different growth conditions, then changes in \(\Delta^{13}C\) are primarily driven by variation in \(C/\text{Ca}\), which is influenced by both the net rates of CO₂ fixation (\(A_{\text{net}}\)) and stomatal conductance (\(g_{s}\)). For example, increasing \(A_{\text{net}}\) can draw down \(C_{a}\) relative to \(C_{s}\) and a reduction in \(g_{s}\) can decrease the supply of atmospheric CO₂ to the intercellular air space for photosynthetic assimilation. Since TE is also related to \(A_{\text{net}}\) and \(g_{s}\), this means that TE and \(\Delta^{13}C\) are linked through their relationship with \(C/\text{Ca}\), which makes \(\Delta^{13}C_{BL}\) a potential proxy for TE (Farquhar et al., 1989; Henderson et al., 1998).

Alternatively, oxygen isotopic enrichment above source water in leaf tissue (\(\Delta^{18}O_{BL}\)) comes partly from oxygen isotopic enrichment in leaf lamina water, a component of leaf water enrichment (\(\Delta^{18}O_{WL}\)), and where organic compounds are synthesized within the leaf. At these sites of carboxyl oxygen isotope exchange, the leaf water oxygen isotope signal is passed on to photosynthetic intermediates and consequently passed on to bulk leaf tissue (Barbour et al., 2000a, b; Gan et al., 2003; Barbour and Farquhar, 2004; Barbour, 2007). The model of Craig and Gordon (1965), which describes the evaporative isotopic enrichment of water from the surface of a water body, was modified to explain how oxygen isotopes in leaf water are enriched:

\[
\Delta^{18}O_{w} = e^{e^{e}} + \frac{e^{e}}{e^{e}} \left(\Delta^{18}O_{s} - e^{e}\right) \tag{3}
\]

where isotopic enrichment of water at the evaporation sites (\(\Delta^{18}O_{s}\)) is influenced by the isotopic enrichment above source water vapor (\(\Delta^{18}O_{v}\)), temperature-dependent gradient in the molar ratio of ambient to intercellular water vapor (\(e^{e}/e^{e}\)), temperature-dependent equilibrium fractionation (\(e^{e}\)), and kinetic fractionation (\(e^{e}\)), which is a function of stomatal and boundary layer conductances (Eq. 3 and more in-depth explanation in Appendix).
The Craig–Gordon model describes $\Delta^{18}O_e$, but tends to overestimate $\Delta^{18}O_{WL}$. To account for this overestimation, the Craig–Gordon model was modified to account for unenriched leaf xylem water and the mixing behavior between xylem and laminar water pools (Péclat and two-pool models: Farquhar et al., 1998; Roden and Ehleringer, 1999). The Péclat model suggests that $\delta^{18}O_{WL}$ reflects the relative isotopic contributions of advection of source water in the xylem and back diffusion of water from the sites of evaporation. The proportional mixing of source water and water from the evaporation sites is primarily determined by the transpiration rate ($E$) and the mean effective path length ($L$) through which water passes from the xylem to the stomates (Farquhar and Lloyd, 1993; Barbour and Farquhar, 2004). Therefore, if $L$ remains constant, $\Delta^{18}O_{WL}$ decreases as $E$ increases by decreasing the influence of $\Delta^{18}O_e$ on $\Delta^{18}O_{WL}$. Because $\Delta^{18}O_{BL}$ partly reflects $\Delta^{18}O_{WL}$, in the Péclat model where $\Delta^{18}O_{WL}$ is related to $E$, $\Delta^{18}O_{BL}$ can potentially provide an integrated proxy of $E$ over the life of the leaf (Barbour et al., 2000b; Barbour, 2007), and when coupled with biomass measurements can be a proxy for TE (Barbour et al., 2000a). However, support for the Péclat effect has not been found in many instances (Song et al., 2013; Roden et al., 2015; Song et al., 2015; Cernusak et al., 2016; Holloway-Phillips et al., 2016). Therefore, it is uncertain if $\Delta^{18}O_{BL}$ can be used as a proxy for $E$ in C₄ grasses.

In this study we tested the relationship between $\delta^{13}C_{BL}$ and $\Delta^{18}O_{BL}$ with TE and $E$, respectively, in the model C₄ grasses Setaria viridis (L.) P. Beauv. and S. italica (L.) P. Beauv. These species are part of the C₄ panicoid grass clade and are closely related to important food and biofuel crops, such as sugar cane, maize, miscanthus, and sorghum (Brutnell et al., 2010; Li and Brutnell, 2011). S. viridis is a unique model organism for this clade because it has a short lifespan, a sequenced genome and a single nucleotide polymorphism (SNP) map for quantitative trait locus analysis (Doust et al., 2009; Bennetzen et al., 2012). Additionally, S. viridis and S. italica are drought-resistant species, growing in areas that cannot support sorghum, sugar cane, or maize production (Li and Wu, 1996). In the current study, both species were grown under well-watered and water-limited conditions to determine the effect that water limitations had on $\delta^{13}C_{BL}$ and $\Delta^{18}O_{BL}$ to evaluate their use as proxies for $g_e$, $E$, and TE.

### Material and methods

#### Growth and greenhouse conditions

**Experiment with Setaria viridis**

*Setaria viridis* (L.) P. Beauv. (accession B-100; Li and Brutnell, 2011) was grown in a controlled-environment growth cabinet (Enconair Ecological GC-16). Growth conditions were set at 16 h photoperiod including a 2 h ramp at dawn and dusk and maximum PPFD of 1000 μmol quanta m⁻² s⁻¹. Day and night temperatures were maintained at 28 ± 1 and 18 ± 1 °C, respectively and a mean relative humidity of 59 ± 6%. Pot location was randomized every day. A total of 33 S. italica seedlings (11 plants per treatment with one seedling per pot) were transplanted into 7.5-liter pots at 15 d after germination. The potting soil was the same as was used with *S. viridis*. Plants received 15-5-15 CalMag (JR Peters Inc., Allentown, PA, USA) twice weekly at a rate of 2.5 g l⁻¹ water. The Scotts Soluble Trace Element Mix, 10.0 mg l⁻¹ biweekly (The Scotts Co., OH, USA).

After initial watering after transplanting, the GWC of the well-watered, moderately and severely water-limited treatments was maintained at 4.0, 0.9, and 0.5 g water g⁻¹ soil⁻¹, respectively. Pots were covered with plastic similar to *S. viridis*. Six and five plants from each treatment were randomly selected to be harvested in the first and second collections, respectively. Leaf gas exchange measurements were made at six time points (31, 34, 40, 43, 53, and 54 d after germination). Plant material for stable isotope analysis was only collected at the final harvest, immediately following gas exchange measurements. At all collection times, panicles had begun to emerge.

**Gas exchange measurements**

Measurements were made on the uppermost fully expanded leaf between 11:00 h and 15:00 h. Leaves were placed in a 2 X 3 chamber of an LI-6400XT open gas exchange system (LI-COR Biosciences, Inc., Lincoln, NE, USA). The leaf was allowed to acclimate at 1500 μmol m⁻² s⁻¹ PPFD, leaf temperature of 29 °C, flow rate of 300 μmol s⁻¹, 21% O₂, 35 Pa CO₂ for *S. viridis*. The same conditions were used for *S. italica* except that light intensity was 900 μmol m⁻² s⁻¹ PPFD to reflect the light intensity of their growing conditions. Relative humidity (RH) in the LI-COR chamber was within 10% of the RH under growth conditions, and the implications of this difference in RH is explained in the next section.
Sample collection for stable isotope analysis

To obtain sufficient leaf water to measure δ18O of leaf water (δ18O\textsubscript{LW}) from S. viridis, an aggregate of five to eight leaves (including the leaf used to measure gas exchange) was collected from each plant at the time of harvest. However, in S. italic\(\)a the leaf used for gas exchange measurements was sufficient to analyse δ18O\textsubscript{LW}. The same leaf samples that were analysed for δ18O\textsubscript{LW} were also analysed for leaf carbon isotopic composition (δ13C\textsubscript{C\textsubscript{\text{cell}}} and oxygen isotopic composition of bulk leaf tissue (δ18O\textsubscript{BL}). All leaves collected for stable isotope analysis were the youngest, fully expanded leaves, which developed 15–20 d after soil water content reached the treatment set point. For both species, leaves were removed from the plant and photographed to measure leaf area using ImageJ software (Schneider et al., 2012). Photographing each leaf only took approximately 20 s, and then the leaf was stored in sealed glass tubes awaiting water extraction.

In S. italic\(\)a only, using the same leaf for gas exchange measurements and stable isotope analysis could have an effect on δ13O\textsubscript{LW} because RH and the oxygen isotope ratio in the growth cabinet air (δ18O\textsubscript{G}) could differ between growth and gas exchange chambers (Loucos et al., 2015). However, gas exchange measurements were conducted within the growth chamber, and the mean±SE proportion of the total leaf area continuously exposed to growth cabinet conditions during gas exchange measurements was 94.0 ± 0.6%, 91.0 ± 0.8%, and 89.5 ± 0.8% for well-watered, moderately water-limited, and severely water-limited treatments, respectively. Therefore, the δ18O of the growth cabinet air was the most appropriate measure of δ18O\textsubscript{BL}. The difference in RH between the growth cabinet and the gas exchange chamber could contribute a 1.9–5.5‰ shift in δ18O, for the leaf section in the gas exchange chamber, assuming, however unlikely, that 10–20 min was adequate time for the leaf section to acclimate. This would represent a shift in δ18O for the entire leaf of 0.24–0.43‰, and δ18O\textsubscript{LW} would shift by a fraction of this. Nonetheless, taking into account this potential shift did not significantly influence observed Δ18O values across treatments. Differences between greenhouse and LI-COR chamber conditions for S. viridis were irrelevant because leaf water was extracted from an aggregate of five to eight leaves.

The δ18O of water vapor in the greenhouse and growth chambers was measured every 30 min during gas exchange measurements by collecting air in 5-liter Supel inert foil gas sampling bags (Supelco, Bellefonte, PA, USA). Bags were flushed several times with air before filling, and δ18O of water vapor was immediately measured on the cavity ringdown spectrometer (L1102-i water analyser, Picarro Inc, Santa Clara, CA, USA). Following the gas exchange measurements, root crowns were cleaned of soil and stored at −20 °C in air-tight tubes. Root crowns are considered the bottom 1 cm of the culm or tiller, but no actual roots were collected. Additionally, two soil samples were collected at the top and bottom of each pot, and stored using the same method as with the root crowns. Leaves, soils, and root crowns were distilled using a cryogenic vacuum distillation method (Vendramini and Sternberg, 2007). Additionally, daily samples of irrigation water (IW) were collected to measure δ18O\textsubscript{IW}.

Biomass measurements

After collection of plant tissue for stable isotope analysis, the entire aboveground biomass was collected and weighed for fresh weight. Samples were dried at 65 °C for 3 d before weighing dry biomass.

Stable isotope analysis

The stable isotope composition of carbon and oxygen (δ18O and δ13C, respectively) were reported in δ notation in parts per thousand (%),

\[
\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 10^3
\]

where \(R_{\text{sample}}\) and \(R_{\text{standard}}\) are the molar ratios of heavy to light isotope (δ18O/δ16O and δ13C/δ12C) of the sample and international standard, respectively. The international standard used for oxygen was Vienna Standard Mean Ocean Water (VSMOW) and for carbon was Vienna Pee Dee Belemnit\(\)e (VPDB).

Stable isotope analysis by isotope ratio mass spectrometer

Leaf tissue was analysed for oxygen isotopic analysis by converting to CO with a pyrolysis elemental analyser (TC/EA, Thermo Finnigan, Bremen, Germany) and analysed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Thermo Finnigan; Brenna et al., 1997; Gehre and Strauch, 2003). For carbon isotopic analysis, leaf tissue was converted to CO\textsubscript{2} with an elemental analyser (ECS 4010, Costech Analytical, Valencia, CA, USA) and analysed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP; Brenna et al., 1997; Qi et al., 2003). Isotopic standards, Standard Light Antarctica Precipitation (SLAP; Gonfiantini, 1978) and Puerto Rico water (Qi et al., 2014), were analysed alongside samples to calculate δ18O based on the VSMOW scale. Lab standards, calibrated to international standards, were used to calculate δ13C relative to VPDB. Standard error for δ13C values for the S. viridis and S. italic\(\)a experiments was 0.11 and 0.05‰, respectively. The standard error for δ18O was 0.2‰ for both experiments.

Leaf and root crown water were analysed for oxygen isotope composition by equilibrating 0.5 ml of water at room temperature with 0.3% CO\textsubscript{2}/He mixture for 48 h on a Thermo Finnigan GasBench II (Thermo Electron Corp., Bremen, Germany). CO\textsubscript{2} was analysed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP; Brenna et al., 1997; Qi et al., 2003).

Stable isotope analysis by isotope ratio infrared spectroscopy

Soil and irrigation water were measured by isotope ratio infrared spectroscopy (model L1102-i, Picarro, Sunnyvale, CA, USA) connected to a vaporization chamber (V1102-i). The mean δ18O was calculated of the last three of six consecutive analyses on each sample. Three laboratory standards, calibrated to the VSMOW scale, were interspersed among the samples and were used to correct the sample δ18O values to the VSMOW scale. Water vapor was analysed for at least 15 min, but the mean of the last 5 min was used for δ18O and correct to the VSMOW scale.

Calculations of transpiration efficiency

\(TE_{\text{instantaneous}} (A_{\text{ref}}/E)\) and \(TE_{\text{instantaneous}} (A_{\text{ref}}/g)\) are derived from gas exchange measurements, but they are independent of both \(TE_{\text{plant}}\) and \(TE_a\), which are independent of each other. \(TE_{\text{plant}}\) was derived from whole-plant measures of biomass and transpiration. \(TE_a\) was calculated using \(C/\bar{C}\) derived from δ13C\textsubscript{BL} and the calculations are independent of the gas exchange measurements (described below).

Discrimination (Δ13C\textsubscript{BL}) and \(C/\bar{C}\) are related in Eq. 2 and were used to calculate the integrated \(C/\bar{C}\) over the life of the leaf (Henderson et al., 1992, 1998). A constant leakiness (ϕ) of 0.21 was assumed for all plants. The Δ13C of ambient CO\textsubscript{2} in the greenhouse and growth chamber that was used to calculate Δ13C\textsubscript{BL} was −10.7 ± 0.8‰, which was collected various times over a period of several months that included the time that the experiment was conducted. Air samples were collected in 5-liter Tedlar gas sampling bags using the same collection procedure used to collect air vapor. The gas was analysed by introducing air directly into either the isotope ratio mass spectrometer or the tunable diode laser absorption spectroscopy (model TGA 200A, Campbell Scientific, Inc., Logan, UT, USA; Ubierna et al., 2013). Transpiration efficiency (\(TE_a\)), defined as the ratio of dry matter produced per unit of water transpired, was calculated from the δ13C\textsubscript{BL} as described in Henderson et al. (1998) as:
where $v$ is the leaf to air vapor pressure difference and $\phi_v$ was calculated as the measured ratio of respiration and photosynthetic rate (0.08 for all plants). This parameter was calculated from gas exchange measurements made during the experiment. The parameter $\phi_w$ was calculated as the measured ratio of whole-plant night to day transpiration (0.30 and 0.23 for water-limited and well-watered plants, respectively) measured in this experiment. Both night-time and daytime whole-plant transpiration were measured on $S$. viridis. Both $\phi_v$ and $\phi_w$ were measured for $S$. viridis and assumed to be the same for $S$. italica. $C/\phi_v$ was calculated from $^{18}$CBL using Eq. 2.

Model calculations to determine validity of the Péclet model

To test the applicability of the Péclet model and its relationship to $E$, we used the method described by Holloway-Phillips et al. (2016) and Song et al. (2015) of determining the proportional deviation ($f$) of $\Delta^{18}$O$_{IW}$ from $\Delta^{18}$O$_{p}$ plotted against $E$ where $f$ is calculated as:

$$f = 1 - \frac{\Delta^{18}O_{IW}}{\Delta^{18}O_{p}}$$

Effective path length ($L$) was calculated using the equations described in Song et al. (2013).

Statistical analysis

In both experiments statistical analyses were conducted in R v. 3.3.0 (R Core Team, 2013), using car (v. 2.0-26) and agricolae (version 1.2-2) packages for statistical tests. Model II regressions (standard major axis regression) were calculated, using the lmmodel2 (v. 1.7-2) package, because neither variable was controlled, both varied naturally with their own associated error, and the physical units of both variables were not the same. Homogeneity was tested based on plotting predicted fit vs residuals. Using the extRemes package (v. 2.0-8), normality was tested by plotting residuals on quantiles-quantiles plots. In all cases, where normality was questionable, transforming the data did not change the statistical results, so the data were not transformed. One- and two-way analysis of variance (ANOVA) was used to determine differences across treatments in the experiment with $S$. italica and between treatments and collection periods for $S$. viridis. Two-sample Student’s $t$-tests were performed to determine the difference between $\delta^{18}$O$_{NW}$ and $\delta^{18}$O$_{RC}$ values. One-sample $t$-tests were performed to determine if the difference of both $\delta^{18}$O$_{NW}$ and $\delta^{18}$O$_{RC}$ with $\delta^{18}$O$_{PL}$ was significantly different from 0. Repeated measures ANOVAs were conducted on the following parameters: total plant water use and GWC for $S$. viridis and on daily water use, GWC, $A_{net}$, $E$, and $g_s$ for $S$. italica.

Results

Plant growth and treatment effect

Maintaining water-limited plants at a GWC 71% lower than the well-watered plants significantly reduced total water use by 59% (Table 2 and Fig. 1). Additionally, at all collection times fresh and dry aboveground biomass were lower in the water-limited treatment relative to the well-watered treatment (Table 3). The results for $S$. italica were similar to $S$. viridis in that the GWC was reduced by 72% and 82%, and total water use by 70% and 80% in the moderately and severely water-limited treatments, respectively (Table 2; Fig. 1). In $S$. italica, fresh and dry aboveground biomass were reduced in the moderately water-limited, 71% and 68%, respectively, and in the severely water-limited treatment, 79% and 74%, respectively (Table 4; Fig. 1). Additionally, leaf length in the water-limited treatments was shorter compared with the well-watered leaves by 18% in $S$. viridis and in $S$. italica by 32% and 57% in the moderately and severely water-limited treatments, respectively (Tables 3 and 4). The number of tillers per plant in the well-watered treatment was 44% greater than the water-limited treatment in $S$. viridis (Table 3); however, the number of tillers per $S$. italica plant did not differ across treatments (Table 4).

For both species, stomatal conductance ($g_s$), rates of transpiration ($E$), and the net rate of CO$_2$ assimilation ($A_{net}$) were generally higher in the well-watered treatment compared with the water-limited treatment. Gas exchange measurements of $S$. viridis were made during the three biomass collections, and $E$ was 33% and 67% greater in the well-watered treatment in collections 2 and 3, respectively, but did not differ between treatments in collection 1 (Table 3). Additionally, $g_s$ was 41% and 76% greater in the well-watered treatment during collection 2 and 3, respectively, but did not differ between treatments in collection 1. However, $A_{net}$ in $S$. viridis was different

Table 2. Statistical summary for repeated-measures ANOVA of variables measured throughout the experiments

Levels of significance were calculated from two-factor repeated measures ANOVA described in ‘Materials and methods’, *$P$<0.05, **$P$<0.01, and ***$P$<0.001, ns not significant ($P$>0.05).
between treatments only in collection 3 when $A_{net}$ of water-limited plants was 61% lower than that of the well-watered treatment (Table 3). In $S. \text{italica}$, $E$, $g_s$, and $A_{net}$ were not different between the severely and moderately water-limited treatments, but both treatments were on average 27%, 32%, and 16% lower, respectively, than the well-watered treatment (Table 4).

**Leaf carbon isotopic composition**

The response in leaf carbon isotopic signature ($\delta^{13}C$) to water limitations was similar for both species. For example, $\delta^{13}C$ values in $S. \text{viridis}$ were consistently lower in the water-limited treatment for all collections by 1.1‰ (Table 3) and in $S. \text{italica}$ the $\delta^{13}C$ values were 0.9‰ lower in the two water-limited treatments compared with the well-watered treatments (Table 4). The $\delta^{13}C$ values of both species were positively correlated with $A_{net}$, $E$, $g_s$, leaf water content, $\Delta^{18}O_{BL}$, and all measurements of TE and plant growth and had stronger correlations with these parameters than either $\Delta^{18}O_{LW}$ or $\Delta^{18}O_{BL}$ (Table 5 and Figs 2 and 3).

Using the simplified equation (Eq. 2) of $\Delta^{13}C$ versus $C_i/C_a$, where $\Delta^{13}C$ was calculated from $\delta^{13}C_{BL}$ and $C_i/C_a$ was measured, the mean leakiness of 0.21 ± 0.02 and 0.19 ± 0.01 was calculated for $S. \text{viridis}$ in the well-watered and water-limited treatments, respectively. In $S. \text{italica}$ leakiness was 0.17 ± 0.01 for well-watered and severely water-limited treatments and 0.20 ± 0.01 in the moderately water-limited. Overall, leakiness did not significantly differ between species or across treatments (0.17–0.21). The difference in leakiness would account for 0.22 ± 0.03‰ and 0.24 ± 0.01‰ of the observed difference in $\Delta^{13}C_{BL}$ between treatments over the observed range of $C_i/C_a$ in $S. \text{italica}$ and $S. \text{viridis}$, respectively.

**Transpiration efficiency (TE) and leaf $\delta^{13}C$-derived transpiration efficiency (TEw)**

Four different methods were used to calculate transpiration efficiency: (i) long term TE (TE$_{\text{plant}}$; grams of aboveground dry biomass per liter water transpired); (ii) instantaneous TE (TE$_{\text{instantaneous}}$; $A_{net}/E$); (iii) intrinsic TE (TE$_{\text{intrinsic}}$; $A_{net}/g_s$); and (iv) $\delta^{13}C_{BL}$-derived TE (TE$_{\text{w}}$; mmoles carbon fixed per mole H$_2$O transpired). For $S. \text{viridis}$ the water-limited plants had higher TE regardless of how it was estimated, except in collection 3 where there was no difference in TE$_{\text{instantaneous}}$ between treatments (Table 3). In $S. \text{italica}$, all four estimates of TE were higher in both water-limited treatments than the well-watered treatment, but the difference was not significant between the moderately and severely water-limited treatments. In both species, the TE$_{\text{intrinsic}}$ had the largest differences between treatments (45% and 32% greater in $S. \text{viridis}$ and $S. \text{italica}$, respectively; Tables 3 and 4). Additionally, TE$_{\text{intrinsic}}$ had the strongest relationship with $\Delta^{18}O_{LW}$, $\Delta^{18}O_{BL}$, and $\delta^{13}C$ (Table 5).

**Leaf oxygen isotopic composition**

The gas exchange measurements and leaf samples were collected between 11:00 and 15:00 h. During this time the vapor oxygen isotope ratios ($\delta^{18}O$) in both the greenhouse for $S. \text{viridis}$ and in the growth chambers for $S. \text{italica}$ were relatively stable. For $S. \text{viridis}$, $\delta^{18}O$ values (mean±SE) were −17.4 ± 0.2, −21.9 ± 0.4, and −24.2 ± 0.3 for the...
three collections, respectively. For *S. italic*α, δ18Ov values were −25.8 ± 0.1 and −20.1 ± 0.8 for the two collections, respectively.

Plants were top irrigated in covered pots with irrigation water (~17.0 ± 0.1‰ and −16.9 ± 0.1‰ for *S. viridis* and *S. italic*, respectively). The mean δ18Osw values (average of δ18O of soil samples from the top and bottom of the pot) of the well-watered and water-limited treatments were 0.8 ± 0.1‰ and 1.9 ± 0.2‰ higher than irrigation water for *S. viridis* (P < 0.0001), respectively. For *S. italic*, δ18Osw values were 0.8 ± 0.2‰, 2.7 ± 0.2‰, and 3.9 ± 0.4‰ higher than irrigation water in the well-watered, moderately and severely water-limited treatments (P < 0.0001), respectively. For *S. italic*, δ18O values were 1.1 ± 0.1‰, 3.1 ± 0.2‰, and 4.3 ± 0.3‰ higher than the irrigation water in the well-watered, moderately and severely water-limited treatments (P < 0.0001), respectively. The δ18O of root crown and soil water was not significantly different in either *S. viridis* or *S. italic* (P > 0.05).

Leaf water enrichment (Δ18OLw) showed a significant treatment effect in *S. viridis* independent of which water was considered source water (irrigation, soil, or root crown water), but the treatment effect on Δ18OLw was greatest with irrigation water. Likewise the strength of the correlation between parameters of gas exchange, water use, and growth depended on which source water was used to calculate Δ18OLw. However, independent of the source water, Δ18OLw negatively correlated with Δ13Cbl and Δ18Obl when irrigation water was used as source water (*S. italic*, Table 5 and Fig. 4). In *S. italic*, Δ18OLw did not have a significant treatment effect or correlate with growth, gas exchange, or TE variables, regardless of source water used (Table 4).

In *S. viridis*, a significant treatment effect in Δ18OLbl was only found and Δ18OLbl only correlated with g, E, fresh aboveground biomass, TEplant and TEintrinsic, Δ18OLw, and Δ13Cbl when irrigation water was used as source water (Table 5 and Fig. 4). The only parameter that correlated significantly with Δ18OLbl when root crown water was considered the source water was Δ18OLw. For *S. italic*, the Δ18OLbl in the well-watered treatment was 2.0‰ greater than the severely water-limited treatment, but Δ18OLbl of the moderately water-limited treatment was not different from either treatment (Table 4). This difference resulted in significant positive correlations with E and g (0.60 and 0.59, respectively) when negative correlations were expected.
The Péclet model was tested by comparing the proportional deviation of \( \Delta^{18}O_{LW} \) from \( \Delta^{18}O_e \) (\( f \); Eq. 6) with \( E \) (see Supplementary Fig. S1 at JXB online). This relationship was significant for \( S. \ viridis \) (\( f =0.086E-0.221, R^2=0.36, P=0.0005 \)) but not for \( S. \ italica \) (\( P=0.93 \)) suggesting that in \( S. \ viridis \) \( \Delta^{18}O_{LW} \) deviated more from \( \Delta^{18}O_e \) as \( E \) increased.
For *S. italica*, the $\Delta^{18}O_{\text{LW}}$ was larger than $\Delta^{18}O_e$ (negative $f$ values), causing $\Delta^{18}O_{\text{LW}}$ to be more enriched than would be expected based on the evaporative environment. For *S. viridis* the estimated effective leaf length ($L$) was small but significantly different between treatments (13.2 ± 2.7 and 8.2 ± 2.4 mm in well-watered and water-limited treatments, respectively; Supplementary Tables S1 and S2).

**Discussion**

**Leaf carbon isotopic composition**

Water-limited C_4 plants consistently have lower $\delta^{13}C_{\text{BL}}$ values than well-watered plants (Henderson *et al.*, 1998; Ghananoum *et al.*, 2002). Water limitations also typically reduce $g_s$ and in C_4 plants low $g_s$ results in depleted $\delta^{13}C_{\text{BL}}$. This is primarily...
because $\Delta^{13}$C_{BL} decreases with $C_{i}/C_{a}$ when $\phi$ is generally below 0.37, and low $g_{s}$ tends to decrease $C_{i}/C_{a}$ (Cernusak et al., 2013; von Caemmerer et al., 2014). Alternatively, a decreased photosynthetic capacity in the water-limited plants could increase $C_{i}/C_{a}$, which would decrease $\Delta^{13}$C_{BL} and lead to an increase in $\delta^{13}$C_{BL} values. Therefore, the decrease in $\delta^{13}$C_{BL} observed in the water-limited plants is mostly due to changes in $g_{s}$ and its influence on $C_{i}/C_{a}$. However, it is possible that water limitations increase $\phi$, and lower $\delta^{13}$C_{BL} values.

Models of $C_{4}$ isotope exchange suggest that a decrease in the capacity of the CO_{2} concentrating mechanism, as would occur with a stomatal limitation in CO_{2} supply, could decrease $\phi$ (Ellsworth and Cousins, 2016). Additionally, leaf level measurements of CO_{2} exchange have demonstrated that $\phi$ remains fairly constant under various environmental conditions (Henderson et al., 1998; Ubierna et al., 2011, 2013; Sun et al., 2012; Cernusak et al., 2013; Kromdijk et al., 2014; von Caemmerer et al., 2014). The ability of $C_{4}$ plants to maintain and minimize $\phi$ in response to long-term changes in growth conditions is not surprising as the $C_{4}$ and $C_{3}$ cycles are metabolically coordinated between the mesophyll and bundle sheath cells, causing them to function as integrated and not independent cycles (Furbank et al., 2013).

Using the simplified model of $\Delta^{13}$C (Eq. 2), the calculated $\phi$ from $\delta^{13}$C_{BL} and $C_{i}/C_{a}$ produced similar values (0.17–0.21) to what has been published previously (see list of studies in Kromdijk et al., 2014). This difference in $\phi$ could account for a mean 22% and 24% of the measured difference in $\delta^{13}$C_{BL} across treatments in $S.$ italicu and $S.$ viridis, respectively. In a separate study, under well-watered conditions $\Delta^{13}$C_{instantaneous} at similar $C_{i}/C_{a}$ was approximately 4.4 ± 0.2‰ for both $S.$ viridis and $S.$ italicu, giving evidence that $\phi$ is not inherently different between these species (Ellsworth et al., unpublished data). Additionally, the relationship between $\Delta^{13}$C_{BL} and $C_{i}/C_{a}$ was similar for both species under all treatments, potentially falling on the same line where $\phi$ controls the slope (see Fig 1; Ellsworth and Cousins, 2016). Granted these calculations of $\phi$ provide only an approximation because instantaneous measures of $\Delta^{13}$C and $\delta^{13}$C_{BL} are known to differ because of post-photosynthetic fractionations, as discussed below (von Caemmerer et al., 2014; Ellsworth and Cousins, 2016).

### Post-photosynthetic fractionation

Post-photosynthetic fractionations of carbon compounds could influence $\delta^{13}$C_{BL}; however, to influence $\delta^{13}$C_{BL} there must be a change in the leaf carbon mass balance by isotopic flux into or out of the leaf (von Caemmerer et al., 2014). Potentially, water-limited plants differ in which carbon pools are exported from the leaf. For example, if enriched amino acids are transported from the leaf at a greater rate than in well-watered plants (Melzer and O’Leary, 1987), then $\delta^{13}$C_{BL}

---

**Fig. 4.** Relationship between $\Delta^{18}$O_{LW} (A, B) and $\Delta^{18}$O_{BL} (C, D) and transpiration rate (A, C) and stomatal conductance (B, D) in $S.$ viridis. Gas exchange measurements were made at time of plant harvest. Circles represent the water-limited treatment and squares represent well-watered plants. Open, gray-filled and black-filled symbols represent collection 1, 2, and 3, respectively. The points represent $\Delta^{18}$O_{LW} and $\Delta^{18}$O_{BL} when they were calculated using $\delta^{18}$O_{RC} as source water ($\Delta^{18}$O_{LW} or $\Delta^{18}$O_{BL} minus $\delta^{18}$O_{BL}). The dashed regression line represents the regression when $\delta^{18}$O_{BL} or $\delta^{18}$O_{RC} were used to calculate $\Delta^{18}$O_{LW} and $\Delta^{18}$O_{BL}. The resulting regression line between $\Delta^{18}$O_{LW} or $\Delta^{18}$O_{BL} with transpiration rate ($E$) and stomatal conductance ($g_{s}$) did not differ when $\delta^{18}$O_{BL} or $\delta^{18}$O_{RC} was used to calculate $\Delta^{18}$O_{LW} and $\Delta^{18}$O_{BL}. Therefore, the shaded region represents the variation associated with which source water was used. Lines represent Model II regressions.
could decrease. However, enriched amino acids are a small pool of carbon compared with sucrose and cellulose, so their export would have to be extremely large to account for the observed depletion in $\delta^{13}$CBL. Additionally, the export or consumption by respiration of sucrose from starch degradation instead of from triose phosphate synthesis could also affect $\delta^{13}$CBL because sucrose from starch degradation is more enriched in $^{13}$C (Hobbie and Werner, 2004; Tcherkez and Farquhar, 2005; von Caemmerer et al., 2014). However, the respiratory carbon flux out of the leaf is relatively small compared with photosynthetic flux into the leaf, so water-limited respiratory carbon flux out of the leaf is relatively small compared with photosynthetic fractionation but rather to the treatment effect on leaf gas exchange and TE.

**Leaf carbon isotopic composition across drought experiments**

In both species, $\delta^{13}$CBL in water-limited plants consistently showed lower values by 0.9–1.1‰ than in the well-watered plants. Previous studies also have found a difference in $\delta^{13}$CBL values between well-watered and water-limited plants of 0.2–0.6‰, which probably depended on the type, severity, or duration of the reduction in water availability (Saliendra et al., 1996; Henderson et al., 1998; Brück et al., 2000; Monneveux et al., 2007; Cabrera-Bosquet et al., 2009a). Nonetheless, the consistent depletion in $^{13}$C in response to water limitations persisted across $C_3$ species and experiments, lending further evidence that decreased $g_s$ is driving the response in $\delta^{13}$CBL. Additionally, Gresset et al. (2014) found that $\delta^{13}$CBL in $C_4$ maize was under genetic control, giving support to the potential use of $\delta^{13}$CBL as a genetic screen for TE. However, further research is needed to determine the degree to which $\delta^{13}$CBL can be used to detect subtle differences in TE in $C_4$ plants, if $\delta^{13}$CBL is under similar genetic control as TE, and if it can be used to screen for TE across genotypes.

**Transpiration efficiency estimated from leaf carbon isotopic composition**

Transpiration efficiency (TE) calculated from $\delta^{13}$CBL correlated strongly with leaf-level gas exchange measurements of $g_s$ and TE$_{\text{intrinsic}} (A_{\text{leaf}}/g_s)$. Therefore, calculating TE$_p$ based on $C_i/C_a$ estimated from $\delta^{13}$CBL accurately reflected differences in $g_s$ between treatments. However, in *S. viridis*, TE$_{\text{plant}}$ was more highly correlated with TE$_{\text{intrinsic}}$ and TE$_{\text{instantaneous}}$ than in *S. italica*. This may be because the short and bushy *S. viridis* has proportionally more leaf biomass than the upright *S. italica*, so leaf characteristics would have a greater influence on plant-level estimates of TE (e.g. TE$_{\text{plant}}$) in *S. viridis* than in *S. italica*. Nonetheless, for both species, $\delta^{13}$CBL reflected differences in both whole-plant and leaf-level estimates of TE.

**Leaf water enrichment**

In *Setaria viridis*, $\Delta^{18}$O$_{\text{LW}}$ formed a negative relationship with $E$ as expected based on the Péclet model. In the Péclet model, the Péclet number is proportional to $E$ and $L$, so a positive relationship between $\Delta^{18}$O$_{\text{LW}}$ and $E$ requires $L$ to remain constant across individuals or treatments. $L$ differed only slightly between treatments, and this did not remove the relationship between $\Delta^{18}$O$_{\text{LW}}$ and $f (1-\Delta^{18}$O$_{\text{LW}}/\Delta^{18}$O$_{e})$ with $E$, showing evidence that the Péclet model best describes leaf water isotopic composition (Supplementary Fig. S1 and Fig. 4). The expected relationship existed for *S. viridis*, lending strength to the possible use of leaf oxygen isotopic composition as a proxy of $E$.

Contrary to *S. viridis*, $\Delta^{18}$O$_{\text{LW}}$ and $E$ did not form a significant relationship in *S. italica*, and $\Delta^{18}$O$_{\text{LW}}$ values were more enriched than $\Delta^{18}$O$_{e}$. One potential reason for this disparity in results between the two species is that leaf temperature differed between treatments and across the range of leaf temperature. High transpiration rate can change $\Delta^{18}$O$_{\text{LW}}$ and subsequently $\Delta^{13}$CBL by decreasing leaf temperature through evaporative cooling. Both $\varepsilon^\prime$ and $e_i$ (and therefore $e_i/e$) are temperature-dependent (Barbour, 2007; Barbour et al., 2004). In the experiment with *S. italica*, the maximum difference in leaf temperature across the treatments was less than 2 °C (Ellsworth et al., 2016); $\Delta^{18}$O$_e$ of leaves of water-limited plants would only increase by ~0.6‰, and $\Delta^{18}$O$_{\text{LW}}$, being partly composed of $\Delta^{18}$O$_e$, would change by a fraction of 0.6‰. If leaf temperature in *S. viridis* differed less than 2 °C, as previously observed with *S. italica* (Ellsworth et al., 2016), then the temperature difference between treatments would be insufficient to explain the difference in $\Delta^{18}$O$_{\text{LW}}$ observed in *S. viridis*. As for *S. italica*, $\Delta^{18}$O$_{\text{LW}}$ values showed the opposite trend as would be expected if the differences were based on leaf temperature. Therefore, the relationship between $\Delta^{18}$O$_{\text{LW}}$ and $E$ in *S. viridis* suggests a Péclet effect in *S. viridis* but not in *S. italica*.

A possible reason why $E$ does not affect the mixing of source water with water from the sites of evaporation in *S. italica* (as described in the Péclet model and observed in *S. viridis*) may be because $\Delta^{18}$O$_{\text{LW}}$ increases with leaf length in $C_4$ grasses (Helliker and Ehleringer, 2000). In *S. italica*, longer leaf length in well-watered plants than in water-limited plants apparently increased $\Delta^{18}$O$_{\text{LW}}$ values sufficiently to mask the expected relationship between $\Delta^{18}$O$_{\text{LW}}$ and $E$. This effect of leaf length was strong enough that $\Delta^{18}$O$_{\text{BL}}$ was positively correlated with $E$ and $g_s$. Enrichment up the leaf blade, described as the longitudinal Péclet effect, occurs because the xylem water being supplied to the sites of evaporation becomes progressively more enriched from the base to the tip of the leaf blade (Gan et al., 2003). Therefore, relative to source water entering the leaf base, the water at the sites of evaporation is enriched above what can be attributed to $E$. In contrast, the treatment difference in leaf length in *S. viridis* was minimal and insufficient to mask the transpiration-derived differences in $\Delta^{18}$O$_{\text{LW}}$. 
Bulk leaf enrichment and E

According to theory, bulk leaf enrichment (Δ\(^{18}\)O\(_{BL}\)) should reflect the isotopic signature of leaf water in which bulk tissue is synthesized (Farquhar and Lloyd, 1993; Sternberg et al., 1986). As expected, a weak but significant positive relationship between Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\) was found in S. viridis, but this relationship did not translate into a significant difference in Δ\(^{18}\)O\(_{BL}\) between treatments or significant correlations of Δ\(^{18}\)O\(_{BL}\) with measures of gas exchange or growth. Three possibilities exist that may explain this pattern. First, the relationship between Δ\(^{18}\)O\(_{LW}\) and E primarily exists because of the longitudinal or xylem Péclet effect and not the mesophyll/lamina or radial Péclet effect. Therefore, the oxygen isotope signature of lamina water that is passed onto organic molecules may have little E-related enrichment, so the relationship between Δ\(^{18}\)O\(_{LW}\) and E would not be passed onto the bulk leaf tissue (Holloway-Phillips et al., 2016). Second, Δ\(^{18}\)O\(_{LW}\) was measured once, and a single measurement may not capture all variation in E and climatic conditions such as Δ\(^{18}\)O\(_{s}\), relative humidity, and leaf temperature over the leaf lifespan, which can be difficult to control or account for precisely even in a controlled environment setting (Roden and Siegwolf, 2012; Roden and Farquhar, 2012). Third, leaf length would not affect Δ\(^{18}\)O\(_{BL}\) as much as Δ\(^{18}\)O\(_{LW}\) because Δ\(^{18}\)O\(_{BL}\) would be driven principally by Δ\(^{18}\)O\(_{LW}\) early in leaf construction when oxygen isotope exchange between water and sucrose takes place. Nevertheless, the magnitude of this non-significant difference between treatments was similar to what has been reported in other studies (Cabrera-Bosquet et al., 2009c; Sánchez-Bragado et al., 2016). The oxygen isotopic composition of other plant organs has been proposed as proxies because they produced stronger correlations with grain yield than Δ\(^{18}\)O\(_{BL}\) (Sánchez-Bragado et al., 2016). However, it is necessary to understand how the leaf oxygen isotope enrichment that is related to E is passed onto these organs before their Δ\(^{18}\)O can be used an effective proxy for E.

Another problem that can obscure the relationship that Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\) have with E is misidentifying source water (δ\(^{18}\)O\(_{s}\)) used to calculate Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\). In this study, we measured the isotopic signature of three possible source waters: (i) irrigation water, (ii) mean soil water, and (iii) root crown water. Isotopically soil and root crown water were statistically indistinguishable, confirming previous studies that there is little fractionation of oxygen isotopes upon uptake by roots, so root crown water is a good representation of δ\(^{18}\)O\(_{s}\) at the time of water collection (Ellsworth and Williams, 2007). Irrigation water does not reflect source water in water limitation studies because it undergoes evaporative enrichment, creating isotopically distinct soil water pools for each treatment. As a result, differences in Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\) between treatments could simply be an artefact of incorrectly identifying δ\(^{18}\)O\(_{s}\), and not because of other physiological traits or environmental factors. Therefore, care must be taken to define the real source water of leaves.

Conclusion

Leaf δ\(^{13}\)C had strong relationships with E, g\(_{s}\), water use, aboveground biomass production, and all measures of TE. Although the variation in δ\(^{13}\)C\(_{BL}\) was less than that in C\(_{3}\) species, the overall consistency of the signal between well-watered and water-limited plants suggests that δ\(^{13}\)C\(_{BL}\) may be an effective tool for distinguishing between well-watered and water-limited plants. However, more research is needed to determine if δ\(^{13}\)C\(_{BL}\) can be used to detect differences in TE and g\(_{s}\) across more similar genotypes and serve as an effective proxy of TE in high throughput phenotyping across a range of field growth conditions. Alternatively, the use of Δ\(^{18}\)O\(_{BL}\) as a proxy for transpiration rate in the C\(_{4}\) grass Setaria is problematic for three reasons. First, source water can be isotopically variable across time and different between treatment conditions, making accurate calculations of Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\) difficult. Furthermore, assuming that both well-watered and water-limited plants have the same δ\(^{18}\)O\(_{s}\) may lead to erroneous implications for differences in E. Second, either a small mesophyll Péclet effect where organic molecules are synthesized or leaf water oxygen exchange with lamina water in sucrose synthesis was not sufficient to pass the leaf water isotopic signature on to that of bulk leaf tissue, so that the subtle differences in Δ\(^{18}\)O\(_{BL}\) across a gradient of E were weak. Finally, changes in leaf size in response to water limitations appeared to mask the expected relationship of Δ\(^{18}\)O\(_{LW}\) and Δ\(^{18}\)O\(_{BL}\) with E.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. The relationship between proportional deviation of leaf water (Δ\(^{18}\)O\(_{LW}\)) from evaporative site water (Δ\(^{18}\)O\(_{s}\)) oxygen isotopic enrichment (E) and transpiration rate (E).

Table S1. F values, numerator degrees of freedom (ndf), denominator degrees of freedom (ddf) and P values from two-way ANOVA of the effects of a differential irrigation treatment and collection period on plant water use and growth, leaf water relations, and isotopic composition for S. viridis. Table S2. Plant water relations, growth, and isotopic composition of S. viridis grown under well-watered and water-limited conditions and harvested during three collection periods.

Table S3. F values, numerator degrees of freedom (ndf), denominator degrees of freedom (ddf) and P values from one-way ANOVA of the effects of a differential irrigation treatment on plant water use and growth, leaf water relations, and isotopic composition for S. italica.

Table S4. Plant water relations, growth, and stable isotopes of S. italica grown under well-watered, moderately and severely water-limited treatments and harvested during two collection periods.

Table S5. Correlations between measured parameters of both well-watered and water-limited plants and leaf water enrichment (Δ\(^{18}\)O\(_{LW}\)), bulk leaf enrichment (Δ\(^{18}\)O\(_{BL}\)), and δ\(^{13}\)C for S. viridis.
Table S6. Correlations of measured parameters with $\delta^{13}$C, $\Delta^{18}$O_L, and $\Delta^{18}$O_BL for S. italica.

Acknowledgements
This work was supported by the Office of Biological and Environmental Research in the Department of Energy Office of Science (DE-SC0008769).

Appendix: Theory behind the relationship of $\delta^{18}$O_LW, $\delta^{18}$O.BL, and transpiration

Transpiration has been shown to affect the leaf water oxygen isotope composition ($\delta^{18}$O_LW) in three different ways. The first two deal with the role that transpiration plays in modifying the evaporative environment under which leaf water becomes enriched. At the sites of evaporation near the stomates, the water undergoing evaporation becomes enriched in $^{18}$O relative to the source water entering the leaf via the xylem. The third way considers the effect that transpiration has on the quantitative contribution of enriched water at the sites of evaporation ($\delta^{18}$O_L) to $\delta^{18}$O_LW.

Leaf water isotopic composition ($\delta^{18}$O_LW) can be considered a mixture of source water in the xylem ($\delta^{18}$O_S) and water from the sites of evaporation and mesophyll ($\delta^{18}$O_L):

$$\delta^{18}$O_LW = $f$ $\times$ $\delta^{18}$O_S + $(1 - f)$ $\delta^{18}$O_L$$  \hspace{1cm} (A1)

where $f$ is the fraction of unenriched water in leaf veins. By expressing the oxygen isotopic composition in leaf water as enrichment above source water ($\Delta^{18}$O_LW), any differences between in source water between leaves is accounted for and only changes in enrichment of $^{18}$O within the leaf are considered:

$$\Delta^{18}$O_LW = $\frac{\delta^{18}$O_LW - $\delta^{18}$O_S}{1 - $\delta^{18}$O_LW}$$  \hspace{1cm} (A2)

Equation S1 can be expressed in terms of enrichments of $^{18}$O, where $\Delta^{18}$O_LW is equal to the proportional contribution of oxygen isotope enrichment in water from the sites of evaporation ($\Delta^{18}$O_L) to $\Delta^{18}$O_LW (Barbour; Cernusak et al., 2016):

$$\Delta^{18}$O_LW = $(1 - f)$ $\Delta^{18}$O_L$$  \hspace{1cm} (A3)

Considering Eq. A3, $\Delta^{18}$O_LW can vary across a gradient of $E$ if either $\Delta^{18}$O_L or the proportional contribution of $\Delta^{18}$O_L $(1 - f)$ presented in the leaf changes with $E$.

The two ways in which $\Delta^{18}$O_L itself can be influenced by $E$ are based on the effect that $E$ has on leaf temperature and the resulting effect that changing leaf temperature has on the equilibrium fractionation ($\varepsilon^*$) and intercellular air vapor mole fraction ($e_i$) when $e_s$ remains constant:

$$\Delta^{18}$O_L = $\varepsilon^*$ + $\varepsilon_k + (\Delta^{18}$O_L - $\varepsilon_k) e_s / e_i$$  \hspace{1cm} (A4)

This, in turn, influences $\Delta^{18}$O_LW (Eq S3). $\Delta^{18}$O_L is assumed to follow the Craig–Gordon model and can be derived from $\varepsilon^*$, the ambient to intercellular air vapor mole fraction ($e_i/e_s$), the kinetic fractionation ($\varepsilon_k$), and air vapor enrichment ($\Delta^{18}$O_a) (Craig & Gordon, 1965; Dongmann et al., 1974; Flanagan et al., 1991). Based on this equation, increasing $g_s$ and subsequently $E$ can either dry the intercellular area or increase $\varepsilon^*$ by decreasing leaf temperature ($T_L$), both of which increase $\Delta^{18}$O_L. This because $\varepsilon^*$ is inversely related to $T_L$:

$$\varepsilon^* = 2.644 - 3.206 \left( \frac{10^3}{T_L} \right) + 1.534 \left( \frac{10^6}{T_L^2} \right)$$  \hspace{1cm} (A5)

$E$ can decrease leaf temperature by increasing evaporative cooling and consequently increase $\varepsilon^*$ (Gates, 1968). Ascribing changes in $\Delta^{18}$O_L to variation in $E$ assumes that $\Delta^{18}$O_L and $\varepsilon_k$ remain constant.

Contrastingly, $\varepsilon_k$ is inversely proportional to the stomatal ($g_s$) and boundary layer ($g_{BL}$) conductances, so increasing $g_s$ decreases $\varepsilon_k$:

$$\varepsilon_k = \frac{32 g_s^{-1} + 21 g_{BL}^{-1}}{g_s^{-1} + g_{BL}^{-1}}$$  \hspace{1cm} (A6)

The third way in which $E$ can change $\Delta^{18}$O_L and consequently $\Delta^{18}$O_LW is by modifying the contribution $(1 - f)$ in Eq. A3 of $\Delta^{18}$O_L to $\Delta^{18}$O_LW. The Péclet model was developed to explain how $\Delta^{18}$O_LW values were often lower than $\Delta^{18}$O_L values, meaning that something is influencing $\Delta^{18}$O_LW other than the evaporative enrichment (Farquhar et al., 1998). It describes the extent to which the advection of relatively unenriched xylem water mixes with enriched water from the sites of evaporation. This depends on the Péclet number ($\rho$), which is proportional to $E$ and the effective path length ($L$) that water takes to move from the veins to the sites of evaporation, and inversely proportional to the molar density of water ($C$) and the diffusivity of $H_2^{18}$O in water ($D$) (Farquhar et al., 1998; Barbour & Farquhar, 2000; Barbour et al., 2000b; Gan et al., 2003; Barbour & Farquhar, 2004; Barbour, 2007):

$$\rho = \frac{LE}{CD}$$  \hspace{1cm} (A7)

$\rho$ is then related to $\Delta^{18}$O_LW according to:

$$\Delta^{18}$O_LW = $\frac{\Delta^{18}$O_L $\left( 1 - e^{-\rho} \right)}{\rho}$$  \hspace{1cm} (A8)

If $L$ remains constant, then $\rho$ increases with $E$, meaning that back diffusion of water from sites of evaporation decreases relative to the advection of unenriched water from the xylem to the sites of evaporation. Simply stated, the relative contribution of $\Delta^{18}$O_L to $\Delta^{18}$O_LW decreases, and $\Delta^{18}$O_LW decreases with increasing $E$, assuming that $L$ remains constant.

Bulk leaf enrichment above source water ($\Delta^{18}$O_BL) reflects both $\Delta^{18}$O_LW, the biochemical fractionation during the synthesis of organic compounds, and the degree of oxygen exchange during synthesis of cellulose (Craig & Gordon, 1965; Dongmann et al., 1974; Yakir, 1992; Barbour, 2007):

$$\Delta^{18}$O_BL = $\Delta^{18}$O_LW \left( 1 - p_{ex} p_s \right) + \varepsilon_{wc} + \varepsilon_{wp}$$  \hspace{1cm} (A9)

Where $p_{ex}$ is the proportion of exchangeable oxygen in cellulose being formed from glucose, $p_s$ is the proportion of source water at the site of cellulose/tissue synthesis, $\varepsilon_{wc}$ is the biochemical
fractionation factor during the carbonyl oxygen exchange with water during cellulose synthesis, and \( \varepsilon \) is the isotopic difference between cellulose and whole tissue. This equation assumes a constant \( \varepsilon \), although this factor may vary among leaves and with leaf age (Cernusak et al., 2005). Therefore, in the scenario where \( \Delta^{18}O_{LB} \) varies across a gradient of \( E \), \( \Delta^{18}O_{LB} \) integrates this variation over the period of leaf construction. In this case, \( \Delta^{18}O_{LB} \) may be used as a proxy for \( E \) during leaf growth (Barbour et al., 2000a; Barbour, 2007).

The concept behind the Péclét effect theory that the proportional deviation of \( \Delta^{18}O_{LW} \) from \( \Delta^{18}O \) (\( f \) in Eq. A3) increases along a gradient of \( E \) has failed to be observed in several studies (Rodên & Ehleringer, 1999; Cernusak et al., 2003; Loucos et al., 2015; Song et al., 2015; Holloway-Phillips et al., 2016). The failure to find this relationship in many instances has been explained by changes in factors such as \( T_L \) and \( \varepsilon \) with \( E \), as discussed above and (Barbour et al., 2000a,b, 2004; Barbour & Farquhar, 2004). Song et al. (2015) found that the two-pool model proposed by Rodên and Ehleringer (1999) better described the response in \( \Delta^{18}O_{LW} \) to environmental conditions in upland cotton (\textit{Gossypium hirsutum}) than the Péclét model. Additionally, the assumption that \( L \) is constant for species and across a gradient of transpiration rates has not always been supported (Song et al., 2013; Rodên et al., 2015). If \( L \) does not remain constant across a range of \( E \), then it obscures any relationship that may exist between \( E \) and \( \Delta^{18}O_{LW} \) or \( \Delta^{18}O_{LB} \).

Vein density and vein volume fraction affect the enrichment of leaf water because of the strong Péclét effect within the xylem (Helliker & Ehleringer, 2000; Farquhar & Gan, 2003; Gan et al., 2003; Holloway-Phillips et al., 2016). This Péclét has been called the longitudinal or xylem Péclét and is greater in magnitude than the radial or mesophyll Péclét (Farquhar & Gan, 2003; Gan et al., 2003; Holloway-Phillips et al., 2016). Vein volume fraction increases the relative importance of xylem water in \( \Delta^{18}O_{LW} \), which makes the influence of the xylem Péclét increasingly measurable in \( \Delta^{18}O_{LW} \). This means that the xylem and not the mesophyll may be the location of the Péclét effect (Holloway-Phillips et al., 2016). Since the mesophyll is where sugars are synthesized, this may explain why the oxygen isotope signature of leaf water is not always reflected in bulk leaf tissue.

References

Araus JL, Cabrera-Bosquet L, Serret MD, Bort J, Nieto-Taladriz MT. 2013. Comparative performance of \( \delta^{13}C \), \( \delta^{18}O \) and \( \delta^{15}N \) for phenotyping durum wheat adaptation to a dryland environment. Functional Plant Biology 40, 595–608.


Cabrera-Bosquet L, Sánchez C, Araus JL. 2009b. How yield relates to ash content, \( \Delta^{13}C \) and \( \Delta^{18}O \) in maize grown under different water regimes. Annals of Botany 104, 1207–1216.


Furbank RT, von Caemmerer S, Price GD. 2013. CO2-concentrating mechanisms in crop plants to increase yield. Applying photosynthesis research to improvement of food crops. ACIAR Proceedings 140, 130–137.


