18O enrichment of leaf cellulose correlated with 18O enrichment of leaf sucrose but not bulk leaf water in a C3 grass across contrasts of atmospheric CO2 concentration and air humidity

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^{18}O enrichment of leaf cellulose correlated with ^{18}O enrichment of leaf sucrose but not bulk leaf water in a C_3 grass across contrasts of atmospheric CO_2 concentration and air humidity

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Summary

- The ^{18}O composition of plant cellulose is often used to reconstruct past climate and plant function. However, uncertainty remains regarding the estimation of the leaf sucrose ^{18}O signal and its subsequent attenuation by ^{18}O exchange with source water during cellulose synthesis.
- We grew *Lolium perenne* at three CO_2 concentrations (200, 400 or 800 μmol mol^{-1}) and two relative humidity (RH) levels (50% or 75%), and determined ^{18}O enrichment of leaf sucrose ($\Delta^{18}O_{Sucrose}$), bulk leaf water ($\Delta^{18}O_{LW}$), leaf cellulose ($\Delta^{18}O_{Cellulose}$) and water at the site of cellulose synthesis ($\Delta^{18}O_{CelSynW}$).
- $\Delta^{18}O_{Cellulose}$ correlated with $\Delta^{18}O_{Sucrose}$ (R^2=0.87) but not with $\Delta^{18}O_{LW}$ (R^2=0.04), due to a variable ^{18}O discrepancy (range 2.0-9.0‰) between sucrose synthesis water ($\Delta^{18}O_{SucSynW}$, estimated from $\Delta^{18}O_{Sucrose}$) and bulk leaf water. The discrepancy resulted mainly from an RH effect. The proportion of oxygen in cellulose that exchanged with medium water during cellulose formation (p_{ex}), was near-constant when referenced to $\Delta^{18}O_{SucSynW}$ ($p_{ex\text{-}SucSynW}$ = 0.52±0.02 SE), but varied when related to bulk leaf water ($p_{ex\text{-}LW}$ = –0.01 to 0.46).
- We conclude that previously reported RH-dependent variations of $p_{ex\text{-}LW}$ in grasses are related to a discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ that may result from spatial heterogeneity in ^{18}O gradients of leaf water and photosynthetic sucrose synthesis.

Key words: *Lolium perenne* (perennial ryegrass), Barbour-Farquhar model of ^{18}O-enrichment in cellulose, ^{18}O in leaf water, sucrose and cellulose, atmospheric CO_2 concentration, relative humidity of air.

Introduction

The oxygen isotope $^{18}O/^{16}O$ ratio of plant cellulose ($\delta^{18}O_{Cellulose}$) and its enrichment above source water ($\Delta^{18}O_{Cellulose}$, with $\Delta^{18}O_{Cellulose} \approx \delta^{18}O_{Cellulose} - \delta^{18}O_{Source}$) contain important environmental and physiological information (see e.g. Roden *et al.*, 2000; Barbour, 2007; Werner *et al.*, 2012; Gessler *et al.*, 2014 for reviews). This is based on the fact that all oxygen in cellulose ultimately originates from water (DeNiro & Epstein, 1979; Liu *et al.*, 2016), and that evaporative ^{18}O enrichment of water in leaves (Dongmann *et al.*, 1974; Flanagan *et al.*, 1991; Roden & Ehleringer, 1999; Farquhar & Cernusak, 2005; Cernusak *et al.*, 2016) imprints an ^{18}O signal onto photosynthetic products (Sternberg & DeNiro, 1983; Sternberg *et al.*, 1986; Farquhar *et al.*, 1998) used for cellulose synthesis in growing sink tissues (Barbour *et al.*, 2000; Helliker & Ehleringer, 2002; Cernusak *et al.*, 2005). However, the exact isotopic identity of the water that dictates the ^{18}O signal of primary photosynthetic products is still uncertain (Lehmann *et al.*, 2017) and a variable proportion ($p_{ex}p_x$, see below) of the original ^{18}O signal in photosynthetic products appears to be subsequently lost by exchange with source water (Helliker & Ehleringer, 2002; Lehmann *et al.*, 2017; Hirl *et al.*, 2021), so that the relationship between the ^{18}O signal in cellulose and evaporative events determining the ^{18}O signal in photosynthetic products is still unsettled. The present paper is addressing these uncertainties, and explores the underlying mechanisms, using perennial ryegrass (*Lolium perenne*, C_3) grown in contrasting CO_2 and atmospheric humidity levels as a model plant.

Current mechanistic understanding of the relationship between evaporative ^{18}O enrichment of water at the site of sucrose synthesis ($\Delta^{18}O_{SucSynW}$) – the most ubiquitous primary photosynthetic product and translocated sugar – and $\Delta^{18}O_{Cellulose}$ can be summarized quantitatively for steady-state conditions (Barbour & Farquhar, 2000) as:

$$\Delta^{18}O_{Cellulose} = \Delta^{18}O_{SucSynW}\,(1 - p_{ex}p_x) + \varepsilon_{bio}, \qquad \text{Eqn 1}$$

where p_x denotes the proportion of unenriched source water at the site of cellulose synthesis, p_{ex} is the proportion of oxygen atoms in cellulose that have exchanged with medium water during cellulose formation at that site, and ε_{bio} is the average biochemical fractionation between carbonyl oxygen and water. The term $\Delta^{18}O_{SucSynW} + \varepsilon_{bio}$ represents the ^{18}O enrichment of leaf sucrose above source water. In field conditions, Eqn 1 requires consideration of non-steady-states and necessitates computation of flux-weighted signals (Cernusak *et al.*, 2005; Barbour, 2007).

When applying Eqn. 1, it is generally assumed that p_x, p_{ex} and ε_{bio} are constant parameters: p_x is often set to unity while p_{ex} is often assumed to vary within a narrow range

between 0.4 and 0.5 (Barbour, 2007; Liu *et al.*, 2016) and ε_{bio} is equal to 27‰ (Sternberg & DeNiro, 1983; Yakir & DeNiro, 1990). Another, almost general, assumption of previous works has been that $\Delta^{18}O_{SucSynW}$ equals the average ^{18}O enrichment of bulk or lamina leaf water ($\Delta^{18}O_{LW}$), so that (assimilation-weighted) $\Delta^{18}O_{SucSynW}$ can be replaced by $\Delta^{18}O_{LW}$ in Eqn 1. This assumption is practical, as measurements (and modelling) of $\Delta^{18}O_{LW}$ are relatively straightforward in comparison to $\Delta^{18}O_{SucSynW}$. However, this assumption is often hard to validate from cellulose ^{18}O data because p_{ex} cannot be measured directly, and can only be estimated as a fitted parameter in Eqn 1. Values of p_{ex} estimated in this way therefore absorb all the uncertainty in the other parameters of the equation, including any possible error in the $\Delta^{18}O_{LW} \approx \Delta^{18}O_{SucSynW}$ assumption.

The assumption that $\Delta^{18}O_{LW} \approx \Delta^{18}O_{SucSynW}$ has received direct support in only two studies that compared ^{18}O enrichment in phloem sap dry organic matter and assimilation-weighted bulk leaf water: one on *Ricinus communis* during steady-state leaf cuvette measurements (Cernusak *et al.*, 2003) and another on *Eucalyptus globulus* in the field (Cernusak *et al.*, 2005). Both studies found good agreement between the two signals, provided that the biochemical fractionation ε_{bio} was set at 27‰. However, phloem sap is not only composed of sucrose and recent work by Lehmann *et al.* (2017) with two C_3 grasses in controlled environments found that sucrose extracted from leaves was substantially more ^{18}O enriched than 27‰ relative to bulk leaf water, questioning the universal validity of the $\Delta^{18}O_{LW} \approx \Delta^{18}O_{SucSynW}$ assumption.

Several other recent studies seem to agree that most simplifying assumptions often applied to the Barbour-Farquhar model (i.e. $\Delta^{18}O_{LW} = \Delta^{18}O_{SucSynW}$; ε_{bio} = 27‰; $p_x \approx 1$; $p_{ex} \approx$ 0.4-0.5) should be questioned. First, there are good indications that the biochemical fractionation ε_{bio} decreases with increasing temperature, with a virtually identical temperature-dependence in aquatic plants and in heterotrophically grown wheat seedlings (Sternberg & Ellsworth, 2011) and a value of *ca*. 26.7‰ at 20°C. This temperature dependence of ε_{bio} was also required to explain interannual and seasonal variations of leaf $\delta^{18}O_{cellulose}$ in a temperate grassland ecosystem (Hirl *et al.*, 2021). In addition, although p_x has been shown to be close to unity in trees (Cernusak *et al.*, 2005) and in the leaf growth-and-differentiation zone of grasses (Liu *et al.*, 2017) that is protected from evaporation, p_x is less certain in dicot species because the leaves are directly exposed to evaporative conditions during their growth. Several recent studies (Song *et al.*, 2014; Liu *et al.*, 2016; Cheesman & Cernusak, 2017; Szejner *et al.*, 2020; Hirl *et al.*, 2021) also indicated large variations in p_{ex}, when p_{ex} was estimated using Eqn 1 with $\Delta^{18}O_{SucSynW}$ replaced by $\Delta^{18}O_{LW}$, measured (or well-constrained) estimates of p_x and a

temperature-dependent ε_{bio} from Sternberg & Ellsworth (2011). Thus far, variations of p_{ex} have been mainly attributed to (1) hexose phosphates going through a futile cycle with triose phosphates before cellulose synthesis (Hill *et al.*, 1995) and an increased probability for an oxygen atom derived from sucrose going through an exchangeable carbonyl group with each turn of the futile cycle (Barbour & Farquhar, 2000; Barbour, 2007), (2) unaccounted participation of non-structural carbohydrate stores in cellulose synthesis (Pfanz *et al.*, 2002; Cernusak & Cheesman, 2015) and (3) changes in turnover of non-structural carbohydrate pools (Song *et al.*, 2014).

Estimates of p_x are also affected by any error in the $\Delta^{18}O_{SucSynW} = \Delta^{18}O_{LW}$ assumption. This is because true p_x is calculated from determinations of ^{18}O-enrichment of water at the site of cellulose synthesis ($\Delta^{18}O_{CelSynW}$), source water ($\Delta^{18}O_{Source}$, with $\Delta^{18}O_{Source} = 0$ by definition) and $\Delta^{18}O_{SucSynW}$, using a two-member mixing model that has $\Delta^{18}O_{Source}$ and $\Delta^{18}O_{SucSynW}$ as its endmember:

$$p_x = 1 - \Delta^{18}O_{CelSynW}/\Delta^{18}O_{SucSynW}. \quad \text{Eqn 2}$$

Importantly, reported variation of p_{ex} seems to follow environmental patterns across plant functional groups, particularly with respect to relative humidity of air (RH) (Offermann *et al.*, 2011; Liu *et al.*, 2016; Hirl *et al.*, 2021). Relative humidity is known to generally affect the ^{18}O enrichment of bulk leaf water but also its spatial variations in leaf blades (Cernusak *et al.*, 2016). In particular, very large variations of ^{18}O enrichment have been found in several monocot leaves, from base to tip and center to edge (Helliker & Ehleringer, 2000; Gan *et al.*, 2002; Helliker & Ehleringer, 2002; Gan *et al.*, 2003), that may underlie variation of the $\Delta^{18}O_{LW}$ versus $\Delta^{18}O_{SucSynW}$ relationship (Lehmann *et al.*, 2017).

Another environmental factor that deserves attention when applying Eqn 1 to biological archives is atmospheric CO_2 concentration, because its rise over the last century may have affected the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$ and resultant estimates of p_{ex} and p_x based on the use of $\Delta^{18}O_{LW}$ (termed $p_{ex\text{-}LW}$ in the following) instead of $\Delta^{18}O_{SucSynW}$ ($p_{ex\text{-}SucSynW}$) in Eqn 1. Atmospheric CO_2 concentration has been shown to have a strong effect on stomatal conductance (Ainsworth & Rogers, 2007; Franks *et al.*, 2013) and consequently on transpiration (Leakey *et al.*, 2009), storage of non-structural carbohydrates (Poorter & Navas, 2003) and on the diurnal oscillation of leaf elongation (Baca Cabrera *et al.*, 2020). All these factors can affect, directly or indirectly, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{SucSynW}$, $\Delta^{18}O_{Cellulose}$, and p_{ex} and p_x, estimated with either $\Delta^{18}O_{LW}$ or $\Delta^{18}O_{SucSynW}$.

In this study, we explored the combined effects of atmospheric CO_2 concentration (200, 400 or 800 μmol mol^{-1}), relative humidity (RH, 50% or 75% during daytime) and their

interactions on: $\Delta^{18}O_{Cellulose}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{CelSynW}$ (estimated as the $\Delta^{18}O$ of water in the leaf growth-and-differentiation zone, $\Delta^{18}O_{LGDZ}$, Liu *et al.*, 2017), $\Delta^{18}O_{SucSynW}$ (estimated as $\Delta^{18}O_{Sucrose} - \varepsilon_{bio}$) and p_{ex} and p_x referenced to average leaf water ($p_{ex\text{-}LW}$ and $p_{x\text{-}LW}$) and sucrose synthesis water ($p_{ex\text{-}SucSynW}$ and $p_{x\text{-}SucSynW}$). In this, we asked specifically: (1) Do atmospheric CO_2 concentration and relative humidity or their interactions affect $\Delta^{18}O_{SucSynW}$ and its relationship with $\Delta^{18}O_{LW}$? (2) Do these environmental factors influence $\Delta^{18}O_{CelSynW}$? (3) How do $\Delta^{18}O_{SucSynW}$- and $\Delta^{18}O_{LW}$-based p_{ex} and p_x respond to these environmental factors? Finally, (4) do we find diurnal variation in these parameters, i.e. between light and dark periods?

Materials and Methods

Plant material and growth conditions

Perennial ryegrass (cv. 'Acento') plants were grown in four plant growth chambers (PGR15, Conviron, Winnipeg, Canada) in a 16 h : 8 h, day : night cycle (temperature 20/16°C), under a 3 × 2 factorial design: three atmospheric CO_2 concentration levels ('half-ambient' = 200, 'ambient' = 400 or 'double-ambient' = 800 µmol mol^{-1}) and two daytime relative humidity levels (low RH = 50%, high RH = 75%; nighttime RH was 75% for all treatments), as previously described in Baca Cabrera *et al.* (2020). In brief, *L. perenne* plants were grown individually in plastic tubes (350 mm height, 50 mm diameter) filled with washed quartz sand (0.3–0.8 mm grain size) and arranged in plastic containers (770 × 560 × 300 mm) at a density of 383 plants m^{-2}. Plants were supplied 4 times a day with a Hoagland type nutrient solution with reduced nitrate-N content (Baca Cabrera *et al.*, 2020). Light was supplied by cool-white fluorescent tubes and warm-white LED bulbs with a constant photosynthetic photon flux density (PPFD) of 800 µmol m^{-2} s^{-1} at plant height during the 16 h-long light period. A total of five sequential experimental runs were performed, resulting in five chamber scale replicates for the so-called 'reference treatment' (400 µmol mol^{-1} CO_2 / 50% RH) and three replicate mesocosm-scale runs for the other treatments.

CO_2 and RH treatments were installed on the 13th day after seed imbibition. For this, the air supplied to the chambers was mixed from dry CO_2-free air and tank CO_2 (from Linde AG, Unterschleißheim, Germany or CARBO Kohlensäurewerke, Bad Hönningen, Germany), using mass flow controllers. RH and temperature were controlled by the chamber control system (CMP6050, Conviron, Winnipeg, Canada). CO_2 concentration and RH were measured every 30 min by an infrared gas analyzer (IRGA; Li-840; Li-Cor) and never deviated more than ± 5 µmol mol^{-1} and ± 2.0% relative to the set nominal value, respectively.

Sampling design and extraction of tissue water, cellulose and sucrose

Plants from each chamber scale replicate were sampled when plant canopies were closed (leaf area index >5.5, at 7-9 weeks after the beginning of the experiment). Sampling took place at c. 2 h before the end of the light and dark periods. Each time, 12 plants were randomly selected, dissected and the sampled plant material of six plants pooled in one subsample (providing two subsamples per chamber and per sampling occasion).

For tissue water extraction, the two youngest fully expanded leaf blades and the leaf growth-and-differentiation zone (LGDZ, see Fig. 1 in Baca Cabrera *et al*., 2020) of three mature tillers per plant were excised, sealed in 12 mL Exetainer vials (Labco, High Wycombe, UK), capped, wrapped with Parafilm and stored at –18°C until water extraction. Tissue water was extracted for 2 h using cryogenic vacuum distillation as in Liu *et al.* (2016).

For cellulose and sucrose extraction, the two youngest fully expanded leaf blades of another two mature tillers from the same plants were excised, placed into paper bags, frozen in liquid nitrogen, stored at –18 °C until freeze-drying, milled and stored again at –18 °C until cellulose and sucrose extraction. α-cellulose was extracted from 50 mg of dry sample material by following the Brendel *et al.* (2000) protocol as modified by Gaudinski *et al.* (2005). Water-soluble carbohydrates were extracted from 50 mg aliquots of dry material from to the youngest fully-expanded leaf blade and sucrose separated from other compounds using a preparative HPLC technique similar to that described by Gebbing & Schnyder (2001).

Isotope analysis

Oxygen isotope composition was expressed in per mil (‰) as:

$$\delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000, \qquad \text{Eqn 3}$$

with R_{sample} the $^{18}O/^{16}O$ ratio of the sample and $R_{standard}$ that in the international standard (Vienna Standard Mean Ocean Water, V-SMOW). $\delta^{18}O$ was measured in the following compartments: tissue water of leaf blades ($\delta^{18}O_{LW}$) and of the LGDZ (designated $\delta^{18}O_{CelSynW}$); and cellulose and sucrose of leaf blades ($\delta^{18}O_{Cellulose}$ and $\delta^{18}O_{Sucrose}$). Furthermore, the nutrient solution (the source water for plants, $\delta^{18}O_{Source}$) was sampled 1-2 times per week. $\delta^{18}O_{Source}$ was near constant throughout the experiment (–9.7 ± 0.2‰ standard deviation). $\delta^{18}O_{Source}$ was used to calculate ^{18}O-enrichment above source water ($\Delta^{18}O_X$) of the different samples (X) as:

$$\Delta^{18}O_{\chi} = \frac{\delta^{18}O_{\chi} - \delta^{18}O_{Source}}{1 + \delta^{18}O_{Source}/1000}, \qquad \text{Eqn 4}$$

Water samples were analyzed by cavity ring-down spectroscopy as described in Liu *et al.* (2016). 1 µL of water sample was injected into a A0211 high precision vaporizer coupled to a L2110-i-CRDS (both Picarro Inc., Sunnyvale, Ca, USA). Each sample was measured five to twelve times depending on memory effects. After every 15–25 samples, heavy and light laboratory water standards, spanning the range of $\delta^{18}O$ values in the dataset and previously calibrated against V-SMOW, V-GISP and V-SLAP, were measured for SMOW-scaling and possible drift correction. Analytical uncertainty was <0.2‰.

Cellulose and sucrose samples were measured by isotope ratio mass spectrometry (IRMS) as in Baca Cabrera *et al.* (2021). Each sample (sucrose or cellulose) was measured against a laboratory working standard carbon monoxide gas, previously calibrated against a secondary isotope standard (IAEA-601, accuracy of calibration ±0.25‰ standard deviation). Solid internal laboratory standards (cotton powder) were run each time after the measurement of four samples for possible drift correction and for SMOW-scaling. The precision for the laboratory standard was <0.3‰.

Additionally, $\delta^{18}O$ of water vapor in the growth chambers ($\delta^{18}O_{Vapor}$) was measured by cavity ring-down spectroscopy as described in Liu *et al.* (2016). Here, we measured $\delta^{18}O_{Vapor}$ continuously during two weeks when canopies were closed, both during the light and the dark periods. $\delta^{18}O_{Vapor}$ was constant across experimental runs and treatments, but was c. 1‰ more enriched during the dark period (−14.2‰ ± 0.5‰ standard deviation) than during the light period (−15.2‰ ± 0.6‰ standard deviation). Interestingly, the $\delta^{18}O_{Vapor}$ and $\delta^{18}O_{Source}$ in the chambers were quite similar to the multi-season average observed in a nearby grassland ecosystem study (Hirl *et al.*, 2019).

Statistics

In a first step, linear mixed models were fitted to test the effect of the diel period (day vs. night) on $\Delta^{18}O_{CelSynW}$ (n=80), $\Delta^{18}O_{LW}$ (n=160), $\Delta^{18}O_{Sucrose}$ (n=70) and $\Delta^{18}O_{Cellulose}$ (n=76). All available subsamples (pseudo-replicates) were included in the analysis, with growth chamber and experimental run defined as the random factors. As a significant diel trend was only detected for $\Delta^{18}O_{LW}$, day and night data of $\Delta^{18}O_{CelSynW}$, $\Delta^{18}O_{Sucrose}$ and $\Delta^{18}O_{Cellulose}$ were pooled for further analysis. In the case of $\Delta^{18}O_{LW}$, only end of day data were used in further calculations, i.e. to estimate $p_{x\text{-}LW}$, $p_{ex\text{-}LW}$ or the discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$. Data from individual chamber scale replications were pooled and two-way ANOVA tests used to assess the effects of CO_2, RH and their interaction on $\Delta^{18}O_{CelSynW}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Sucrose}$, $\Delta^{18}O_{Cellulose}$, $p_{x\text{-}LW}$, $p_{x\text{-}SucSynW}$, $p_{ex\text{-}LW}$, $p_{ex\text{-}SucSynW}$, and the discrepancy between $\Delta^{18}O_{SucSynW}$ and

$\Delta^{18}O_{LW}$ ($\Delta^{18}O_{SucSynW} - \Delta^{18}O_{LW}$). All statistical analyses were conducted in R v.4.0.2 (R Core Team, 2020). The R packages nlme (Pinheiro *et al.*, 2019) and ggplot2 (Wickham, 2016) were used for fitting linear mixed models and data plotting, respectively.

Results

^{18}O enrichment of sucrose, bulk leaf water and the discrepancy between sucrose synthesis- and bulk leaf-water

^{18}O enrichment of sucrose in leaf blades ($\Delta^{18}O_{Sucrose}$) differed significantly between RH levels, with low RH resulting in a higher $\Delta^{18}O_{Sucrose}$ (+6.4‰ on average) ($P < 0.001$, Fig. 1a, Table 1). An effect of atmospheric CO_2 concentration was also detected: $\Delta^{18}O_{Sucrose}$ decreased significantly with increasing CO_2 from 45.7‰ to 42.9‰ at low RH and from 39.0‰ to 37.0‰ at high RH ($P = 0.03$). Across treatments, $\Delta^{18}O_{Sucrose}$ did not differ significantly between samples collected near the end of the day and the end of the night ($P > 0.05$, Fig. 1a).

Unexpectedly, the ^{18}O enrichment of bulk leaf water ($\Delta^{18}O_{LW}$) was not affected by RH or the interaction between atmospheric CO_2 concentration and RH (Fig. 1b and Table 1). However, we did observe an effect of atmospheric CO_2 concentration on $\Delta^{18}O_{LW}$. That effect involved a decrease of $\Delta^{18}O_{LW}$ with increasing atmospheric CO_2 concentration both when measured near the end of the day ($P < 0.01$) and end of the night ($P < 0.001$). On average, $\Delta^{18}O_{LW}$ decreased by 1.7‰ between the 'half ambient' and 'double ambient' CO_2 concentrations. Besides, we observed a significant diurnal trend for $\Delta^{18}O_{LW}$ ($P < 0.001$): $\Delta^{18}O_{LW}$ was higher at the end of the day (7.9-10.2‰) than at the end of the night (6.7-8.8‰). That diurnal trend was similar for all treatments.

To estimate ^{18}O enrichment of sucrose synthesis water ($\Delta^{18}O_{SucSynW}$) from $\Delta^{18}O_{Sucrose}$ ($\Delta^{18}O_{SucSynW} = \Delta^{18}O_{Sucrose} - \varepsilon_{bio}$; Barbour 2007) a constant ε_{bio} was assumed (26.7‰, as estimated from Sternberg & Ellsworth, 2011, at 20°C) for all samples collected near the end of the light period. These data indicated that sucrose synthesis water was always more ^{18}O-enriched than bulk leaf water (Fig.1c). The discrepancy, that is $\Delta^{18}O_{SucSynW} - \Delta^{18}O_{LW}$, seemed unaffected by CO_2 ($P > 0.05$), but was much higher at low than at high RH (8.5‰ vs. 2.2‰; $P < 0.001$).

^{18}O enrichment of water in the leaf growth-and-differentiation zone and p_x

The ^{18}O enrichment of water in the leaf growth-and-differentiation zone ($\Delta^{18}O_{LGDZ}$ taken here as a proxy for $\Delta^{18}O_{CelSynW}$) was small for all treatments (0.1-0.9‰) (Fig. 1b). It decreased slightly with increasing atmospheric CO_2 ($P = 0.04$, 0.4‰ decrease between 200 and 800 µmol mol^{-1}, on average), but did not respond to RH or the interaction of CO_2 and RH (Table 1). Also, we observed no significant differences in $\Delta^{18}O_{CelSynW}$ between day and night ($P > 0.05$).

Table 1 Results of a two-way ANOVA testing the effect of atmospheric CO_2 concentration, RH and their interaction on: $\Delta^{18}O$ of sucrose ($\Delta^{18}O_{Sucrose}$), $\Delta^{18}O$ of bulk water in the leaf blades ($\Delta^{18}O_{LW}$) and in the leaf growth-and-differentiation zone ($\Delta^{18}O_{CelSynW}$), the discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ ($\Delta^{18}O_{SucSynW} - \Delta^{18}O_{LW}$), $\Delta^{18}O$ of cellulose in leaf blades ($\Delta^{18}O_{cellulose}$) and p_{ex} and p_x referenced to average leaf water ($p_{ex\text{-}LW}$ and $p_{x\text{-}LW}$) and sucrose synthesis water ($p_{ex\text{-}SucSynW}$ and $p_{x\text{-}SucSynW}$), determined for closed canopies of *L. perenne*.

	CO_2		RH		CO_2 : RH	
Parameter	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
$\Delta^{18}O_{Sucrose}$ (*n*=18)	5.6	**0.03**	63.3	**<0.001**	0.2	0.68
$\Delta^{18}O_{LW}$ (day) (*n*=20)	10.7	**<0.01**	0.4	0.55	0.6	0.47
$\Delta^{18}O_{LW}$ (night) (*n*=20)	21.2	**<0.001**	3.2	0.09	1.0	0.34
$\Delta^{18}O_{SucSynW} - \Delta^{18}O_{LW}$ (*n*=18)	0.4	0.56	74.0	**<0.001**	1.0	0.34
$\Delta^{18}O_{CelSynW}$ (*n*=20)	4.7	**0.04**	1.7	0.22	0.0	0.95
$p_{x\text{-}LW}$ (*n*=20)	3.6	0.07	2.4	0.14	0.0	0.97
$p_{x\text{-}SucSynW}$(*n*=18)	3.4	0.09	11.6	**<0.01**	0.4	0.55
$\Delta^{18}O_{Cellulose}$ (*n*=19)	0.0	0.88	79.4	**<0.001**	0.5	0.47
$p_{ex\text{-}LW}$ (*n*=19)	8.3	**0.01**	55.4	**<0.001**	0.1	0.72
$p_{ex\text{-}SucSynW}$ (*n*=18)	3.3	0.09	0.1	0.71	0.2	0.70

The number of total canopy scale replicates (*n*) is presented for each parameter, individually. Significant *P*-values are highlighted in bold print.

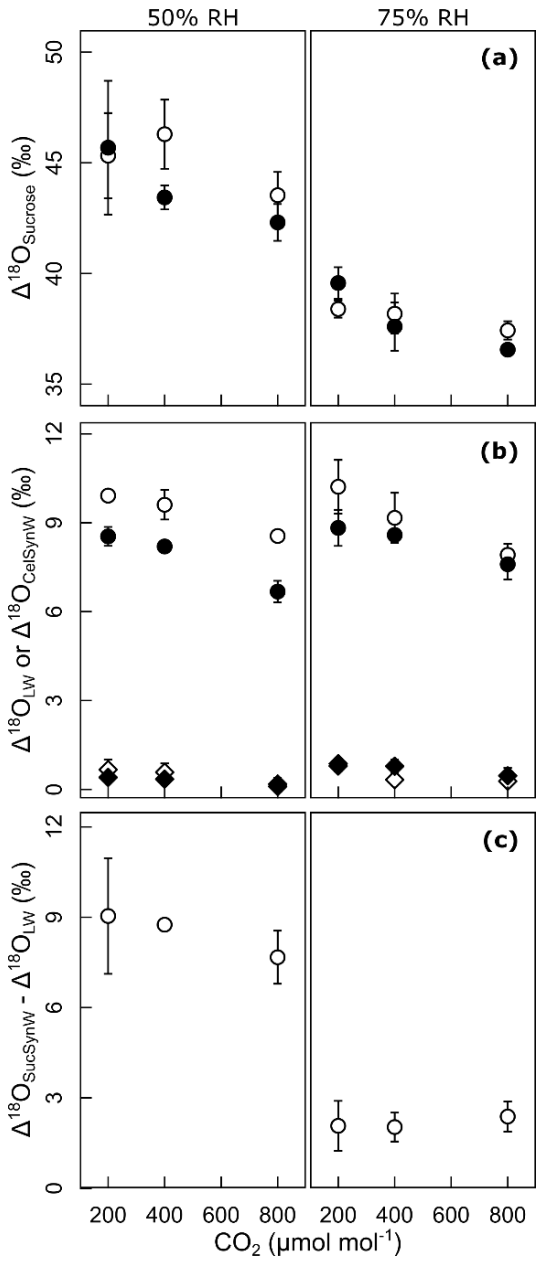

Fig. 1: $\Delta^{18}O$ of leaf blade sucrose ($\Delta^{18}O_{Sucrose}$) (a) $\Delta^{18}O$ of bulk water of leaf blades ($\Delta^{18}O_{LW}$, circles) or $\Delta^{18}O$ of water at the site of cellulose synthesis ($\Delta^{18}O_{CelSynW}$, diamonds) (b), and difference between $\Delta^{18}O$ of sucrose synthesis water and $\Delta^{18}O$ of bulk leaf water ($\Delta^{18}O_{SucSynW} - \Delta^{18}O_{LW}$) in the light period (c), as influenced by atmospheric CO_2 concentration at low RH and high RH. Full symbols represent values near the end of the dark period and empty symbols near the end of the light period. Measurements were performed in closed canopies of *L. perenne*. Data points and error bars represent the mean ± SE (n = 3-5).

The proportion of source water in the leaf growth-and-differentiation zone (p_x) was calculated using Eqn 2, with either $\Delta^{18}O_{Sucrose} - \varepsilon_{bio}$ ($p_{x\text{-}SucSynW}$) or $\Delta^{18}O_{LW}$ ($p_{x\text{-}LW}$) as alternative proxies for $\Delta^{18}O_{SucSynW}$. $p_{x\text{-}LW}$ varied in a narrow range between 0.92-0.98, but was not significantly affected by CO_2, RH or their interaction (Table 1). In comparison, $p_{x\text{-}SucSynW}$ was slightly higher, but also varied in a narrow range (0.93-0.99) that was also not affected by CO_2 or its interaction with RH, but was slightly smaller at low RH compared to high RH (0.95 vs. 0.98, $P < 0.01$, Table 1).

^{18}O enrichment of leaf cellulose and p_{ex}

279 ^{18}O enrichment of cellulose in leaf blades ($\Delta^{18}O_{Cellulose}$) was significantly affected by RH
280 (+3.1‰ at low RH relative to high RH, $P < 0.001$), but effects of CO_2 concentration or the
281 interaction of CO_2 concentration and RH were not significant (Fig. 2a, Table 1).

282 Using the data presented above and Eqn 1, we calculated p_{ex} alternatively as p_{ex}
283 referenced to sucrose synthesis water ($p_{ex\text{-}SucSynW}$) or leaf water ($p_{ex\text{-}LW}$). This showed that $p_{ex\text{-}SucSynW}$ was not significantly affected by CO_2 concentration, RH or their interaction and
285 averaged 0.52 (±0.02 SE) (Fig. 2b). In contrast, $p_{ex\text{-}LW}$ varied strongly between treatments from
286 −0.01 at 800 µmol CO_2 mol^{-1} and 50% RH to 0.46 at 200 µmol CO_2 mol^{-1} and 75% RH. $p_{ex\text{-}LW}$
287 was significantly affected by both RH ($P < 0.001$) and CO_2 concentration ($P = 0.01$) (Fig. 2c).

288 Across all treatments, $\Delta^{18}O_{cellulose}$ was closely related to $\Delta^{18}O_{SucSynW}$ ($R^2 = 0.87$, $P <$
289 0.01, Fig. 3), but a relationship with $\Delta^{18}O_{LW}$ was not evident ($R^2 = 0.04$, $P > 0.05$).
290

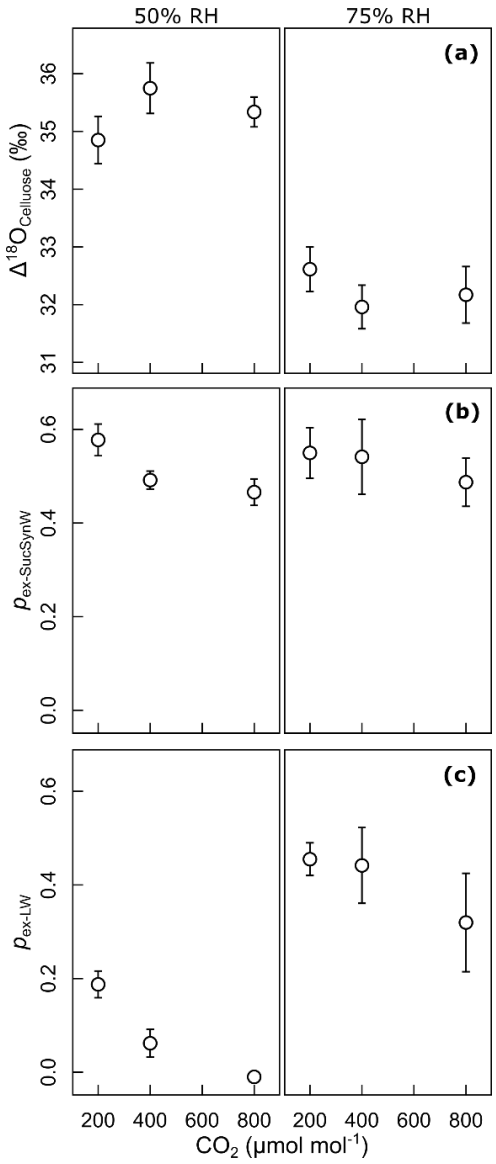

Fig. 2: $\Delta^{18}O$ of leaf blade cellulose ($\Delta^{18}O_{Cellulose}$) (a) and p_{ex}, calculated based on $\Delta^{18}O$ of sucrose synthesis water ($p_{ex\text{-}SucSynW}$) (b) or $\Delta^{18}O$ of bulk leaf water ($p_{ex\text{-}LW}$) (c), as influenced by atmospheric CO_2 concentration at low and high relative humidity. $\Delta^{18}O$ measurements were performed in closed canopies of *L. perenne*. Data points and error bars represent the mean ± SE (n = 3-5).

291

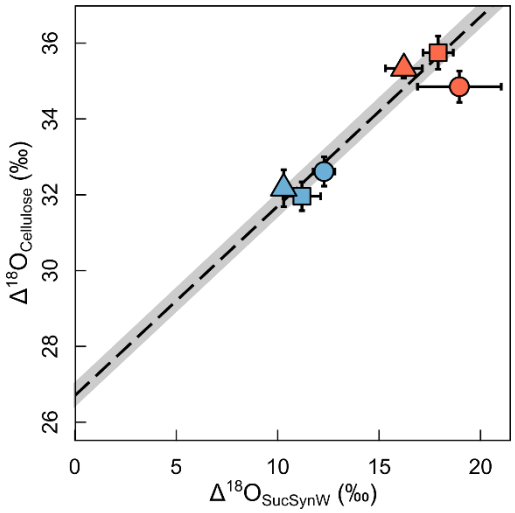

Fig. 3 Relationship between $\Delta^{18}O$ of sucrose synthesis water ($\Delta^{18}O_{SucSynW}$) and ^{18}O-enrichment of cellulose ($\Delta^{18}O_{Cellulose}$) as influenced by atmospheric CO_2 concentration (circles, 200 µmol mol^{-1}; squares, 400 µmol mol^{-1}; triangles, 800 µmol mol^{-1}), at high (blue symbols) and low relative humidity (red symbols). The dashed line and the shadowed area indicate the values predicted with the Barbour-Farquhar model with $p_{ex}p_x = 0.5$ ($p_{x\text{-}SucSynW}$ = 0.96 and $p_{ex\text{-}SucSynW}$ = 0.52) and ε_{bio} at 18 °C (upper limit, ε_{bio} = 27.0‰), 20°C (dashed line, ε_{bio} = 26.7‰) or 22°C (lower limit, ε_{bio} = 26.4‰). Data points and error bars represent the mean ± SE.

Discussion

Isotopic discrepancy between average leaf water and sucrose synthesis water

This work found no negative effect of RH on ^{18}O enrichment of bulk leaf water ($\Delta^{18}O_{LW}$), which was unexpected (Helliker & Ehleringer, 2002; Gan *et al.*, 2003; Xiao *et al.*, 2012; Cernusak *et al.*, 2016; Liu *et al.*, 2016; Liu *et al.*, 2017; Hirl *et al.*, 2019). However, that result was highly reproducible in replicate (n=3-5) mesocosm-scale experiments with different CO_2 concentrations. Also, the result was not a peculiarity of the experimental equipment, as we previously found more typical, negative RH-effects on $\Delta^{18}O_{LW}$ in a range of C_3 and C_4 grasses, including a *Lolium* sp., in the same system (Liu *et al.*, 2016; Liu *et al.*, 2017). Although we are not aware of previous reports noting complete absence of an RH effect on $\Delta^{18}O_{LW}$, the effect is notoriously variable, with significant variation between plant species and stands, including in grasses (Helliker & Ehleringer, 2002; Xiao *et al.*, 2012; Liu *et al.*, 2017). Also, we note that the treatments affected canopy and leaf morphophysiological properties, that may have indirectly influenced $\Delta^{18}O_{LW}$, affecting the apparent RH sensitivity of $\Delta^{18}O_{LW}$. For instance, the high RH

treatments led to a significantly smaller leaf area index (LAI) and lower nitrogen content per unit leaf area (both $P<0.01$; Table S1). Both these differences could reduce the apparent RH sensitivity of $\Delta^{18}O_{LW}$ as noted in previous investigations with an isotope-enabled, process-based soil-plant-atmosphere model of a grassland ecosystem (Hirl *et al.*, 2019). In those investigations, sensitivity analysis indicated that both a decrease of photosynthetic capacity – which correlates with nitrogen content per unit leaf area (Kattge *et al.*, 2009) – and LAI generate an increase of $\Delta^{18}O_{LW}$, elevating $\Delta^{18}O_{LW}$ of the stands grown at high RH relative to low RH (Hirl *et al.*, 2019). Although we cannot prove that these indirect mechanisms explained the absence of an RH effect on $\Delta^{18}O_{LW}$ observed here, we note that such an absence was not a necessary condition for the discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ as a similar discrepancy was also noted by Lehmann *et al.* (2017) in conditions with a more common (negative) RH response of $\Delta^{18}O_{LW}$.

The negative effect of atmospheric CO_2 on $\Delta^{18}O_{LW}$ was similarly non-intuitive, since leaf transpiration decreased with increasing CO_2 (Baca Cabrera *et al.*, 2020), a factor that could drive an increase of $\Delta^{18}O_{LW}$ due notably to a Péclet effect (Farquhar & Lloyd, 1993; Barbour *et al.*, 2000; Farquhar *et al.*, 2007). However, Hirl *et al.* (2019) found no evidence of a Péclet effect in mixed species leaf samples from a temperate grassland ecosystem and in *L. perenne* and *Dactylis glomerata* in controlled conditions. Also, Cooper & Norby (1994) did not find consistent effects of atmospheric CO_2 on $\Delta^{18}O_{LW}$ of two deciduous tree species. Besides, we know of no other studies of the effect of growth under different atmospheric CO_2 levels on $\Delta^{18}O_{LW}$, which also limits opportunities for discussion. Importantly, effects of atmospheric CO_2 on $\Delta^{18}O_{LW}$ and associated mechanisms at stand scale, including interactive effects of nutrient limitation (as observed here; Table S1), have not been investigated in any detail.

In contrast to $\Delta^{18}O_{LW}$, ^{18}O enrichment of sucrose ($\Delta^{18}O_{Sucrose}$) reflected very closely the anticipated negative RH effect on ^{18}O enrichment of water at the site of photosynthetic sucrose synthesis, i.e. $\Delta^{18}O_{SucSynW}$. That RH sensitivity was –0.25‰ per %RH on average of all treatments. The effect appeared to be stable throughout diurnal cycles as we found no significant difference between $\Delta^{18}O_{Sucrose}$ sampled near the end of the light and dark periods. Near-constancy of $\Delta^{18}O_{Sucrose}$ and assimilation-weighted $\Delta^{18}O_{SucSynW}$ was likely related to (1) the constant environmental conditions that led to virtually constant daytime stand-scale CO_2 assimilation (Fig. S1) and transpiration rates (Fig. S6 in Baca *et al.*, 2020) and (2) the small day-night variation of $\Delta^{18}O_{LW}$ in all treatment combinations. Additionally, (3) we observed diurnal variation of sucrose contents in leaf blades (Fig. S2), suggesting presence of a diurnal

sucrose store (Sicher *et al.*, 1984; Schnyder, 1993) but no starch, which may have also helped to maintain a near-constant $\Delta^{18}O_{Sucrose}$.

The fact that $\Delta^{18}O_{SucSynW}$ was significantly higher than bulk leaf $\Delta^{18}O_{LW}$ must have resulted from sucrose synthesis being closer to the evaporative sites or a greater proportion of sucrose synthesis in the distal half of the leaf blades, where ^{18}O enrichment of leaf water is much greater (Helliker & Ehleringer, 2000; Helliker & Ehleringer, 2002; Gan *et al.*, 2003; Affek *et al.*, 2006; Ogée *et al.*, 2007). Indeed, all plants grew in a dense canopy situation (with a LAI >5.5), which must have determined a significant decrease of incident radiation and probably also of photosynthetic sucrose synthesis rate between the tip and the base of leaf blades.

p_x, the proportion of source water at the site of cellulose synthesis, was close to 1

When expressed relative to irrigation water, i.e. nutrient solution, ^{18}O enrichment of water at the site of cellulose synthesis in the leaf growth-and-differentiation zone ($\Delta^{18}O_{CelSynW}$) was very low in all treatments. This implied that p_x, the proportion of source water at the site of cellulose synthesis, was close to 1, consistent with prior findings of Liu *et al.* (2017) for several C_3 and C_4 grasses. Referencing p_x to $\Delta^{18}O_{LW}$ ($p_{x\text{-}LW}$) instead of $\Delta^{18}O_{SucSynW}$ ($p_{x\text{-}SucSynW}$) caused only a small underestimation of $p_{x\text{-}LW}$ (–0.013 ±0.004 SE), due to the small leverage effect of any discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ on estimates of p_x when $\Delta^{18}O_{CelSynW}$ is small. However, if $\Delta^{18}O_{CelSynW}$ were higher, as may be expected for leaves of dicot species (Kahmen *et al.*, 2011; Song *et al.*, 2014), any difference between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ should exert a greater effect on the difference between $p_{x\text{-}SucSynW}$ and $p_{x\text{-}LW}$.

Is true p_{ex} a constant?

This work found a near-constant $p_{ex\text{-}SucSynW}$ of 0.52 (± 0.02 SE) across contrasting environmental conditions. This near-constancy of $p_{ex\text{-}SucSynW}$ was also conserved when we altered temperature-dependent ε_{bio} (Sternberg & Ellsworth, 2011) within the limits of uncertainty for leaf temperature in our controlled environment experiments (Table S2) and contrasted sharply with estimates of $p_{ex\text{-}LW}$ in the different treatments which varied between −0.01 and 0.46. Clearly, the error made in replacing $\Delta^{18}O_{SucSynW}$ with $\Delta^{18}O_{LW}$ in Eqn 1 was the principal (if not the only) cause of variation of $p_{ex\text{-}LW}$. This indicates that the treatment-related variation of $p_{ex\text{-}LW}$ was virtually fully-independent of actual variation of substrate-oxygen exchange with medium water during transport to and at the site of cellulose synthesis as its

variation was eliminated almost entirely when the discrepancy between $\Delta^{18}O_{SucSynW}$ and $\Delta^{18}O_{LW}$ was accounted for in the analysis. So, the principal mechanism underlying variation of $p_{ex\text{-}LW}$ resided in the (source) leaf and not in the growing sink tissue. The primary data of Lehmann *et al.* (2017) are also consistent with that conclusion. However, Lehmann *et al.* (2017) made a mistake in the estimation of $p_{ex}p_x$ (their p_{sc}), due to an error in Eqn 2 of their paper (compare Eqn 1 with their Eqn 2). If we calculate $p_{ex\text{-}SucSynW}$ from their primary data using our Eqn 1, taking tap water instead of crown water as the source water $\delta^{18}O$ (–10.9‰), a temperature-dependent ε_{bio} (Sternberg & Ellsworth, 2011) of 26‰ for 28 °C, the temperature in their growth chamber, and a $p_{x\text{-}SucSynW}$ of 0.96 as observed here – Lehmann *et al.* (2017) did not determine $p_{x\text{-}LW}$ or $p_{x\text{-}SucSynW}$ –, then we obtain a mean $p_{ex\text{-}SucSynW}$ for *Dactylis glomerata* and *Lolium perenne* of *ca*. 0.55, close to our observation. Interestingly, our estimate of $p_{ex\text{-}SucSynW}$ also matches closely the mean p_{ex} estimate (0.53) calculated by Barbour & Farquhar (2000) from the data of Hill *et al.* (1995). That estimate was based on an alternative approach, that is measurements of randomization of ^{14}C-labelled hexose phosphates during cellulose synthesis in oak stem tissue.

The virtual constancy of $p_{ex\text{-}SucSynW}$ in contrasting environmental conditions is also remarkable given its theoretical range of 0.2-1.0 (Barbour & Farquhar, 2000). Clearly, the $p_{ex\text{-}LW}$ <0.2 observed at 400 and 800 μmol mol^{-1} CO_2 at a RH of 50% are outside that theoretical expectation. Although many studies have converged to a p_{ex} estimate of 0.4-0.5, if the original substrate for cellulose synthesis is carbohydrates (see compilation in Cernusak *et al.*, 2005), Song *et al.* (2014) suggested that true p_{ex} may vary significantly depending on turnover time of non-structural carbohydrates. When using the same approach as Song *et al.* (2014), we found only minor variation of turnover time of non-structural carbohydrates in our data set (Fig. S3), perhaps also contributing to the near constancy of $p_{ex\text{-}SucSynW}$. Moreover, *L. perenne* uses different fructan series, including mixed-linkage fructans, as the primary non-structural carbohydrate store (Pavis *et al.*, 2001) and all plants had very high fructan contents (>35% of dry wt) in both the leaf growth-and-differentiation zone (Baca Cabrera *et al.*, 2020) and leaf blades of fully-expanded leaves in all treatments (Fig. S2). Futile cycling of sucrose appears to be very active in *L. perenne* (Lattanzi *et al.*, 2012), and a high fraction of the substrate used for leaf structural biomass synthesis likely first passes through the fructan pool in the growth-and-differentiation zones of leaves (Schnyder *et al.*, 1988). These factors may have also contributed to the magnitude and relative constancy of $p_{ex\text{-}SucSynW}$ in this study.

Both RH and atmospheric CO_2 concentration were strong determinants of $p_{ex\text{-}LW}$ variation in our experiments. While effects of atmospheric CO_2 concentration during

plant/stand growth have not been studied previously, a very similar effect of RH on $p_{ex\text{-}LW}$ was also observed in a multi-seasonal, ecosystem-scale study of modelled and observed $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cellulose}$ in a temperate grassland (Hirl *et al.*, 2021), showing that the same effect can also occur in natural conditions. A similar RH effect on $p_{ex\text{-}LW}$ in the C_4 grass *Cleistogenes squarrosa* (Liu *et al.*, 2016) and in several C_3 and C_4 species (Helliker & Ehleringer, 2002) was discussed by Liu *et al.* (2017). Moreover, a tendency for a similar RH effect on $p_{ex\text{-}LW}$ is also apparent in the data from *R. communis* presented by Song *et al.* (2014). It is tempting to also interpret these effects in terms of a disagreement between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$. The present analysis shows that the divergence between $p_{ex\text{-}LW}$ and $p_{ex\text{-}SucSynW}$ was essentially a direct result of the discrepancy between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$, with $\Delta^{18}O_{SucSynW}$ well approximated by:

$$\Delta^{18}O_{SucSynW} \approx \Delta^{18}O_{LW}\,(1 - p_{ex\text{-}LW})/\,(1 - 0.52), \text{ since} \qquad \text{Eqn 5a}$$

$$\Delta^{18}O_{SucSynW}\,(1 - p_{ex\text{-}SucSynW}\,p_{x\text{-}SucSynW}) = \Delta^{18}O_{LW}\,(1 - p_{ex\text{-}LW}\,p_{x\text{-}LW}). \qquad \text{Eqn 5b}$$

Such a rough calculation only requires knowledge of (assimilation-weighted) $\Delta^{18}O_{LW}$, ε_{bio}, p_x, $\Delta^{18}O_{Cellulose}$ (to estimate $p_{ex\text{-}LW}$) and a theoretically- or empirically-based estimate of $p_{ex\text{-}SucSynW}$ and can provide a quantitative guess for the magnitude of the discrepancy between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$. We suggest that this hypothetical interpretation (Eqn 5a, b) should be tested more widely across plant functional groups and environmental conditions to evaluate the magnitude of eventual discrepancies between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$ and on their implication in the interpretation of the relationship between (assimilation-weighted) leaf water ^{18}O enrichment and ^{18}O enrichment of cellulose. Most certainly, a better understanding of the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{SucSynW}$ will require a better knowledge of the spatio-temporal dynamics of convective and diffusive water fluxes and associated patterns of ^{18}O-enrichment in leaves – both at subcellular and tissue level – and corresponding spatio-temporal patterns of (photosynthetic) sucrose synthesis rates in the different parts of leaves.

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Author contributions

HS, JCBC and RTH designed the study. JCBC, RTH and JZ performed the experiments, sampling, and sample processing, with technical assistance (see above). RS performed the isotope analyses. JCBC analyzed the data and wrote the first draft. JCBC, HS, JO, RTH, RS, JZ and HL contributed to the discussion and revision of the manuscript.

Competing interests

The authors declare that they have no competing interests

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