Received: 6 November 2023 | Revised: 2 February 2024 | Accepted: 1 March 2024

DOI: 10.1111/pce.14881

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

Half of the ^{18}O enrichment of leaf sucrose is conserved in leaf cellulose of a C_3 grass across atmospheric humidity and CO_2 levels

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Funding information
Deutsche Forschungsgemeinschaft (DFG SCHN 557/9-1)

Abstract

The ^{18}O enrichment ($\Delta^{18}O$) of cellulose ($\Delta^{18}O_{Cel}$) is recognized as a unique archive of past climate and plant function. However, there is still uncertainty regarding the proportion of oxygen in cellulose (p_{ex}) that exchanges post-photosynthetically with medium water of cellulose synthesis. Particularly, recent research with C_3 grasses demonstrated that the $\Delta^{18}O$ of leaf sucrose ($\Delta^{18}O_{Suc}$, the parent substrate for cellulose synthesis) can be much higher than predicted from daytime $\Delta^{18}O$ of leaf water ($\Delta^{18}O_{LW}$), which could alter conclusions on photosynthetic versus post-photosynthetic effects on $\Delta^{18}O_{Cel}$ via p_{ex}. Here, we assessed p_{ex} in leaves of perennial ryegrass (*Lolium perenne*) grown at different atmospheric relative humidity (RH) and CO_2 levels, by determinations of $\Delta^{18}O_{Cel}$ in leaves, $\Delta^{18}O_{LGDZW}$ (the $\Delta^{18}O$ of water in the leaf growth-and-differentiation zone) and both $\Delta^{18}O_{Suc}$ and $\Delta^{18}O_{LW}$ (adjusted for ε_{bio}, the biosynthetic fractionation between water and carbohydrates) as alternative proxies for the substrate for cellulose synthesis. $\Delta^{18}O_{LGDZW}$ was always close to irrigation water, and p_{ex} was similar (0.53 ± 0.02 SE) across environments when determinations were based on $\Delta^{18}O_{Suc}$. Conversely, p_{ex} was erroneously and variably underestimated (range 0.02–0.44) when based on $\Delta^{18}O_{LW}$. The photosynthetic signal fraction in $\Delta^{18}O_{Cel}$ is much more constant than hitherto assumed, encouraging leaf physiological reconstructions.

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

1 | INTRODUCTION

The ^{18}O enrichment of cellulose above source water ($\Delta^{18}O_{Cel}$, for definitions and specifications of symbols, see Table 1) is believed to contain important environmental and physiological information (Barbour, 2007; Gessler et al., 2014; Helliker & Richter, 2008; Roden & Lin, & Ehleringer, 2000; Song et al., 2022; Werner et al., 2012), such as past atmospheric relative humidity (RH) (Barbour et al., 2004; Helliker & Ehleringer, 2002a, 2002b; Hirl et al., 2021; Liu et al., 2016, 2017b) or differences in transpiration or stomatal

TABLE 1 Definitions of symbols and specifications.

Symbol	Definition	Specification
$\delta^{18}O_X$	The relative abundance of ^{18}O in a sample *X*	Calculated as $\delta^{18}O_X = (R_X/R_s - 1) \times 1000$, with R_X and R_s the molar abundance ratios, $^{18}O/^{16}O$ of the sample and of Vienna standard mean ocean water (VSMOW), thus expressed in per mil (‰)
$\delta^{18}O_{Source}$	The average $\delta^{18}O$ of the water taken up by the root system of a plant, often termed $\delta^{18}O$ of xylem water	Here defined as the $\delta^{18}O$ of nutrient solution (−9.7 ± 0.2‰ SD), which was slightly depleted relative to the $\delta^{18}O$ of water extracted from the leaf growth-and-differentiation zone (see Table 2 and Baca Cabrera et al., 2023)
$\delta^{18}O_{Vapour}$	$\delta^{18}O$ of water vapour in the growth chamber atmosphere	
$\Delta^{18}O_X$	The ^{18}O enrichment above source water of a sample *X*	Calculated as $\Delta^{18}O_X = (\delta^{18}O_X - \delta^{18}O_{Source})/(1 + \delta^{18}O_{Source}/1000)$, thus expressed in per mil (‰)
$\Delta^{18}O_{LW}$	The $\Delta^{18}O$ of leaf water	Based on total water extracted from fully expanded leaf blades, including veins
$\Delta^{18}O_{LGDZW}$	The $\Delta^{18}O$ of water in the leaf growth-and-differentiation zone	Collected as in Liu et al. (2017a)
$\Delta^{18}O_{Cel}$	The $\Delta^{18}O$ of leaf blade cellulose	Cellulose extracted from fully expanded leaf blades
$\Delta^{18}O_{Suc}$	The $\Delta^{18}O$ of sucrose	Sucrose extracted from fully expanded leaf blades and isolated by preparative HPLC
$\Delta^{18}O_{SSW}$	The assimilation-weighted $\Delta^{18}O$ of medium water at the site of photosynthesis and associated sucrose synthesis, before the p_{ex}-process occurs during assimilate (sucrose) transport and cellulose synthesis	Calculated as $\Delta^{18}O_{Sucrose} - \varepsilon_{bio}$
p_{ex}	The proportion of oxygen atoms in cellulose that exchanged with medium water during cellulose formation	Defined as $p_{ex} = (\Delta^{18}O_{Suc} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{SSW} - \Delta^{18}O_{LGDZW})$
$p_{ex\text{-}Suc}$	p_{ex} based on measurements of $\Delta^{18}O_{Suc}$	Calculated as $p_{ex\text{-}Suc} = (\Delta^{18}O_{Suc} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{Suc} - \varepsilon_{bio} - \Delta^{18}O_{LGDZW})$
$p_{ex\text{-}LW}$	p_{ex} based on the assumption that $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$	Calculated as $p_{ex\text{-}LW} = (\Delta^{18}O_{LW} + \varepsilon_{bio} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{LW} - \Delta^{18}O_{LGDZW})$
p_x	the proportion of source water at the site of cellulose synthesis, which is the leaf growth-and-differentiation zone in grass tillers	Defined as $p_x = 1 - \Delta^{18}O_{LGDZW}/\Delta^{18}O_{SSW}$
$p_{x\text{-}Suc}$	p_x based on measurements of $\Delta^{18}O_{Suc}$	Calculated as $p_x = 1 - \Delta^{18}O_{LGDZW}/(\Delta^{18}O_{Suc} - \varepsilon_{bio})$
$p_{x\text{-}LW}$	p_x based on the assumption that $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$	Calculated as $p_x = 1 - \Delta^{18}O_{LGDZW}/\Delta^{18}O_{LW}$
ε_{bio}	The average biochemical fractionation between carbonyl oxygen and water	26.7‰, according to the temperature dependence of ε_{bio} for cellulose synthesis in aquatic plants as reported by Sternberg & Ellsworth (2011), taken as constant for all treatments and closely similar to the constant ε_{bio} = 27‰ used in most studies (Barbour, 2007)

conductance among plant species and genotypes in the same environment (e.g., Baca Cabrera et al., 2021; Barbour et al., 2000a; Lin et al., 2022; Scheidegger et al., 2000; Siegwolf et al., 2023). These relationships are grounded in the fact that virtually all of the oxygen in cellulose originates from water (DeNiro & Epstein, 1979; Liu et al., 2016) and evaporative conditions lead to an ^{18}O enrichment of leaf water above source water ($\Delta^{18}O_{LW}$, Table 1) (Cernusak et al., 2016, 2022; Dongmann et al., 1974; Farquhar & Cernusak, 2005; Farquhar et al., 2007; Flanagan et al., 1991; Roden & Ehleringer, 1999). The latter affects the ^{18}O enrichment of photosynthetic products (e.g., Baca Cabrera et al., 2023; Barbour et al., 2000b; Cernusak et al., 2003, 2005; Farquhar et al., 1998; Lehmann et al., 2017; Sternberg & DeNiro, 1983; Sternberg et al., 1986), which are subsequently used in cellulose synthesis (Barbour & Farquhar, 2000; Helliker & Ehleringer, 2002a, 2002b; Cernusak et al., 2005). Leaf sucrose plays a central role in the transfer of the photosynthetic ^{18}O signal onto cellulose (Barbour et al., 2000b; Cernusak et al., 2003; Gessler et al., 2013, 2014; Lehmann et al., 2017; Roden & Lin, & Ehleringer, 2000). It is the main form of carbohydrate synthesized in the cytosol of photosynthetically active mesophyll cells and an important diurnal storage carbohydrate in leaves (e.g., Farrar & Farrar, 1986; Gerhardt et al., 1987; Kaiser et al., 1982). Also, it is the predominant carbohydrate translocated to sink tissues in most plants (e.g., Braun, 2022; Lalonde et al., 2003). Therefore, it is the main

original substance from which cellulose is synthesized (Delmer et al., 1995; Haigler et al., 2001). Nevertheless, there is a great scarcity of studies on the quantitative relationship between ^{18}O enrichment of sucrose ($\Delta^{18}O_{Suc}$, Table 1) and $\Delta^{18}O_{Cel}$, a relationship we are scrutinizing here with the C_3 grass perennial ryegrass (*Lolium perenne* L.).

It is well accepted that a significant fraction of the ^{18}O enrichment signal in cellulose is not inherited from the parent photosynthetic substrate but is exchanged post-photosynthetically with oxygen from source (i.e., xylem) water during sugar metabolism (Barbour & Farquhar, 2000; Roden & Ehleringer, 1999; Roden & Lin, & Ehleringer, 2000; Helliker & Ehleringer, 2002a, 2002b; Barbour, 2007; Martínez-Sancho et al., 2023). This phenomenon is the product of two parameters: (i) the proportion (fraction) of substrate-oxygen incorporated in cellulose that has exchanged post-photosynthetically with medium water (p_{ex}, see Equations 1a and 1b below) and (ii) the proportion of unenriched source water in the medium water (p_x) (Barbour & Farquhar, 2000) (Figure 1). In that, carbohydrate metabolism along the transport path, including cycling through stores, may perhaps contribute to oxygen exchange with source water (Gessler et al., 2013, 2014). Furthermore, there is a distinct (possibly temperature-dependent) offset in ^{18}O content of the substrate oxygen that is exchanged with water during the reactions leading to cellulose synthesis (Hirl et al., 2021; Sternberg & Ellsworth, 2011; Sternberg et al., 1986). This offset is termed average biochemical ^{18}O fractionation (ε_{bio}) (Barbour, 2007) and results from an isotope effect during carbonyl hydration reactions (Sternberg et al., 1986). Barbour & Farquhar (2000) summarized understanding of the controls of ^{18}O enrichment of water at the site of photosynthesis and associated sucrose synthesis ($\Delta^{18}O_{SSW} = \Delta^{18}O_{Suc} - \varepsilon_{bio}$) and $\Delta^{18}O_{Cel}$ in the following quantitative model (see also Figure 1):

$$\Delta^{18}O_{Cel} = \Delta^{18}O_{SSW}(1 - p_{ex}\,p_x) + \varepsilon_{bio}. \quad (1a)$$

Equation 1a omits second-order isotope terms associated with fractionation during cellulose synthesis, as this is generally assumed to be sufficiently close to zero for ^{18}O (Holloway-Phillips et al., 2022; Waterhouse et al., 2002). When expressed in terms of the ^{18}O enrichment of the organic substrate ($\Delta^{18}O_{Suc} = \Delta^{18}O_{SSW} + \varepsilon_{bio}$) Equation 1a becomes:

$$\Delta^{18}O_{Cel} = \Delta^{18}O_{Suc}(1 - p_{ex}\,p_x) + \varepsilon_{bio}\,p_{ex}\,p_x. \quad (1b)$$

Equation 1b is consistent with Song et al. (2014, their Equation 4), but differs from Lehmann et al. (2017, their Equation 2), who omitted the $\varepsilon_{bio}\,p_{ex}\,p_x$ term. The terms $(1 - p_{ex}\,p_x)$ and $p_{ex}\,p_x$ represent the proportional contributions of the photosynthetic signal and of post-photosynthetic oxygen exchange with source water, respectively, to $\Delta^{18}O_{Cel}$. Classically, in applications of this model, it has been assumed that $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$ (Barbour, 2007) based primarily on evidence from studies with dicot species, *Ricinus communis* (Barbour et al., 2000b; Cernusak et al., 2003) and *Eucalyptus globulus* (Cernusak et al., 2005). In studies with

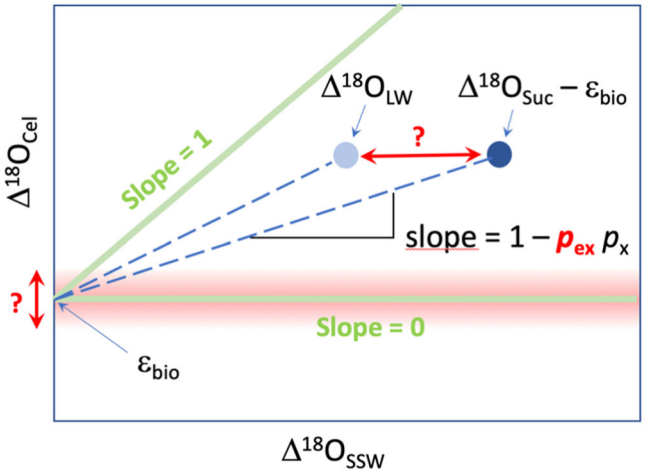

FIGURE 1 Conceptual scheme of the Barbour & Farquhar (2000) model of ^{18}O enrichment of cellulose above source water ($\Delta^{18}O_{Cel}$), illustrating the effect of parameter uncertainty (question marks) on calculation of $(1 - p_{ex}\,p_x)$, the slope parameter which quantifies the proportion of the original photosynthetic ^{18}O signal in cellulose in a given context. $\Delta^{18}O_{Cel} = \Delta^{18}O_{SSW}(1 - p_{ex}\,p_x) + \varepsilon_{bio}$, with $\Delta^{18}O_{SSW}$ the ^{18}O enrichment of the medium water in which photosynthesis and sucrose synthesis take place (calculated as $\Delta^{18}O_{SSW} = \Delta^{18}O_{Suc} - \varepsilon_{bio}$) or inferred from ^{18}O enrichment of leaf water ($\Delta^{18}O_{LW}$) as $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$ (see text); $p_{ex}\,p_x$ is the so-called attenuation (or damping) factor and is the product of p_x (the proportion of source water at the site of cellulose synthesis) and p_{ex} (the proportion of oxygen atoms in cellulose that exchanged with medium water during cellulose formation). ε_{bio} represents the average ^{18}O equilibrium fractionation between carbonyl groups and water. According to Liu et al. (2017a) p_x is close to unity in grass leaves, which implies that variation in the slope parameter is mainly attributable to p_{ex}. The area between the green lines delimits the theoretical range of the $\Delta^{18}O_{Cel}$ versus $\Delta^{18}O_{SSW}$ relationship. A hypothetical data point on the slope = 1 line means that all of the primary substrate ^{18}O ('photosynthetic signal') is conserved in cellulose. Such a situation arises if either p_{ex} or $p_x = 0$ (or both p_{ex} and $p_x = 0$). Conversely, a data point falling on the slope line = 0 (implying $\Delta^{18}O_{Cel} = \varepsilon_{bio}$) means that $\Delta^{18}O_{Cel}$ is fully independent of the ^{18}O signal in the original photosynthetic substrate, which is used to manufacture cellulose. For the latter, $p_{ex}\,p_x = 1$, which requires that both p_{ex} and $p_x = 1$, that is all oxygen in the substrate is exchangeable and all water at the site of cellulose synthesis is source water ($\Delta^{18}O_{LGDZ} = 0$). In studies with intact autotrophic terrestrial plants, uncertainty is greatest for p_{ex} (red) as this parameter cannot be determined directly, and hence can only be estimated by solving the Barbour & Farquhar model with knowledge of all other parameters. Classically, $\Delta^{18}O_{SSW}$ has been equated with $\Delta^{18}O_{LW}$ (see text); however, recent work with C_3 grasses has determined an RH-dependent underestimation of $\Delta^{18}O_{SSW}$ (estimated as $\Delta^{18}O_{Suc} - \varepsilon_{bio}$) by $\Delta^{18}O_{LW}$ (Lehmann et al., 2017; Baca Cabrera et al., 2023), which always causes an underestimation of p_{ex} when $\Delta^{18}O_{SSW}$ is set equal to $\Delta^{18}O_{LW}$ and p_x is close to unity (see slope change in Figure 1). In addition, there is some uncertainty about ε_{bio} (red-shaded horizontal band) which may be temperature-dependent (Hirl et al., 2021; Sternberg & Ellsworth, 2011). Here, we determine p_{ex} in grass leaves using a complete data set of the remaining parameters of the Barbour & Farquhar model (including $\Delta^{18}O_{Suc} - \varepsilon_{bio}$ and $\Delta^{18}O_{LW}$ as alternative proxies of $\Delta^{18}O_{SSW}$) and a consensus estimate of ε_{bio} obtained by growing plants in a thermal environment for which standard and temperature-corrected estimations of ε_{bio} converge closely.

grasses, $\Delta^{18}O_{LW}$ has generally been equated with the $\Delta^{18}O$ of bulk leaf water, including the main vein (Helliker & Ehleringer, 2002a; Hirl et al., 2021; Lehmann et al., 2017; Liu et al., 2016).

In grasses (Figure 2), p_x is determined from $\Delta^{18}O_{SSW}$ and $\Delta^{18}O_{LGDZW}$, the $\Delta^{18}O$ of water at the site of cellulose synthesis in the leaf growth-and-differentiation zone, as (Liu et al., 2017a):

$$p_x = 1 - \Delta^{18}O_{LGDZW}/\Delta^{18}O_{SSW}. \quad (2)$$

In investigations with several C_3 and C_4 grasses exposed to low (50%) and high (80%) RH in a controlled environment, Liu et al. (2017a) found that p_x was very high (0.9–1.0) in all species × RH combinations, when they assumed that $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$. Similar determinations were made earlier in the cambium of woody stems and also found a $p_x \sim 1$ (Cernusak et al., 2005; Roden & Lin, & Ehleringer, 2000; Yakir, 1998).

There is a wide consensus that p_{ex} is likely the most uncertain term in the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ (e.g., Hirl et al., 2021; Martínez-Sancho et al., 2023; Roden & Ehleringer, 2000; Song et al., 2022). This is because p_{ex} cannot be determined directly in an intact terrestrial autotrophic plant for a given environmental context. Instead, the value of p_{ex} has been either (i) extrapolated from findings in heterotrophic systems, e.g., seedlings grown in the dark (Cernusak et al., 2005; Luo & Sternberg, 1992; Yakir & DeNiro, 1990), or (ii) obtained by solving Equation 1. For approach (i), and when the main substrate for cellulose synthesis was a carbohydrate, the estimates of p_{ex} ranged between 0.31 and 0.50 among studies compiled by Cernusak et al. (2005). Additionally, estimates of p_{ex} between 0.49 and 0.57 were calculated by Barbour & Farquhar (2000) from the data of Hill et al. (1995), who explored randomization of ^{14}C-labelled hexose phosphates during cellulose synthesis in oak stem tissue. For approach (ii), solving Equation 1 and substituting p_x with $(1 - \Delta^{18}O_{LGDZW}/\Delta^{18}O_{SSW}$; Equation 2) yields:

$$p_{ex} = (\Delta^{18}O_{Suc} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{SSW} - \Delta^{18}O_{LGDZW}). \quad (3)$$

The advantage of the latter approach is that it permits the estimation of a context-specific p_{ex} value (a 'single-point' determination). That is, it can principally detect possible environment- or metabolism-dependent variations in p_{ex}. However, this approach is prone to errors in the quantification of p_{ex} and p_x, as it is affected by the uncertainty of all the parameter estimates needed for its calculation (Figure 1). In addition, this also includes uncertainty in ε_{bio}, which is often fixed at 27‰ (e.g., Barbour, 2007; Helliker & Richter, 2008), but may be temperature-dependent, particularly below ~15°C (Hirl et al., 2021; Sternberg & Ellsworth, 2011). p_{ex} estimates are also sensitive to errors in the estimation of the ^{18}O signature of the source water taken up by roots ($\delta^{18}O_{Source}$), as this parameter is needed for the calculations of $\Delta^{18}O_{Cel}$, $\Delta^{18}O_{SSW}$, and $\Delta^{18}O_{LGDZW}$ in Equations 1–3 (Table 1). Particularly, $\delta^{18}O_{Source}$ can vary strongly over time and space in field studies (Allen et al., 2022; Hirl et al., 2019; Martínez-Sancho et al., 2023; Song et al., 2022) and, therefore, must be integrated in an assimilation- and allocation-weighted manner over the period during which a given cellulose sample is synthesized (Hirl et al., 2021). To date, as far as we are aware, there has been no study in any terrestrial autotrophic plant

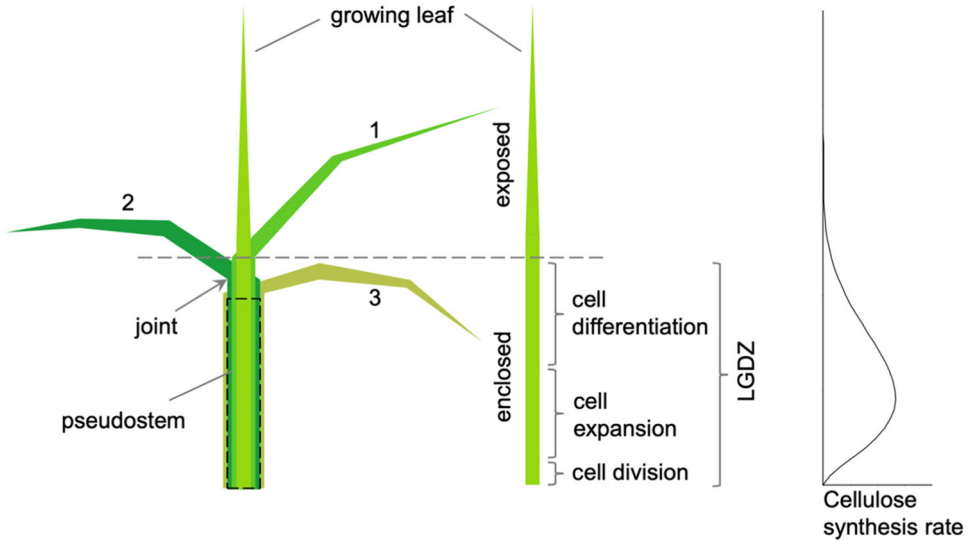

FIGURE 2 Scheme of a vegetative grass tiller (Hirl, 2021, adapted from Liu et al., 2017a). A vegetative tiller of a C_3 grass usually comprises three fully-expanded mature leaves, with leaf 1 the youngest and leaf 3 the oldest (senescing) leaf, plus one growing leaf. The joint marks the transition between leaf blade and leaf sheath. The pseudostem represents the basal part of the tiller and consists of the sheaths of the expanded leaves and the leaf growth and differentiation zone (LGDZ). The LGDZ comprises zones of cell division, expansion, and differentiation, including cellulose synthesis, and is completely enclosed inside the leaf sheaths of the next older leaf (Allard & Nelson, 1991; Liu et al., 2017b; MacAdam & Nelson, 2002; Schnyder & Nelson, 1987; Schnyder et al., 1990, 2000; Stebler, 1876; Volenec & Nelson, 1981). Thus, the LGDZ is not directly exposed to evaporative conditions in the surrounding air. The spatial distribution of cellulose synthesis rate along the LGDZ is schematically displayed on the right. We used water extracted from the bulk LGDZ tissue to determine ^{18}O enrichment of the medium water for cellulose synthesis in leaf blades (termed $\Delta^{18}O_{LGDZW}$). [Color figure can be viewed at wileyonlinelibrary.com]

system that determined both $\Delta^{18}O_{Suc}$ and $\Delta^{18}O$ of water at the location of cellulose synthesis (here denoted $\Delta^{18}O_{LGDZW}$) in the same experiment.

Importantly, the variation of p_{ex} across studies with terrestrial autotrophic systems—deducible from the application of Equation 3 with widely accepted simplifying assumptions—appears to be extremely large (Figure 1). In studies with grasses (Helliker & Ehleringer, 2002a, 2002b; Hirl et al., 2021; Lehmann et al., 2017; Liu et al., 2016; Liu, 2017), a range of p_{ex} from ~0.2 to 1.0 is estimated across studies if we set p_x to 0.95 (Liu et al., 2017a) and $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$. In most cases (Hirl et al., 2021; Lehmann et al., 2017; Liu et al., 2016; Liu, 2017), a positive correlation emerges between p_{ex} and RH. A similar environmentally-related range of p_{ex} (0.0–0.9) is suggested for cellulose synthesis in studies on tree-rings if p_x is assumed to be ~1 and $\Delta^{18}O_{SSW}$ is set to (daytime assimilation-weighted) $\Delta^{18}O_{LW}$ (Kagawa & Battipaglia, 2022; Offermann et al., 2011; Song et al., 2022; see also Holloway-Phillips et al., 2023). However, the directionality of the relationship between RH and p_{ex} was not the same in all studies (Martínez-Sancho et al., 2023).

Variation of p_{ex} is plausible based on biochemical theory because of the variable futile cycling of triose phosphates derived from hexose phosphates during cellulose synthesis: on the premise that all carbonyl oxygens formed during futile cycling exchange with medium water, the theoretically possible range of p_{ex} varies from 0.2 (no triose phosphate cycling) to 1 (all hexose phosphates recycled trough triose phosphates) (Barbour & Farquhar, 2000).

Most importantly, erroneous assumptions could bias estimates of p_{ex} and its RH sensitivity. For example, in investigations with two grasses, Lehmann et al. (2017) first observed that $\Delta^{18}O_{Suc}$ was much greater than $\Delta^{18}O_{LW} + \varepsilon_{bio}$ (with ε_{bio} set to 27‰), particularly at low RH. This finding was recently corroborated by Baca Cabrera et al. (2023) and suggests that the RH-dependent variation of p_{ex} may be the result of replacing $\Delta^{18}O_{SSW}$ by $\Delta^{18}O_{LW}$ in Equation 1. Nevertheless, one still finds an RH-dependent p_{ex} when the data from Lehmann et al. (2017) is re-evaluated with Equation 3, using their primary data (including $\Delta^{18}O_{Suc}$) and an estimate of $p_x = 0.95$ (as presented by Liu et al., 2017a). Clearly, given all uncertainties, there is a need for joint determinations under controlled conditions of all experimentally accessible parameters ($\Delta^{18}O_{Cel}$, $\Delta^{18}O_{Suc}$, $\Delta^{18}O_{LW}$, and $\Delta^{18}O_{LGDZW}$) in Equation 3, as such an exercise has not been performed hitherto.

Here, we test the hypothesis that sucrose ^{18}O-based estimates of p_{ex} (termed $p_{ex\text{-}Suc}$, Equation 6a below) in leaves of perennial ryegrass (*Lolium perenne*, C_3) are less variable and closer to p_{ex} values determined in heterotrophic systems than estimates based on $\Delta^{18}O_{LW}$ ($p_{ex\text{-}LW}$, Equation 6b). In doing so, we also verify previous estimations of p_x (Figure 2) which have been made only in one study and with other C_3 grasses (Liu et al., 2017a). To this end, we also assessed the eventual environmentally-driven variability of p_{ex} by performing replicated (n = 3–5) mesocosm-scale experiments at three different constant atmospheric CO_2 concentrations (200, 400, or 800 µmol mol^{-1}) combined with constant daytime conditions of either low (50%) or high (75%) RH, environmental parameters which were known (or expected) to affect the ^{18}O enrichment of leaf water, sucrose synthesis water and cellulose in grasses (Baca Cabrera et al., 2021, 2023; Cernusak et al., 2016, 2022; Hirl et al., 2019; Lehmann et al., 2017). All experiments were performed in a thermal environment for which temperature-dependent estimates of ε_{bio} (26.7‰ at 20°C; Sternberg & Ellsworth, 2011) converged closely with the standard ε_{bio} = 27‰ assumption to reduce the uncertainty of parameter estimation. All other parameters included in Equations 1–3 (Table 1 and Section 2) were determined jointly in the same experiments. In this analysis, we took advantage of determinations of $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Suc}$, which have already been discussed in terms of mechanism controlling ^{18}O enrichment of water in the photosynthetic and non-photosynthetic tissue water fractions of leaf blades (Baca Cabrera et al., 2023).

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Experimental details have been previously described by Baca Cabrera et al. (2020, 2023). The experiments were performed in a gas exchange facility which included four plant growth chambers (PGR15, Conviron, Winnipeg, and Canada) as in Schnyder et al. (2003). Briefly, perennial ryegrass (cv. 'Acento') plants were grown in the different chambers in five sequential runs (cycles) in a 16 h/8 h, day/night cycle (temperature 20°C/16°C) with a 3 × 2 factorial design: three atmospheric CO_2 concentration [CO_2] levels ('half-ambient' = 200, 'ambient' = 400 or 'double-ambient' = 800 µmol mol^{-1}) and two daytime RH levels (low RH = 50%, high RH = 75%; nighttime RH was 75% for all treatments). Each mesocosm-scale treatment combination was run with three independent replications, except for the so-called 'reference treatment' (400 µmol mol^{-1} CO_2 at 50% RH), which was replicated five times (Table S1). 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%, respectively, relative to the set nominal value.

In all treatments, 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}. A Hoagland-type nutrient solution with reduced nitrate-N content was supplied to plants four times per day by using a flood and drain-type hydroponic system (Baca Cabrera et al., 2020). The solution was refreshed every 3 weeks. A constant photosynthetic photon flux density of 800 µmol m^{-2} s^{-1} was maintained at plant height during the 16 h-long light period.

2.2 | Sampling design and extraction of tissue water, cellulose, and sucrose

Plants from each chamber scale replicate were sampled when 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 was pooled in one subsample (providing two subsamples per chamber and per sampling occasion).

For tissue water extraction, the two most recently fully expanded leaf blades (from leaves 1 and 2 in Figure 2) and the leaf growth-and-differentiation zone (LGDZ, see Liu et al., 2017a and Baca Cabrera et al., 2020) of three mature tillers per plant were excised, sealed in 12 mL Exetainer vials (Labco), 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 set of two similar 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 (i.e., the water-modified Brendel method) by Gaudinski et al. (2005), including a treatment with a 17.5% NaOH solution. Samples obtained with this protocol consistently contained at most traces of nitrogen (<0.05% of dry mass). Water-soluble carbohydrates were extracted from 50 mg aliquots of dry material from the youngest fully-expanded leaf blade, and sucrose was separated from other compounds using a preparative HPLC technique similar to that described by Gebbing & Schnyder (2001) (Baca Cabrera et al., 2023).

2.3 | Isotope analysis

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

$$\delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000, \quad (4)$$

with R_{sample} the $^{18}O/^{16}O$ ratio of the sample and $R_{standard}$ (=0.0020052 according to IAEA Tecdoc 825) 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 ($\delta^{18}O_{LGDZW}$); and cellulose and sucrose of leaf blades ($\delta^{18}O_{Cel}$ and $\delta^{18}O_{Suc}$). Furthermore, the nutrient solution (defined as the source water for plants, $\delta^{18}O_{Source}$) was sampled 1–2 times per week. $\delta^{18}O_{Source}$ was virtually constant during the 3 weeks-long nutrient solution use (i.e., a nutrient solution refreshment cycle) throughout the experiments (−9.7 ± 0.2‰ SD). $\delta^{18}O_{Source}$ was used to calculate ^{18}O enrichment above source water ($\Delta^{18}O_X$) of the different sample types (X) as:

$$\Delta^{18}O_X = \frac{\delta^{18}O_X - \delta^{18}O_{Source}}{1 + \delta^{18}O_{Source}/1000}. \quad (5)$$

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.). 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 data set 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, as in Baca Cabrera et al. (2021, 2023). 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 (fine ground cotton fibre) were run each time after the measurement of four samples for possible drift correction and for SMOW-scaling. The long-term precision for the laboratory standard was <0.3‰.

Additionally, $\delta^{18}O$ of water vapour in the growth chambers ($\delta^{18}O_{Vapour}$) was measured by cavity ring-down spectroscopy as described by Liu et al. (2016). Here, we measured $\delta^{18}O_{Vapour}$ continuously during 2 weeks when canopies were closed, both during the light and the dark periods. $\delta^{18}O_{Vapour}$ was constant across experimental runs and treatments but was c. 1‰ more enriched during the dark period (−14.2‰ ± 0.5‰ SD) than during the light period (−15.2‰ ± 0.6‰ SD).

2.4 | Calculation of p_{ex}

According to Equation 3, p_{ex} was determined for each mesocosm-scale replicate with two alternative proxies of $\Delta^{18}O_{Suc}$, one based on leaf sucrose (denoted $p_{ex\text{-}Suc}$) and the other on bulk leaf water (denoted $p_{ex\text{-}LW}$) extracted from fully expanded leaf blades under the (common) assumption that $\Delta^{18}O_{Suc}$ equals the assimilation-weighted $\Delta^{18}O_{LW} + \varepsilon_{bio}$ (or $\Delta^{18}O_{SSW} = \Delta^{18}O_{LW}$) as in Helliker & Ehleringer (2002a), Liu et al. (2016), and Lehmann et al. (2017). $p_{ex\text{-}Suc}$ and $p_{ex\text{-}LW}$ were thus calculated as:

$$p_{ex-Suc} = (\Delta^{18}O_{Suc} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{Suc} - \varepsilon_{bio} - \Delta^{18}O_{LGDZW}), \quad (6a)$$

and

$$p_{ex-LW} = (\Delta^{18}O_{LW} + \varepsilon_{bio} - \Delta^{18}O_{Cel})/(\Delta^{18}O_{LW} - \Delta^{18}O_{LGDZW}), \quad (6b)$$

with $\varepsilon_{bio} = 26.7$ in all cases, according to the temperature-dependence of ε_{bio} for cellulose synthesis in aquatic plants as reported by Sternberg and Ellsworth (2011), which is closely similar to the constant $\varepsilon_{bio} = 27$‰ used in most studies (Barbour, 2007).

2.5 | Calculation of p_x

p_x was estimated for each replicate based on a two-member mixing model that has $\Delta^{18}O_{Source}$ (with $\Delta^{18}O_{Source} = 0$ by definition) as one member and photosynthesis and sucrose synthesis medium water as the other member. The latter was estimated by either $\Delta^{18}O_{SSW}$

(calculated as $\Delta^{18}O_{Suc} - \varepsilon_{bio}$) or $\Delta^{18}O_{LW}$. Again, according to the above, these variants of p_x are thus designated $p_{x\text{-}Suc}$ and $p_{x\text{-}LW}$ and were calculated as:

$$p_{x-Suc} = 1 - \Delta^{18}O_{LGDZW}/(\Delta^{18}O_{Suc} - \varepsilon_{bio}), \quad (7a)$$

and

$$p_{x-LW} = 1 - \Delta^{18}O_{LGDZW}/\Delta^{18}O_{LW}. \quad (7b)$$

2.6 | Statistics

In a first step, linear mixed models were fitted to test the effect of the diel period (day vs. night) on $\Delta^{18}O_{LGDZW}$ ($n = 80$), $\Delta^{18}O_{LW}$ ($n = 160$), $\Delta^{18}O_{Suc}$ ($n = 70$) and $\Delta^{18}O_{Cel}$ ($n = 129$). 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 $\Delta^{18}O_{LW}$, the day and night data of $\Delta^{18}O_{LGDZW}$, $\Delta^{18}O_{Suc}$, 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, that is, to estimate $p_{x\text{-}LW}$ and $p_{ex\text{-}LW}$. Data from individual chamber scale replications were pooled, and two-way ANOVA tests were used to assess the effects of CO_2, RH, and their interaction on $\Delta^{18}O_{LGDZW}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Suc}$, $\Delta^{18}O_{Cel}$, $p_{x\text{-}LW}$, $p_{x\text{-}Suc}$, $p_{ex\text{-}LW}$, and $p_{ex\text{-}Suc}$. Additionally, ordinary least-squares linear regressions were performed to test the relationship of $\Delta^{18}O_{Cel}$ with $\Delta^{18}O_{Suc}$ and $\Delta^{18}O_{LW}$ (based on treatment averages). All statistical analyses were conducted in R v.4.3.1 (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.

3 | RESULTS

The results of the p_{ex} analysis agreed with the tested hypothesis: when calculations were based on $\Delta^{18}O_{Suc}$ (Table 2) p_{ex} (i.e., $p_{ex\text{-}Suc}$; Equation 6a) varied comparatively little, averaged 0.53 (±0.02 SE) and was not influenced significantly by $[CO_2]$, daytime RH, or the interaction of daytime RH and $[CO_2]$ (Table 3, Figure 3). Conversely, when calculations were based on $\Delta^{18}O_{LW}$ (Table 2), the estimates of p_{ex} (i.e., $p_{ex\text{-}LW}$; Equation 6b) varied strongly (range 0.02–0.44), increasing significantly with RH ($p < 0.001$) and decreasing with $[CO_2]$ ($p = 0.01$) (Table 3, Figure 3).

Meanwhile, $\Delta^{18}O_{LGDZW}$ was very low in all treatments (Table 2). Thus, total tissue water in the LGDZ was generally only minimally ^{18}O enriched relative to irrigation water. The small variation of $\Delta^{18}O_{LGDZW}$ was unrelated to RH or the interaction of $[CO_2]$ and RH levels, but the effect of $[CO_2]$ was just significant ($p = 0.04$) and implied a very small decrease of $\Delta^{18}O_{LGDZW}$ with $[CO_2]$ (Table 3). Accordingly, p_x was close to unity in all treatments and differed only slightly when ^{18}O enrichment in photosynthetic and sucrose synthesis water was used as the enriched end-member in the mixing model (average $p_{x\text{-}Suc}$ of 0.97) instead of bulk leaf water (average $p_{x\text{-}LW}$ of 0.95) (Figure 3).

TABLE 3 Significance (p-value) of treatment (CO_2 and RH levels and their interaction) effects on the parameters of the Barbour and Farquhar (2000) model of ^{18}O enrichment ($\Delta^{18}O$) of cellulose ($\Delta^{18}O_{Cel}$).

Parameter	Effect significance (p-value)		
	CO_2	RH	CO_2:RH
$p_{ex\text{-}Suc}$	0.08	0.95	0.61
$p_{ex\text{-}LW}$	**0.01**	**<0.001**	0.59
$p_{x\text{-}Suc}$	0.09	**<0.01**	0.58
$p_{x\text{-}LW}$	0.09	0.14	0.99
$p_{ex\text{-}Suc}$ $p_{x\text{-}Suc}$	0.11	0.55	0.54
$p_{ex\text{-}LW}$ $p_{x\text{-}LW}$	**0.01**	**<0.001**	0.52
$\Delta^{18}O_{Cel}$ (‰)	0.88	**<0.001**	0.34
$\Delta^{18}O_{Suc}$ (‰)[a]	**0.02**	**<0.001**	0.74
$\Delta^{18}O_{LGDZW}$ (‰)	**0.04**	0.22	0.95
$\Delta^{18}O_{LW}$ (‰)[a]	**<0.01**	0.55	0.47

Note: p values of a two-way ANOVA for multiple parameters measured in replicated ($n = 3$–5) mesocosm experiments. Significant effects are given in bold type.

[a]reported in Baca Cabrera et al. (2023).

TABLE 2 ^{18}O enrichment ($\Delta^{18}O$) of cellulose ($\Delta^{18}O_{Cel}$), sucrose ($\Delta^{18}O_{Cel}$), bulk water ($\Delta^{18}O_{LW}$) and water in the leaf growth-and-differentiation zone ($\Delta^{18}O_{LGDZW}$) for leaf blades of *Lolium perenne* plants grown at different atmospheric CO_2 concentrations (200, 400 or 800 µmol mol^{-1}) in combination with low (50%) or high (75%) daytime relative humidity. Averages for each treatment (mean ± SE) were calculated based on replicated mesocosm scale experiments ($n = 3$–5).

	Daytime RH (%)					
	50			75		
	Atmospheric CO_2 concentration (µmol mol^{-1})					
Parameter	200	400	800	200	400	800
$\Delta^{18}O_{Cel}$ (‰)	34.6 (0.3)	35.3 (0.4)	35.1 (0.4)	32.8 (0.6)	32.0 (0.3)	32.2 (0.5)
$\Delta^{18}O_{Suc}$ (‰)[a]	45.4 (1.9)	44.2 (0.7)	42.6 (0.8)	39.1 (0.5)	38.0 (1.0)	37.0 (0.2)
$\Delta^{18}O_{LW}$ (‰)[a]	9.9 (0.2)	9.6 (0.5)	8.6 (0.2)	10.2 (0.9)	9.2 (0.8)	7.9 (0.4)
$\Delta^{18}O_{LGDZW}$ (‰)	0.5 (0.3)	0.5 (0.2)	0.1 (0.2)	0.8 (0.1)	0.6 (0.05)	0.4 (0.2)

[a]reported in Baca Cabrera et al. (2023).

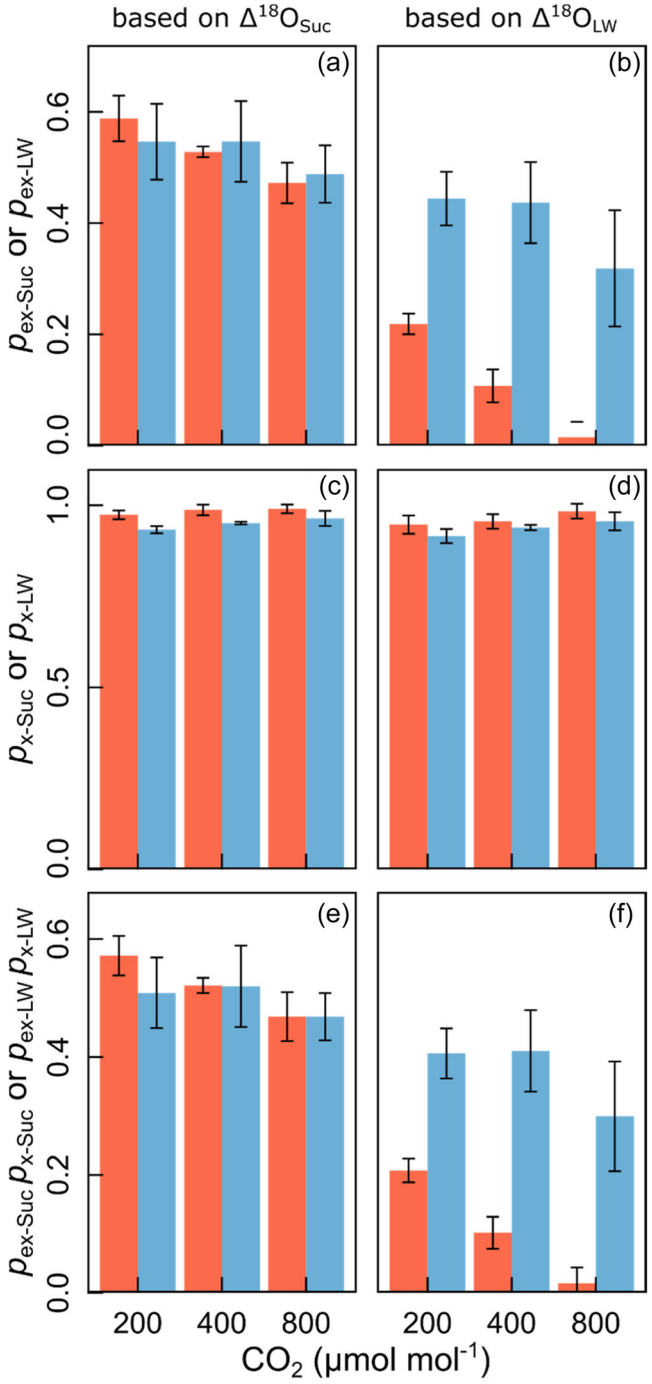

FIGURE 3 Effects of atmospheric CO_2 concentration and daytime relative humidity (RH) on the proportion of oxygen atoms in cellulose that exchanged with medium water during cellulose formation, p_{ex} (a, b); the proportion of source water at the site of cellulose synthesis, p_x (c, d); and the product of p_{ex} and p_x, the so-called attenuation (or damping) factor, which represents the proportion of ^{18}O from source water in cellulose (e, f). Red bars, low RH (50%); blue bars, high RH (75%). p_{ex}, p_x, and p_{ex} p_x were calculated based on $\Delta^{18}O_{Suc}$ (a, c, and e) or $\Delta^{18}O_{LW}$ with Equations as presented in Table 1. Data points and error bars represent the mean ± SE. [Color figure can be viewed at wileyonlinelibrary.com]

The fraction of the photosynthetic signal in cellulose, given by $1 - p_{ex}$ p_x (the slope parameter in Figure 1), did not vary significantly between treatments and averaged 0.49 (±0.02 SE) when calculated based on the ^{18}O enrichment of photosynthetic and sucrose synthesis water (Figure 4), and was determined essentially by the variation of $p_{ex\text{-}Suc}$ (Figure 3). Conversely, when based on $\Delta^{18}O_{LW}$, $1 - p_{ex\text{-}LW}$ $p_{x\text{-}LW}$ varied strongly (range 0.59–0.98) between treatments, primarily due to a positive effect of daytime RH and a negative effect of $[CO_2]$ on $p_{ex\text{-}LW}$ (Figure 3).

The small and treatment-independent variation of $1 - p_{ex\text{-}Suc}$ $p_{x\text{-}Suc}$ implied that variation of $\Delta^{18}O_{SSW}$ caused a largely proportional variation of $\Delta^{18}O_{Cel}$ ($R^2 = 0.85$; $p < 0.01$) (Figure 4a). Interestingly though, a small but significant CO_2 effect on $\Delta^{18}O_{Suc}$ ($p = 0.02$) disappeared during translation in $\Delta^{18}O_{Cel}$ ($p = 0.88$) (Table 3). In contrast to the close relationship between $\Delta^{18}O_{SSW}$ and $\Delta^{18}O_{Cel}$ (Figure 4a), we observed no significant relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ (Figure 4b).

4 | DISCUSSION

4.1 | Variation of $\Delta^{18}O$ in cellulose is driven by $\Delta^{18}O$ of leaf sucrose

This work separated and quantified the components p_{ex} and p_x of the so-called damping (or attenuation) factor of the Barbour & Farquhar (2000) model of ^{18}O incorporation in cellulose in an autotrophic/photosynthesizing terrestrial plant species in vivo. Based on the joint determination of ^{18}O enrichment of leaf sucrose ($\Delta^{18}O_{Suc}$) and water in the cellulose-synthesizing sink tissue ($\Delta^{18}O_{LGDZW}$) in controlled constant conditions, this study demonstrated that p_x in the LGDZ of grasses was always close to unity (0.97 ± 0.01 SE) across contrasting $[CO_2]$ and RH conditions. As $\Delta^{18}O_{LGDZW}$ was always very close to source water and $p_{ex\text{-}Suc}$ (0.53 ± 0.02 SE) varied little across atmospheric environments, variation of $\Delta^{18}O_{Cel}$ was determined by variation of $\Delta^{18}O_{SSW}$ ($R^2 = 0.85$) dampened by a near-constant proportionality (represented by $1 - p_{ex\text{-}Suc}$ $p_{x\text{-}Suc}$) of ~0.49. That is, nearly half of the oxygen in cellulose originated from leaf sucrose. Accordingly, variation of $\Delta^{18}O_{Cel}$ was chiefly determined by changes of $\Delta^{18}O$ of the medium water for photosynthesis and sucrose synthesis. This is an encouraging finding for continued endeavours to identify and reconstruct photosynthetic information archived in $\Delta^{18}O_{Cel}$. For instance, in the present experiments, we also observed a close correspondence between $\Delta^{18}O_{SSW}$ and average ^{18}O enrichment of evaporative site water ($\Delta^{18}O_e$) (Baca Cabrera et al., 2023). Since $1 - p_{ex\text{-}Suc}$ $p_{x\text{-}Suc}$ was very similar in all environments (but see below), the close relationship between $\Delta^{18}O_{SSW}$ and $\Delta^{18}O_e$ implied that there existed also a close relationship between $\Delta^{18}O_e$ and $\Delta^{18}O_{Cel}$ with a very similar p_{ex} p_x ($R^2 = 0.79$; $p < 0.05$; Figure 5).

Sucrose-based p_{ex} ($p_{ex\text{-}Suc}$) was a bit larger than p_{ex} determined in heterotrophic systems that used a carbohydrate source (Cernusak et al., 2005), but almost identical to the mean and range of p_{ex}

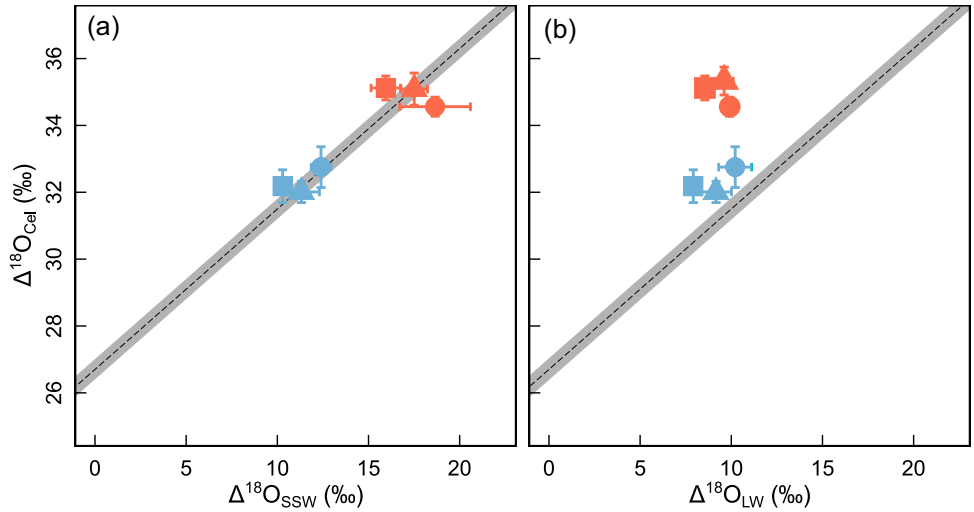

FIGURE 4 Relationship between $\Delta^{18}O$ of sucrose synthesis water ($\Delta^{18}O_{SSW}$) in leaf blades and $\Delta^{18}O_{Cel}$ (a) and between $\Delta^{18}O$ of total leaf blade water and $\Delta^{18}O_{Cel}$ (b) in plants of *L. perenne* grown at low (50%, red symbols) or high (75%) daytime relative humidity at low (200 µmol mol^{-1}, circles), ambient (400 µmol mol^{-1}, triangles) or high (800 µmol mol^{-1}, squares) atmospheric CO_2 concentration. The dashed line and the shadowed area in (a) and (b) indicate the values predicted with the Barbour–Farquhar model with $1 - p_{ex}\, p_x = 0.48$ 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. [Color figure can be viewed at wileyonlinelibrary.com]

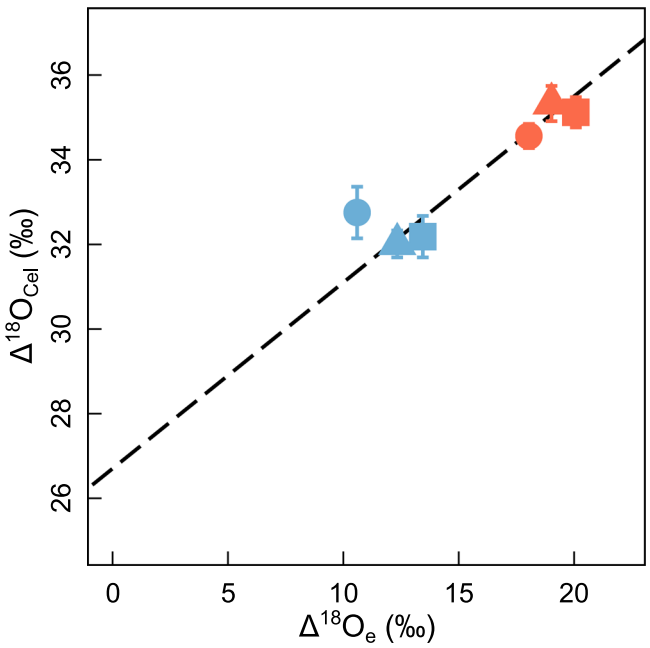

FIGURE 5 Relationship between leaf-scale $\Delta^{18}O$ of water at the evaporative sites in the same plants, as calculated with the Craig–Gordon model ($\Delta^{18}O_e$; see Baca Cabrera et al., 2023), and $\Delta^{18}O_{Cel}$ in plants of *L. perenne* grown at low (50%, red symbols) or high (75%, blue) daytime relative humidity at low (200 µmol mol^{-1}, circles), ambient (400 µmol mol^{-1}, triangles) or high (800 µmol mol^{-1}, squares) atmospheric CO_2 concentration. The broken line (R^2 = 0.79) corresponds to the Barbour–Farquhar model with a slope ($1 - p_{ex}\, p_x$) of 0.44 and intercept (ε_{bio}) of 26.7‰. Data points and error bars represent the mean ± SE. [Color figure can be viewed at wileyonlinelibrary.com]

estimates derived from the data of Hill et al. (1995) by Barbour & Farquhar (2000). However, the $p_{ex\text{-}Suc}$ estimates of the present work differ in several respects from those calculated from the primary data of Lehmann et al. (2017): the estimates were less variable, smaller on average (0.76 in Lehmann et al., 2017 vs. 0.53 in this study) and insensitive to RH, if the estimation for their study was based on the same average $p_{x\text{-}Suc}$ (0.97) as observed here. While we do not know the cause for the difference, we find it interesting that a much better agreement between the studies exists (average $p_{ex\text{-}Suc}$ 0.51 in Lehmann et al., 2017, vs. 0.53 in this work) if both studies consider a temperature-dependent ε_{bio} as presented by Sternberg & Ellsworth (2011) (i.e., a ε_{bio} = 26‰ for 28.5°C of Lehmann et al., 2017) and both define source water as irrigation water. In their work, Lehmann et al. (2017) assumed that ε_{bio} = 27‰, in agreement with the 'constant ε_{bio}' assumption, and defined the $\delta^{18}O$ of source water as the $\delta^{18}O$ of water extracted from the root crown, an approach described by Barnard et al. (2006). Source water defined in this way was ^{18}O-enriched by several per mil relative to irrigation water.

Variation of $p_{ex\text{-}Suc}$ among treatments was small (coefficient of variation of 8%) and not significant at the 5% probability level for either the effects of RH or [CO_2] or their interaction. Yet, $p_{ex\text{-}Suc}$ tended to decrease with increasing [CO_2], an effect that was marginally significant (p = 0.09) (Figure 3a). This may explain that we did not observe a [CO_2] effect on $\Delta^{18}O_{Cel}$, although $\Delta^{18}O_{Suc}$ was significantly affected by [CO_2] (compare Tables 2, 3 and Figure 4a). Interestingly, $p_{ex\text{-}Suc}$ tended to correlate negatively with day-night variations of sucrose concentration (p = 0.07; Supporting Information S1: Figure S1a) in the LGDZ reported for the same experiment (Baca Cabrera et al., 2020). In the same work, Baca Cabrera et al. (2020)

also analyzed diurnal changes of leaf water potential, and osmotic potential in the LGDZ at 200 and 800 µmol mol^{-1} CO_2 at both high and low RH. Comparisons with these data also revealed a positive relationship with $p_{ex\text{-}Suc}$ for both diurnal variation of leaf water potential and osmotic potential (both $p = 0.04$; Supporting Information S1: Figure S1b,c). Therefore, it may be that metabolism associated with greater diurnal variation of leaf elongation (connected with hydraulic limitation and stored growth effects, and related stronger osmotic adjustment in the LGDZ; Baca Cabrera et al., 2020) could perhaps cause a small increase of $p_{ex\text{-}Suc}$, although the possible mechanism(s) underlying such an effect are unknown.

Given that $p_{ex\text{-}Suc}$ values were based on sucrose extracted from leaf blades, the $p_{ex\text{-}Suc}$ also integrated eventual ^{18}O exchange during metabolism associated with transport, including storage and mobilization of carbohydrates along the path (Gessler et al., 2013). Actually, the concentration of fructan (a vacuolar, water-soluble and sucrose-based storage carbohydrate; Pollock & Cairns, 1991; Versluys et al., 2018; Wagner et al., 1983) was very high in whole-shoot tissue (>35% of dry mass; Zhu, 2023) and in the LGDZ (Baca Cabrera et al., 2020) of the same plants, and metabolism associated with sucrose re-cycling through the fructan pool (Lattanzi et al., 2012) was also very active at the whole shoot level (Zhu, 2023). Again, however, it is unknown if—and how—fructan metabolism in leaves and storage tissue along the path (specifically the leaf sheaths; Borland & Farrar, 1988) and in the cellulose synthesizing leaf growth zones could affect oxygen exchange between substrate oxygen and water at the respective locations. As an intermediate step in a future analysis of this phenomenon, it would be certainly worthwhile to analyse changes of $\Delta^{18}O_{Suc}$ between the leaf blade, leaf sheath, and LGDZ as well as of $\Delta^{18}O$ of water in these tissues, to separate eventual effects of metabolism during transport, storage along the path and sink cell metabolism on the integral $p_{ex\text{-}Suc}$.

4.2 | A false RH dependence of p_{ex} based on $\Delta^{18}O_{LW}$

p_{ex} calculated on the basis of $\Delta^{18}O_{LW}$ ($p_{ex\text{-}LW}$, Equation 6b) variably underestimated $p_{ex\text{-}Suc}$ (Equation 6a). As p_x varied very little, the discrepancy between $p_{ex\text{-}LW}$ and $p_{ex\text{-}Suc}$ was virtually entirely attributable to a variable and RH-dependent underestimation of the ^{18}O enrichment of photosynthesis and associated sucrose synthesis medium water ($\Delta^{18}O_{SSW}$) by $\Delta^{18}O_{LW}$ (Figure 4a,b). Baca Cabrera et al. (2023) interpreted this mismatch by (1) the presence of a very large non-photosynthetic tissue water fraction in leaf blades (approx. 53% in *Lolium perenne*, based on the anatomical studies of Charles-Edwards et al., 1974 and Dengler et al., 1994) coupled with (2) an environmentally-driven radial Péclet effect in the non-photosynthetic tissue water fraction of the leaf blade, while (3) the photosynthetic tissue water fraction was relatively close to theoretical estimates of average ^{18}O enrichment of water at the evaporative sites (see above). In particular, at low RH, source water accounted for a very large proportion (likely >80%) of the water contained in the non-photosynthetic tissue (Baca Cabrera et al., 2023), which was mostly (~77%) comprised of epidermis water according to the anatomical data of Charles-Edwards et al. (1974) and Dengler et al. (1994). Quite certainly, therefore, $\Delta^{18}O_{SSW}$ is a much better measure of the $\Delta^{18}O$ of (assimilation-weighted) photosynthetic medium water than $\Delta^{18}O_{LW}$, which has been the traditional proxy of the $\Delta^{18}O$ of photosynthetic medium water in grasses (e.g., Helliker & Ehleringer, 2002a; Liu et al., 2016). Modelling the discrepancy between $\Delta^{18}O_{SSW}$ and $\Delta^{18}O_{LW}$ requires improvements in our mechanistic understanding of the controls of $\Delta^{18}O$ of the (large) non-photosynthetic leaf water fraction (Baca Cabrera et al., 2023), and its main components, the epidermis and vascular tissues, as well as leaf hydraulic architecture (see discussions in Barbour et al., 2021; Holloway-Phillips et al., 2016).

Concerning the observed greater disparity between $p_{ex\text{-}LW}$ and $p_{ex\text{-}Suc}$ at low than at high RH, a similar effect was also apparent, when the primary data of Lehmann et al. (2017) were recalculated under the assumption that $p_x = 0.97$. Accordingly, $p_{ex\text{-}LW}$ increased from 0.3 at low RH to 0.88 at high RH on average of the two species; meanwhile $p_{ex\text{-}Suc}$ increased from 0.66 (average of two species) at low RH to 0.95 at high RH. Although the very high p_{ex} value seems unrealistic (Lehmann et al., 2017, see also 4.1 above and Figure 1), these observations also support qualitatively the present observation of a RH dependent underestimation of $p_{ex\text{-}Suc}$ by $p_{ex\text{-}LW}$ especially at low RH.

Interestingly, Helliker & Ehleringer (2002a) found a very similar average $p_{ex\text{-}LW}$ of 0.25 for five C_3 and five C_4 grass species across a range of RH as our work (average $p_{ex\text{-}LW}$ of 0.26). All else equal, this result would also suggest that $\Delta^{18}O_{Suc}$ was similarly underestimated by $\Delta^{18}O_{LW} + \varepsilon_{bio}$ in Helliker & Ehleringer (2002a), although a conspicuous relationship between RH and $p_{ex\text{-}LW}$ was not evident in their work (but see discussion in Liu et al., 2016). Again, this underscores that further attempts for a better understanding of $p_{ex\text{-}LW}$ will require studies on the drivers and mechanisms underlying divergent $\Delta^{18}O$ of photosynthetic and non-photosynthetic leaf tissue water (Baca Cabrera et al., 2023).

Most importantly, the present analysis may explain why Hirl et al. (2021) had to assume a RH-dependent p_{ex} p_x to match predictions of grassland leaf $\Delta^{18}O_{Cel}$—made with a meticulously validated, physically- and physiologically-based, ^{18}O-enabled soil-plant-atmosphere transfer model (MuSICA; Ogée et al., 2003, 2009; Hirl et al., 2021; Hirl, 2021)—with multi-seasonal observations of leaf $\Delta^{18}O_{Cel}$ in a pasture paddock. The model validation of Hirl et al. (2019) also lends confidence to the idea that observations made here in controlled and artificial environments are equally relevant in natural conditions at the scale of grassland ecosystems.

5 | CONCLUSIONS

This work demonstrated that p_{ex}—the most uncertain parameter in the Barbour & Farquhar model of ^{18}O enrichment of cellulose—is actually far less variable when based on measurements of $\Delta^{18}O_{Suc}$ in

photosynthetically active leaves than was previously assumed on the basis of $\Delta^{18}O_{LW}$. Also, the small and treatment-independent variation of p_{ex} may be taken to suggest that opportunities for metabolically-driven variation of oxygen exchange along the path and inside the sink tissue are much smaller than may be suspected based on the evaluation of the $\Delta^{18}O_{LW}$ vs $\Delta^{18}O_{Cel}$ relationship. Also, $p_{ex\text{-}Suc}$ was much closer to estimates of p_{ex} obtained in heterotrophic systems (Cernusak et al., 2005) and in studies of the randomization of ^{14}C-labelled hexose phosphates during cellulose synthesis in stem tissue (Barbour & Farquhar, 2000; Hill et al., 1995) than estimates based on $\Delta^{18}O_{LW}$. This finding is consequential, as it identified a critical error that has caused confusion of photosynthetic ($1 - p_{ex}\ p_x$) and post-photosynthetic ($p_{ex}\ p_x$) contributions to variation of $\Delta^{18}O_{Cel}$ (Figure 1), which arose primarily from the underestimation of $\Delta^{18}O_{Suc}$ by $\Delta^{18}O_{LW} + \varepsilon_{bio}$ (compare Equations 6a and 6b). In the present work, the variation of $\Delta^{18}O_{Cel}$ was closely related to the variation of ^{18}O enrichment at the site of photosynthesis and related sucrose synthesis in leaves (corrected for the near-constant proportion of post-photosynthetic oxygen exchange with source water). $\Delta^{18}O_{Cel}$ also correlated closely with theoretical predictions of average evaporative site water ^{18}O enrichment (Figure 5). Given that this observation can be generalized, it would imply that ^{18}O enrichment of grass leaf cellulose collected from herbaria, sample archives (e.g., the Rothamsted Park Grass Experiment; Baca Cabrera et al., 2021) or macrofossils in bogs and fens (e.g., Mauquoy et al., 2010) can be used to reconstruct historical and paleoenvironmental changes of ^{18}O enrichment of water at the site of photosynthesis. Such works would be interesting for both treeless sites (to expand spatially our paleoenvironmental and -physiological understanding) and sites with trees present in the records (to explore eventual plant functional differences in responses to environmental changes).

Beyond this, however, more work is needed, comparing $p_{ex\text{-}Suc}$ and $p_{ex\text{-}LW}$ in contrasting (controlled) environments and with different plant functional groups, including species with different leaf hydraulic design and proportions of non-photosynthetic and photosynthetic leaf water fractions. In that, also uncertainty concerning (and factors underlying variation of) ε_{bio}—including intramolecular variation of ^{18}O enrichment of the substrate and metabolic intermediates of cellulose synthesis—should be addressed with greater analytical and mechanistic detail, as it affects the estimated contribution of photosynthetic and post-photosynthetic oxygen exchange in a given cellulose sample. Importantly, however, variation of ε_{bio} would have similar effects on the magnitude of $p_{ex\text{-}Suc}$ and $p_{ex\text{-}LW}$ in the present work, so it would not alter its main conclusions.

ACKNOWLEDGEMENTS

We thank Anja Schmidt, Monika Michler, Angela Ernst-Schwärzli, Laura Dorn, Wolfgang Feneis, Richard Wenzel and Hans Vogl for assistance in the sampling and sample processing (A. S., M. M. and A. E.-S.), maintenance of the mesocosm facility and gas exchange equipment (W. F. and R. W.) and carbohydrate analyses (A. S. and L. D.). This research was supported by the Deutsche Forschungsgemeinschaft (DFG SCHN 557/9-1). Jianjun Zhu was supported by the China Scholarship Council (CSC). Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Cabrera, J. C. B., Hirl, R. T., Schäufele, R., Zhu, J., Liu, H. T., Gong, X. Y. et al. (2024) Half of the ^{18}O enrichment of leaf sucrose is conserved in leaf cellulose of a C_3 grass across atmospheric humidity and CO_2 levels. *Plant, Cell & Environment*, 47, 2274–2287. https://doi.org/10.1111/pce.14881