On the effect of heavy water (D$_2$O) on carbon isotope fractionation in photosynthesis

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Abstract. Internal conductance to carbon dioxide is a key aspect of leaf photosynthesis although it is still not well understood. It is thought that it comprises two components, namely, a gas phase component (diffusion from intercellular spaces to cell walls) and a liquid phase component (dissolution, diffusion in water, hydration equilibrium). Here we use heavy water (D$_2$O), which is known to slow down CO$_2$ hydration by a factor of nearly three. Using $^{12}$C/$^{13}$C stable isotope techniques and Xanthium strumarium leaves, we show that the on-line carbon isotope discrimination ($\Delta^{13}$C, or $\Delta_{on}$) associated with photosynthesis is not significantly decreased by heavy water, and that the internal conductance, estimated with relationships involving the deviation of $\Delta^{13}$C, decreased by 8–40% in 21% O$_2$. It is concluded that in typical conditions, the CO$_2$-hydration equilibrium does not exert an effect on CO$_2$ assimilation larger than 9%. The carbon isotope discrimination associated with CO$_2$ addition to ribulose-1,5-bisphosphate by Rubisco is slightly decreased by heavy water. This effect is proposed to originate from the use of solvent-derived proton/deuteron during the last step of the catalytic cycle of the enzyme (hydration/cleavage).

Introduction

Leaf photosynthesis is driven by the gradient of CO$_2$ mole fraction between the atmosphere and the intercellular spaces, by stomatal conductance and also by the internal gradient of CO$_2$ from intercellular spaces to carboxylation sites, which depends on internal conductance. Internal conductance is not infinite, so can be seen as a photosynthetic limitation (Evans et al. 1986). Many studies investigating the components and the origin of the internal conductance can be found in the literature (for a recent example see Warren and Dreyer 2006). Briefly, it is assumed that leaf internal conductance is made of two parts, a gas phase and a liquid phase component. Much uncertainty remains regarding the nature and the contribution of the components of the liquid part of the internal conductance.

Ever since carbonic anhydrases (CAs) were found in plants (Day and Franklin 1946; Bradfield 1947), their involvement as a component of the liquid phase conductance has been hypothesised. Much debate nevertheless remains on whether CAs, which catalyse the reversible interconversion of CO$_2$ and HCO$_3^-$, are essential to C$_3$ photosynthesis. Chloroplastic CA has been assumed to be a key regulator of CO$_2$ supply to Rubisco carboxylation by favouring hydration of CO$_2$ in the peripheral stroma, thus, facilitating the diffusion of inorganic carbon within the chloroplast and balancing carboxylation by peripheral and central Rubisco molecules in the chloroplast (Enns 1967; Arakelyan et al. 1993). In addition, correlations between the photosynthesis rate or the growth rate and CA activity have been detected (Khan 1994, 2002; Tiwari et al. 2006). Further, under Zn deficiency (which induces a decrease in CA activity) internal CO$_2$ transfer conductance is considerably decreased (Sasaki et al. 1998). However, Price et al. (1994) found that tobacco plants with low CA activity (2% of the wild-type value) showed nearly no effect on CO$_2$ assimilation and only a slight effect on carbon isotope discrimination. Williams et al. (1996) showed that CA antisense plants had only a small decrease in photosynthetic carbon isotope discrimination (of around 1.5 %e) and a drop in the (CO$_2$) oxygen isotope discrimination. As CA activity is responsible for oxygen exchange between CO$_2$ and leaf water (which is $^{18}$O-enriched because of transpiration), the latter result provides evidence that CA activity is decreased in planta. Therefore, it was concluded by those authors that CA suppression has a modest effect only on photosynthetic CO$_2$ transfer. With pea protoplasts, the addition of the soluble CA inhibitor acetazolamide influenced neither the photosynthetic rate nor electron transport (Ignatova et al. 2001). Thus, no clear picture emerges from the literature; it remains plausible that CA is involved in CO$_2$ transfer resistance, although other roles such as pH-buffering might be emphasised (Oja et al. 1999).

In the present paper, we take advantage of the effect of heavy water (D$_2$O) on photosynthesis of Xanthium strumarium (Asteraceae) leaves, to investigate the importance of the liquid phase components of internal conductance. In D$_2$O, all the components of the CO$_2$ conductance in the liquid phase are affected (Table 1). However, the CA-catalysed interconversion of CO$_2$ and bicarbonate is the most reduced, with a H$_2$O/D$_2$O isotope effect on both $k_{cat}$ (turn-over rate) of hydration and $K_e$ (equilibrium constant) – although there is nearly no $k_{cat}/K_m$ isotope effect. The value of the observed isotope effect

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Table 1. The $\text{H}_2\text{O}/\text{D}_2\text{O}$ isotope effects ($\alpha$) considered in the present study

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$\alpha$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylation of RuBP by Rubisco (pH 7.5)</td>
<td></td>
<td>Van Dyk and Schloss (1986)</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ isotope effect</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ isotope effect</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Diffusion of CO$_2$ in water (calculated$^a$)</td>
<td>0.995</td>
<td>Bearman and Jolly (1984)</td>
</tr>
<tr>
<td>Interconversion CO$_2$/HCO$_3^-$ by CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_e$ equilibrium isotope effect</td>
<td>2.7</td>
<td>Silverman and Vincent (1983)</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ isotope effect of CO$_2$ hydration</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ isotope effect of CO$_2$ hydration</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$K_e$ equilibrium isotope effect</td>
<td>3.2</td>
<td>Pocker and Bjorkquist (1977)</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ isotope effect of CO$_2$ hydration</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ isotope effect of CO$_2$ hydration</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Uncatalysed hydration of CO$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinetic isotope effect</td>
<td>1.8</td>
<td>Pocker and Bjorkquist (1977)</td>
</tr>
<tr>
<td>Dissolution of CO$_2$ in water</td>
<td>0.994</td>
<td>Wilhelm et al. (1977)</td>
</tr>
</tbody>
</table>

$^a$The calculation of the isotope effect assumes that water molecules aggregates involve six molecules, as suggested by O’Leary (1984).

depends upon the kinetic status of the reaction (close to or far from equilibrium). CO$_2$ hydration and dehydration are likely to be nearly at equilibrium in the chloroplast; the rate constants of the enzyme-catalysed reaction in both forward and backward directions are very high (in the $10^5-10^6$ molecules site$^{-1}$ s$^{-1}$ range; Pocker and Ng 1973; Silverman and Vincent 1983). The observed isotope effect of a reversible reaction has been mathematically developed by Tcherkez (2004) and Tcherkez and Farquhar (2005) for aldolase. By strict analogy, assuming hydration is a simple reversible step, the net rate of bicarbonate production is a rational function of first-order kinetics (exponential terms). Applying the equation to CO$_2$ hydration (that is, CO$_2$ + H$_2$O $\rightarrow$ H$^+$ + HCO$_3^-$ and CO$_2$ + D$_2$O $\rightarrow$ D$^+$ + DCO$_3^-$), the isotope effect as a function of time is as follows (see Appendix by Tcherkez and Farquhar 2005 for the evidence):

$$\alpha_{\text{obs}} = \frac{1-u_{\text{H}}}{1-u_{\text{D}}} \times \frac{u_{\text{D}} + 1/K_{\text{eD}}}{u_{\text{H}} + 1/K_{\text{eH}}},$$

(1)

where $\alpha_{\text{obs}}$ is the H/D observed isotope effect and $u_{\text{H}}$ is given by (similarly for $u_{\text{D}}$): and

$$u_{\text{H}} = \exp \left(-K_{\text{H}} \left(1 + \frac{1}{K_{\text{eH}}} \right) t\right),$$

(2)

where $K_{\text{H}}$ is the rate of the forward reaction (hydration), $K_{\text{eH}}$ and $K_{\text{eD}}$ are equilibrium constants, and $t$ is time. With the kinetic values $K = k_{\text{cat}}/K_m$ and $K_e$ by Silverman and Vincent (1983) and a site concentration of $\approx 0.1$ mmol L$^{-1}$, the isotopic equilibrium (that is, the equilibrium isotope effect value) is reached in nearly 5 $\mu$s (at pH 8 and an initial amount of 10 $\mu$mol L$^{-1}$ CO$_2$). Such a delay is very short considering the time needed for carboxylation by Rubisco (the overall Rubisco-catalysed carboxylation turnover is near: eight sites per Rubisco molecule $\times$ 1 mmol L$^{-1}$ $\times$ 4s$^{-1}$ site$^{-1}$ = 32 s$^{-1}$, which gives near 31 ms, for a physiological Rubisco amount about 10 times higher than that of CA). In other words, the CO$_2$/HCO$_3^-$ conversion catalysed by carbonic anhydrase is very rapidly at isotopic equilibrium, and the thermodynamic isotope effect of nearly three applies (Table 1). This also accords with the investigation of the $^{18}$O abundance of leaf CO$_2$ efflux, which suggested that CO$_2$ fully exchanged its oxygen with chloroplastic water in the chloroplast in the light (Farquhar et al. 1993; Yakir et al. 1994; Cernusak et al. 2004).

Thus, the comparison of the (total) internal conductance in natural and heavy water provides a simple way to see whether a 3-fold change of carbonic anhydrase activity influences photosynthesis. We also recognise that the carboxylation rate by Rubisco is affected by deuterium (with a H/D isotope effect of around two, Table 1); nevertheless, the specificity factor $S_{\text{iso}}$ is not modified (Kent and Tanny 1984): $S_{\text{iso}}$ is simply the ratio of the rate constant of the carbonylation step to that of the oxygenation step (that is, $k_{\text{o}}/k_3$ in the nomenclature by Farquhar 1979), both being water-independent (Lorimer 1981). Thus, the compensation point in the absence of day respiration (T*) is not affected by heavy water, and neither is the carboxylation to oxygenation ratio, for a given CO$_2$-to-O$_2$ concentration ratio. The carbon isotope discrimination by Rubisco, however, is thought to be decreased by deuterium (Roeske and O’Leary 1984; Table 2).

In the following, we use $^{13}$C/$^{12}$C isotopic techniques to measure internal conductance in H$_2$O and D$_2$O (in which CA activity is slowed down). We show that a difference of internal conductance arises indeed in heavy water, and we also explore the effects of heavy water on other processes associated with gas exchange of leaves.

Materials and methods

Plant material and growth conditions

Xanthium strumarium L. plants were grown (in the greenhouse) from seeds in 100-mL pots of potting mix and transferred to 3-L pots after 2 weeks. Minimum PPFD during a 16-h photoperiod was maintained at ~400$\mu$mol m$^{-2}$ s$^{-1}$ by supplementary lighting. Temperature and vapour pressure deficit were maintained at ~25.5/18.5°C and 1.4/1.2 kPa day/night, respectively. The carbon isotope composition ($\delta^{13}$C) of CO$_2$ in the greenhouse air was $\approx -9.5 \pm 0.3\%$e. The third or fourth leaves (from the apical bud) were used for all measurements. Heavy water (99.8% D$_2$O) was from Eurisotop (Saint-Aubin, France). For experiments using heavy water, leaves were cut (under water) at the end of the previous light period and the petiole placed in D$_2$O for a whole night period of 13 h to renew leaf water before starting gas-exchange measurements.

Gas-exchange measurements

The measurements were made as by Tcherkez et al. (2005). Briefly, the assimilation chamber was connected to the sample air hose of the LI-6400 (Li-Cor Inc.). The chamber was made of aluminium and clear plexiglass. Two fans placed in the chamber...
Table 2. The H2O/D2O isotope effect on the 12C/13C isotope effects involved in water-dependent steps of C4 photosynthesis

<table>
<thead>
<tr>
<th>Step</th>
<th>12C/13C Isotope Effect in H2O</th>
<th>12C/13C Isotope Effect in D2O</th>
<th>Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion of CO2 in water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>1.0007</td>
<td>-</td>
<td>-</td>
<td>O’Leary (1984)</td>
</tr>
<tr>
<td>Calculated</td>
<td>1.0009</td>
<td>1.0009</td>
<td>1</td>
<td>Bearman and Jolly (1984)</td>
</tr>
<tr>
<td>Dissolution of CO2 in water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>1.00107</td>
<td></td>
<td></td>
<td>Vogel et al. (1970)</td>
</tr>
</tbody>
</table>

^A Value obtained on spinach Rubisco in H2O with deuterated ribulose-1,5-bisphosphate as a substrate.

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gave a boundary layer conductance to water of ~6.7 mol m\(^{-2}\) s\(^{-1}\). Leaf temperature was controlled at 21°C with circulating water from a cooling water bath to the jacket of the leaf chamber, and was measured with a copper-constantan thermocouple plugged into the thermocouple sensor connector of the LI-6400 chamber/ infrared gas analyser (IRGA). Ingoing air was dried (to the thermocouple sensor connector of the LI-6400 chamber/measured with a copper-constantan thermocouple plugged into a cooling water bath to the jacket of the leaf chamber, and was measured following the method described by Evans et al. (1986). Artificial air with 2% O2 was supplied by a cylinder (Crystal gas mixture, 2% O2 in N2, Air Liquide). Inlet CO2 was obtained from a gas cylinder (Air Liquide, Grigny, France) with a 18O (minimum water quantity of 0.1 mL) was measured after 48 h equilibration at 21°C with 500 μmol mol\(^{-1}\) CO2 in tight glass flasks; the 18O of CO2 was measured by introducing the gas sample with pure N2 (N2, Alphagaz, Air Liquide) into the loop of the elemental analyser described above. The isotope ratio of CO2 is equal to that of the water (the molar ratio H2O/CO2 is more than 2 × 10\(^3\)) corrected for the equilibrium isotope effect of CO2-water exchange, that is, 1.0419 for H2O (Brenninkmeijer et al. 1983) and 1.0236 for D2O (Majzoub 1966). In the present paper, the δ-values are given with respect to PDB (carbon) and SMOW (oxygen).

The carbon isotope composition of night-respired CO2 and the night-respiration rate were measured using a closed system coupled to the mass spectrometer through the elemental analyser, as described by Tcherkez et al. (2003).

The δ\(^13\)C of inlet and outlet CO2 were measured using the mass spectrometer ‘Optima’ (Micromass, Villeurbanne, France) coupled to an elemental analyser to separate CO2 by chromatography (continuous flow), as described by Tcherkez et al. (2005). As heavy water may also be enriched in 18O, a property that would modify reaction rates further, the oxygen isotope composition of source water was measured. Source water δ18O (minimum water quantity of 0.1 mL) was measured after 48 h equilibration at 21°C with 500 μmol mol\(^{-1}\) CO2 in tight glass flasks; the δ18O of CO2 was measured by introducing the gas sample with pure N2 (N2, Alphagaz, Air Liquide) into the loop of the elemental analyser described above. The isotope ratio of CO2 is equal to that of the water (the molar ratio H2O/CO2 is more than 2 × 10\(^3\)) corrected for the equilibrium isotope effect of CO2-water exchange, that is, 1.0419 for H2O (Brenninkmeijer et al. 1983) and 1.0236 for D2O (Majzoub 1966). In the present paper, the δ-values are given with respect to PDB (carbon) and SMOW (oxygen).

The carbon isotope composition of night-respired CO2 and the night-respiration rate were measured using a closed system coupled to the mass spectrometer through the elemental analyser, as described by Tcherkez et al. (2003).

\(^{12}\)C/\(^{13}\)C isotopic theory

The isotopic theory developed in the following has been described elsewhere (Farquhar et al. 1989) and the main equations only are recalled here. The ‘theoretical’ form of the carbon isotope fractionation associated with photosynthesis
(denoted as $\Delta_i$), which neglects (photo) respiratory fractionation and the boundary layer resistance and assumes an infinite internal conductance, is:

$$\Delta_i = a + (b - a) \frac{c_i}{c_a},$$  (4)

where the diffusional fractionation $a$ is the fractionation associated with diffusion and $b$ is the fractionation associated with carboxylation. $c_a$ and $c_i$ are the outlet and internal mole fractions of CO$_2$. Two different uses of the above equation should be distinguished: that one used for regression analyses (conducted in Fig. 1) and that one used for calculating the deviation of the observed discrimination from the theoretical value (see below). In the first case, the fitted $b$ value integrates internal conductance effects (and so is generally less than 29‰). In the second case, $b$ is purely related to carboxylation.

The more complete expression of the isotope discrimination, usually assumed to explain the ‘observed’ photosynthetic fractionation ($\Delta_{obs}$), is given by:

$$\Delta_{obs} = \Delta_0 - \Delta_e = \frac{c_i - c_h}{c_a} + \frac{a + e_x}{c_a} + \frac{c_m}{c_a} = \frac{eR_d}{k + f \Gamma^*},$$

where the subscripts a, b, i and c refer to atmospheric, leaf surface, intercellular and carboxylation site CO$_2$, respectively. Here, $\Delta_0$ is the gaseous diffusional fractionation associated with the boundary layer (2.9‰), and $a_1$ (1.1‰) and $e_x$ (0.7‰) are the fractionations associated with diffusion in the liquid phase and with CO$_2$ dissolution, respectively. Also, $e$ and $f$ are the fractionations associated with day respiration (rate $R_d$) and photorespiration, respectively, $k$ is the carboxylation efficiency and $\Gamma^*$ the CO$_2$-compensation point in the absence of day respiration, and $b$ is the carbon isotope fractionation associated with Rubisco-catalysed carboxylation. Under the assumption that $b = b = 29$‰ and $a_0 = a_1 / c_a$ is negligible, it can then be shown that the internal CO$_2$-conductance (denoted as $g_m$) is such that (Evans et al. 1986):

$$\Delta_i - \Delta_{obs} = \frac{b - a}{c_a} \times \frac{A}{c_i} + d,$$  (6)

where $A$ is net assimilation and $d = (eR_d / k + f \Gamma^*) / c_a$. A linear regression can be used only if $d$ is constant, that is, if $c_m$ is maintained constant during experiments (in the present study, it was 400 μmol mol$^{-1}$). Such a linear model is used in Fig. 2A (see below).

The internal conductance can also be obtained with the following relationship, which does not assume similar values for $b$ and $b$ (von Caemmerer and Evans 1991):

$$\frac{\Delta_i - \Delta_{obs}}{c_a} = \frac{b - a}{c_i} \times \frac{A}{c_i} + (\tilde{b} - b) + \frac{c_{i}}{c_i}$$  (7)

If $d$ is small (say, 1‰), the intercept of this relationship is close to $\tilde{b} - b$. This linear model (with such an assumption) is used in Fig. 2B (see below).

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**Fig. 1.** The relationship between the on-line carbon isotope discrimination ($\Delta_{obs}$) and $c_i / c_a$ of detached leaves in H$_2$O (closed circles) or D$_2$O with either H$_2$O or D$_2$O in the inlet air (semi-closed and open circles, respectively). Straight lines are linear type II regressions. They are significant ($F = 66.4, P < 0.0001; F = 7.9$, $P < 0.05; F = 20.7, P < 0.02$ for H$_2$O, D$_2$O and D$_2$O + D$_2$O inlet, respectively). Slopes are $22.9 \pm 2.8$‰ and $21.1 \pm 3.0$‰ for H$_2$O and D$_2$O, respectively. Intercepts are $0.72 \pm 2.3$‰ (H$_2$O), $0.69 \pm 3.2$‰ (D$_2$O + inlet H$_2$O) and $-1.6 \pm 4.2$‰ (D$_2$O + inlet D$_2$O). (Inset) same as in the main panel, in 2% O$_2$. Slopes are $22.5 \pm 4.8$‰ and $23.6 \pm 2.8$‰ for H$_2$O and D$_2$O, respectively. Intercepts are $-1.62 \pm 4.2$‰ (H$_2$O) and $-1.12 \pm 2.2$‰ (D$_2$O). Both regressions are significant ($F = 20.7$, $P < 0.02; F = 66.8, P < 0.005$). Data represent means (±s.e.) on three measurements made with independent leaves.
It should be noted that the two linear models described above may be considered as two extreme cases: in Eqn 6, the $b$ values are considered similar so that the offset that may occur between H$_2$O and D$_2$O would be attributed to the (photo)respiratory component, and in Eqn 7, such an offset is attributed to a shift in the $b$ value. Both models are then used here to give a range of $b$ and $d$ values.
Results

Photosynthesis values in heavy water

In heavy water, the net assimilation rate, measured in conditions similar to those during growth, was reduced nearly 2-fold (Table 3), a value close to the $k_{o2}/k_m$ isotope effect (2.3) of Rubisco (Table 1). The inhibition of the photosynthesis rate did not come from the considerable 18O-enrichment in D2O that might occur because of the D2O production techniques such as distillation (Malkov 1959): source D2O had a near-natural 18O-abundance, with a $\delta^{18}O$ value of +27.1‰ (Table 3). In addition, neither the photosynthesis rate nor the isotope discrimination changed significantly when H2O was changed to D2O in the inlet of the open gas-exchange system (Table 3), showing that, with inlet H2O, the possible dilution of D2O by H2O was very small (see also the Appendix 1).

In contrast to the assimilation rate, the carbon isotope discrimination was unchanged in D2O, with values of around 20%e (Table 3), indicating that 12C/13C isotopic properties of photosynthesis might not be influenced by heavy water, unless compensating mechanisms occurred (see below).

The respiration rate was very slightly (not significantly) affected by heavy water, with an additional 13C-depletion of 2.1‰ of night-respired CO2 after 13 h in the dark (Table 3).

The relationship between $\Delta^{13}C$ and $c_i/c_a$ in natural and heavy water

The plot of the on-line carbon isotope discrimination ($\Delta^{13}C$, also usually denoted as $\Delta_{obs}$) vs. the internal-to-external CO2 ratio ($c_i/c_a$) is shown in Fig. 1. Clearly, there was no drastic difference in the slope of the relationship so that the $b$ value of the (simplified, with infinite internal conductance $g_{m}$) model by Farquhar et al. (1982) was only slightly influenced by heavy water: in heavy water, $b$ was 25.5 ± 3.0‰ (H2O in the inlet) and 22.5 ± 4.9‰ (D2O in the inlet) and it was 27.2 ± 2.8‰ in natural water. In 2% O2, the $b$ value was similar in H2O and D2O (Fig. 1, inset). In both 21 and 2% O2, the difference between $b$ values obtained in D2O and H2O was not significant; in other words, at this stage of the analysis, it seems that the carbon isotope effect associated with carboxylation is unaffected by heavy water (but see below).

The internal conductance in natural and heavy water

The relationship between the deviation $\Delta_{b} - \Delta_{obs}$ of the carbon isotope discrimination from the value at infinite conductance with zero (photo)respiratory fractionation ($\Delta_i$) as a function of $A/c_a$ is shown in Fig. 2A. Not surprisingly, data for heavy water are on the left hand side because of low $A$ values (lower $A/c_a$ values). Further, the heavy and light datasets did not appear to be on the same relationship; however, the slopes were quite similar. Using a carboxylation discrimination value of 29‰ for both D2O- and H2O-experiments (see ‘Isotope theory’), we found internal conductance values of 0.166 in H2O, and 0.150 (inlet H2O) and 0.134 (inlet D2O) mol m⁻² s⁻¹ in D2O. The difference between conductance values so obtained in H2O and D2O was not statistically significant (Table 4). By contrast, the intercept was larger in D2O (+3.3 and +4.1‰) compared with H2O (-1.5‰). In the framework of the linear model of Fig. 2A, the intercept value accounts for the (photo)respiratory fractionations. As the specificity factor of Rubisco does not change in heavy water, one might assume that the offset came from the larger 12C/13C isotope effects associated with (photo)respiratory decarboxylations. It could be so if these decarboxylations were slowed down and became more rate-limiting in heavy water (see also Discussion).

However, in 2% O2, the results were similar, in that the deviation $\Delta_i - \Delta_{obs}$ was almost larger in D2O compared with H2O, with a ~3% difference in the intercept (Fig. 2A, inset); the slopes were slightly but non-significantly different (Fig. 2A, legend). This simply indicates that the photosrespiratory fractionation was not likely to be responsible for the offset between the H2O and the D2O lines of Fig. 2A.

When plotting the discrimination deviation times $c_i/c_a$ a difference also emerged in the intercept (which is equal to $b - b + d \times c_i/c_a$ (Fig. 2B) and this difference did not change to a large extent in 2% O2 (Fig. 2B, inset). The (photo) respiratory contribution to the intercept, thus, seems to be (very) small. Now neglecting the photosrespiratory term $d$, the $b$-difference would be −0.5 in H2O and +3.7 in D2O (and −4.1 and +2.2 in 2% O2). In other words, this would indicate a lower carbon isotope fractionation within the range 25–26‰ in heavy water [that is, 25.3‰ (inlet H2O) and 25.0‰ (inlet D2O) in 21% O2 and 26.8 in 2% O2] compared with that in natural water (29.5‰ in 21% O2 and 33.1‰ in 2% O2) (Table 4). Using these values of $b$, the slopes 21% O2 were such that the internal conductance was significantly lower ($P < 0.05$) in heavy water: 0.205 ± 0.048 in H2O and 0.144 ± 0.020 (inlet H2O) 0.114 ± 0.025 (inlet D2O) mol m⁻² s⁻¹ in D2O (Table 4). The same trend occurs in 2% O2 (0.280 ± 0.092 in H2O and 0.130 ± 0.043 mol m⁻² s⁻¹ in D2O, Table 4) although the difference is not statistically significant.

Discussion

Although the importance of internal conductance to CO2 transfer ($g_m$) has been well recognised, studies about it are frequently subject to technical difficulties (Warren 2006), so

Table 3. Photosynthetic and respiratory properties of Xanthium strumarium detached leaves in natural water (H2O) and in heavy water (D2O)

<table>
<thead>
<tr>
<th>Source water ($\delta^{18}O$, ‰)</th>
<th>$R_n$ (µmol m⁻² s⁻¹)</th>
<th>$\delta^{18}C$ (‰)</th>
<th>$A$ (µmol m⁻² s⁻¹)</th>
<th>$g_m$ (µmol m⁻² s⁻¹)</th>
<th>$\Delta^{13}C$ (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (−21.2 ± 1.8)</td>
<td>0.73 ± 0.06</td>
<td>−27.3 ± 0.5</td>
<td>16.5 ± 0.5</td>
<td>0.23 ± 0.01</td>
<td>19.6 ± 0.1</td>
</tr>
<tr>
<td>D2O (−27.1 ± 0.5)</td>
<td>0.69 ± 0.05</td>
<td>−29.4 ± 0.4</td>
<td>9.6 ± 0.5</td>
<td>0.21 ± 0.03</td>
<td>20.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(8.7 ± 0.9)</td>
<td></td>
<td>(0.20 ± 0.08)</td>
<td>(20.6 ± 0.6)</td>
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</tr>
</tbody>
</table>
that the magnitude of the components of $g_m$ are not well known. Here we took advantage of the slowing effect of heavy water on CO$_2$-HCO$_3^-$ equilibrium and carboxylation to investigate their roles in photosynthesis. In fact, heavy water has negligible effects on other steps (dissolution, diffusion) (see Appendix I). Although the effect of D$_2$O on electronic (primary) photosynthetic reactions has been extensively studied (see Soriano and Cramer (2001) and Flores et al. (2006) as recent examples), to our knowledge it is the first time the effect of D$_2$O on both photosynthetic gas exchange and $^{13}$CO$_2$/^12CO$_2$ discrimination has been studied.

**H$_2$O/D$_2$O isotope effect associated with carboxylation**

Heavy water caused an isotope effect of nearly two on the assimilation rate (Table 3), and this is consistent with the H/D ($k_{cat}/k_{ran}$) isotope effect associated with carboxylation (by Rubisco) of 2.3 (Table 1). The effect of heavy water on the $^{12}$C/$^{13}$C fractionation by Rubisco can be seen with (i), a plot showing $A_{obs}$, the slope of which is $b = a$ (but in such a plot, $a$ integrates internal conductance effects, see ‘Theory’ section) (ii), the intercept of the plot showing $A_{obs} = A_{cat} + A_{ran}$, where $A_{cat}$ is the estimated fraction associated with carboxylation seems to be lowered by a few per mille, as revealed by the slightly smaller slope in Fig. 1 and the larger intercept of Fig. 2B. This is also true in 2% O$_2$ (in low-oxygen non-pho-torespiratory conditions, the (photo)respiratory component $d = (eR_d/k + fT^*)/e$ decreases to $eR_d/(ke_a$ which is very small). The drop would be nevertheless small, in the range 1.7-4.2‰ (compared with the *in vitro* Rubisco 29‰ value), so that the relative decrease of the fractionation is between 5 and 14‰.

The effect of D$_2$O on the $^{12}$C/$^{13}$C fractionation can be understood in the light of the mechanism of the catalytic cycle. This has been formalised as (Farquhar 1979; Tcherkez and Farquhar 2005):

$$\text{RuBP} \xrightarrow{k_{cat}} \text{Dieno} \xrightarrow{k_{k10}} \text{[CO}_2] \xrightarrow{k_{k9}} 6C \xrightarrow{k_a} \text{Intermediate} \xrightarrow{k_{8}} 2\text{PGA}$$

where RuBP stands for ribulose-1,5-bisphosphate and PGA for phosphoglyceric acid. Under the assumption that $k_7$ (rate constant associated with decarboxylation) is negligible, the observed $^{12}$C/$^{13}$C isotope effect associated with CO$_2$ addition is $^{12}$k$_8$/^{13}k$_8$. As a result, it is D$_2$O-independent because heavy water slows down enolisation (that is, $k_9$ and $k_{10}$ rate constants) and, perhaps, hydration/cleavage (rate constant $k_{8}$) (Tcherkez and Farquhar 2005). This view is in agreement with the variation of the carbon isotope effect in a phylogenetic range of Rubiscos, which is interpreted as a change in the intrinsic isotope effect (Tcherkez et al. 2006) rather than (i), a change in other limiting steps or (ii), in the prevalence of decarboxylation, which has been found to be negligible in assay conditions (Pierce et al. 1986; and see McNevin et al. 2007; for a discussion).

However, we find here a reduction of a few permil and accordingly, Roeske and O’Leary (1984) also found an effect of deuterated RuBP on the carbon isotope fractionation by Rubisco (Table 2). Such an effect probably comes from a slower hydration/cleavage rate in heavy water, which would artificially induce the reverse reaction of carboxylation (that is, decarboxylation $k_7$) to occur. It would be so because the energy barrier for hydration is higher in heavy water, making decarboxylation more likely. As decarboxylation probably fractionates against $^{13}$C, it would compensate for the isotope effect of CO$_2$ addition by decarboxylating $^{13}$C-depleted CO$_2$. This would explain the diminution of the carbon isotope discrimination by deuterium.

There are several reasons to consider such a scenario as reasonable, if not likely. First, there is a higher H/D isotope effect on $k_{cat}$ (= $k_8$, the rate constant of hydration and cleavage) than on $k_{cat}/k_{ran}$ (overall rate) (2.8 as compared with 2.3, Table 1). Second, this would agree with the catalytic mechanism proposed by Cleland et al. (1998) and the hypothesis of Mauser et al. (2001) that the carbamylated Lysine of the active site (Lys 201), which is involved in proton abstraction during ribulose-1,5-bisphosphate enolisation, is also involved in the hydration of the 6-carbon intermediate formed by CO$_2$ addition (in other words, deuteration of the Lys residue would be responsible for slowing the $k_8$ step, which in turn may promote decarboxylation of the 6C intermediate). Parenthetically, this would explain why D$_2$O and deuterated RuBP have similar effects on $^{12}$C/$^{13}$C fractionation. Third, in the tobacco L335V line in which there is a leucine-to-valine mutation in loop 6 of the large subunit, both the $k_{cat}$ and the $^{12}$C/$^{13}$C fractionation are lower than in the wild type, and this has been interpreted as a change in the probability of decarboxylation (McNevin et al. 2007).

**H$_2$O/D$_2$O isotope effect on photosynthetic fractionation**

The $y$-intercepts of Fig. 1 give the value of $a$ (diffusional fractionation) minus the (photo) respiratory fractionation $d$. In 21% O$_2$, the intercept is 0.72‰ in H$_2$O v. 0.69‰ in D$_2$O v. H$_2$O and −1.6‰ in D$_2$O v. inlet D$_2$O. They are statistically identical, and although a change to low O$_2$ conditions did change the estimated intercepts, there was no significant difference between H$_2$O and D$_2$O conditions. However, there was a clear offset between the relationship of Fig. 2 in H$_2$O and that in D$_2$O in air (main panel), and again in 2% O$_2$ (inset). In 2% O$_2$, the offset was slightly decreased by 1.5‰. Neglecting day respiration (which is indeed small) and assuming a realistic $\Gamma^*/\kappa_a$ value of around 0.1, this 1-1.5‰ decrease (in the offset) would indicate an increase of the isotope effect associated with glycine decarboxylation from 1.020 (Tcherkez 2006) to 1.030-1.045. This corresponds to what would be observed if decarboxylation became more limiting, so that the isotope effect approaches the intrinsic limit (1.060, Tcherkez 2006). We also note that the (photo)respiratory fractionation value obtained in H$_2$O is negative (Fig. 2A). This might come from the large $^{13}$C-enrichment of growth CO$_2$ (−9.5‰) and so, of decarboxylated CO$_2$ compared with inlet CO$_2$ (−50.2‰).

**H$_2$O/D$_2$O isotope effect on CO$_2$ evolved in darkness**

There is an effect of D$_2$O on dark-respired CO$_2$ (that is, CO$_2$ evolved in the night-time) as a 2.1‰ $^{13}$C-depletion of the night-respired CO$_2$ was observed in heavy water (Table 3). The yeast thiamine diphosphate-dependent pyruvate decarboxylase (EC 4.1.1.1) undergoes a (small) H$_2$O/D$_2$O solvent isotope effect.
near unity (Wang et al. 2001) and similarly, little isotope effect may be assumed for the respiratory enzyme pyruvate dehydrogenase (EC 1.2.4.1, also thiamine diphosphate dependent) so that the weak effect of D₂O on the respiratory rate comes as no surprise (Table 3). We also note that the H/D isotope effect associated with the yeast pyruvate decarboxylase is larger on \( k_{\text{cat}} \) than on \( k_{\text{cat}}/K_m \) (Wang et al. 2001). In other words, the CO₂ production step of the catalytic cycle (the rate constant of which is \( k_{\text{cat}} \)) becomes limiting in heavy water, because of the slower protonation of the \( \alpha \)-carbon of pyruvate. Consequently, a larger \(^{13}\text{C}/^{12}\text{C}\) isotope effect associated with CO₂ production is expected and there is indeed a \(^{13}\text{C}\)-depletion of evolved CO₂ in heavy water (Table 3).

**H₂O/D₂O isotope effect on internal conductance**

The effect of heavy water on the internal conductance, given by the slope of the deviation \( \Delta_i - \Delta_{\text{obs}} \), is negligible in the present study when a common \( b = 29\% \) discrimination for carboxylation is assumed (Fig. 2A). When a difference in the \( b \) value (Table 4) is considered to account for the difference in intercepts of \( \Delta_i - \Delta_{\text{obs}} \) × \( c_d/c_o \), a difference occurs (Fig. 2B), which is significant (with \( P < 0.05 \)) in 21% \( \text{O}_2 \): 0.205 in \( \text{H}_2\text{O} \) v. 0.144 and 0.114 mol m⁻² s⁻¹ in \( \text{D}_2\text{O} \) (Table 4). In the usual conditions (our plant growth conditions, Table 3), this decrease in internal conductance would lead to a drop of \( \sim 30 \) μmol mol⁻¹ in intracellular CO₂ mole fraction (\( c_c \)) when leaves are subjected to \( \text{D}_2\text{O} \). We may calculate what the effect on CO₂ assimilation would be when CA activity is slowed down (regardless of the H/D isotope effect on Rubisco) in an electron transport limited mode, if \( c_c \) drops by 30 μmol mol⁻¹ from 200 μmol mol⁻¹. Using \( (c_c - \Gamma^+\Gamma^-) \) as a scaled estimate of \( A \), we get \( (200 - 40)/(200 + 80) = 160/280 = 0.57 \) with full CA-activity. If CA activity goes down and \( c_c = 170 \) μmol mol⁻¹, we have \( (170 - 40)/(170 + 80) = 130/250 = 0.52 \). The resulting difference in \( A \) is near 9%. Although other effects such as reallocation of nitrogen to Rubisco synthesis and larger stomatal conductances occurred (though they were statistically insignificant) in the study by Price et al. (1994), our study is in accord with the findings of these authors, who observed little change in the assimilation rate when CA activity was reduced. Our results are also consistent with the theoretical study by Cowan (1986), in which the rate of CO₂ fixation with optimal partitioning of nitrogen (between Rubisco and CA) is only \( \sim 5\% \) greater than it would be if the same amount of nitrogen were invested in Rubisco alone.

The elasticity value of internal conductance with respect to CA activity is, at most, 

\[
1 - \frac{g_{\text{m}}(\text{D}_2\text{O})}{g_{\text{m}}(\text{H}_2\text{O})} = \frac{1}{1 - \frac{K_c(\text{H}_2\text{O})}{K_c(\text{D}_2\text{O})}} \approx 0.7
\]

In other words, a 3-fold reduction of the CA equilibrium constant has a modest effect (elasticity less than 1) on the CO₂ transfer resistance in the typical conditions of the present study (21% \( \text{O}_2 \), 400 μmol mol⁻¹ CO₂, 21°C). Extremely low (chloroplastic) CA plants exhibit a slight reduction of the carbon isotope discrimination and a larger decrease of the oxygen isotope discrimination because of the strong alteration of the \( \text{CO}_2/\text{H}_2\text{O} \) equilibration efficiency (Price et al. 1994; Williams et al. 1996). The 3-fold reduction in the present study did not induce a measurable effect on \( \Delta^{13}\text{C} \) (Table 3). This is so because CO₂ assimilation was lower, and thus \( c/c_c \) values were higher, compensating for the smaller fractionation associated with carboxylation. This is also explained by the very high catalytic rates of the enzyme (\( k_{\text{cat}} \) values indicate that the enzyme is nearly limited by diffusion only) that make the \( \text{CO}_2/\text{H}_2\text{O} \) equilibration very efficient.

In plants where CA activity reduction is caused by Zn deficiency, Sasaki et al. (1998) found a decrease of the internal conductance that is comparable to our observations, although it could be related to other additional side effects of Zn deficiency. Parenthetically, we emphasise the fact that all cellular CAs are slowed down in our study, indicating that the minor effect of the deficiency in chloroplastic CA on photosynthesis (found by Price et al. 1994; Williams et al. 1996; Sasaki et al. 1998) can be generalised to cell CA activity as a whole.

Our study, which is in agreement with papers using genetic manipulations of CA, indicates that CA enzymatic activity is a limitation to \( \text{C}_3 \) photosynthesis but not a major one. Similarly, in \( \text{C}_4 \) plants, it has been recently shown that a reduced CA activity also has little effect on photosynthesis (while the \( \text{CO}_2/\text{H}_2\text{O} \) oxygen exchange was reduced, as revealed by \( \Delta^{18}\text{O} \) values), unless CA activity was suppressed by antisense manipulation (Cousins et al. 2006a, 2006b). The modest effect of the catalytic activity of CAs on facilitating photosynthetic CO₂ fixation raises the question of the biological significance of these enzymes, and the cost (in terms of energy for synthesis and nitrogen requirement) they may represent. Nevertheless, as suggested by Cowan (1986), an enhancement \( \sim 5\% \) of photosynthesis is not negligible as far as evolution is concerned, particularly when the proteins involved account for no more than 2% of leaf proteins. In addition, while CAs might be involved in PSII-catalysed \( \text{O}_2 \) generation (Villarejo et al. 2002; but see McConnell et al. 2007), we recognise that CAs may have other important roles, such as contributing to the structure of the thylakoid membrane (e.g. Rudenko et al. 2007).

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**Table 4. Internal conductance (\( g_{\text{m}} \)) values (in mol m⁻² s⁻¹) obtained in the present study, from regressions based on Fig. 2 data**

<table>
<thead>
<tr>
<th>Assumption</th>
<th>( g_{\text{m}} ) in H₂O</th>
<th>( g_{\text{m}} ) in D₂O</th>
<th>( g_{\text{m}} ) in D₂O + inlet D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data in 21% O₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( b = 29% )</td>
<td>0.166 ± 0.038</td>
<td>0.150 ± 0.049</td>
<td>0.134 ± 0.026</td>
</tr>
<tr>
<td>( b ) values from regression</td>
<td>0.205 ± 0.048</td>
<td>0.144 ± 0.020</td>
<td>0.114 ± 0.025</td>
</tr>
<tr>
<td><strong>Data in 2% O₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( b = 29% )</td>
<td>n.s.</td>
<td>-</td>
<td>0.166 ± 0.056</td>
</tr>
<tr>
<td>( b ) values from regression</td>
<td>0.280 ± 0.092</td>
<td>-</td>
<td>0.130 ± 0.043</td>
</tr>
<tr>
<td></td>
<td>(33.1 ± 4.2)</td>
<td></td>
<td>(26.8 ± 1.6)</td>
</tr>
</tbody>
</table>
Leaf photosynthesis in heavy water


Soriano GM, Cramer WA (2001) Deuterium kinetic isotope effects in the
Silverman DN, Vincent SH (1983) Proton transfer in the catalytic mechanism
Tcherkez G (2004) Etude de la gestion des re´serves et de la respiration foliaires
anhydrase activity in pea thylakoids: soluble and membrane-bound forms.
activity and CO2 transfer resistance in Zn-deficient rice leaves.
Functional Plant Biology
Planta

Manuscript received 28 November 2007, accepted 18 February 2008
In the present study, water is small, as we explain below. Accordingly, the experiments with H2O or D2O in inlet air do not differ significantly (Fig. 2, semi-exchange system. However, because the isotope ratio of source water is very high (99.8% D2O, that is, D/H = 499), the dilution by inlet water is small, as we explain below. Accordingly, the experiments with H2O or D2O in inlet air do not differ significantly (Fig. 2, semi-exchange system. However, because the isotope ratio of source water is very high (99.8% D2O, that is, D/H = 499), the dilution by inlet water is small. This is consistent with the very small effect of heavy water on internal conductance (as discussed in the main text) is not caused by an artefactual depletion in deuterium in the leaf when H2O is used in the inlet.

Leaf water isotope composition

Robust, the relationship giving the isotopic abundance at the evaporative sites uses proportions instead of delta-values. If $R_S$ is the D/H ratio of source water, the proportion of D is $P_D = R_S/(1+R_S)$ and that of H is $P_H = 1/(1+R_S)$. In transpired water,

$$P_D = \frac{g_D(d_i-d_a)}{g_H(w_i-w_a) + g_D(d_i-d_a)}$$

and

$$P_H = \frac{g_H(w_i-w_a)}{g_H(w_i-w_a) + g_D(d_i-d_a)}$$

where $g_D$ and $g_H$ are D2O and H2O conductance, $d_i$ and $w_i$ are D2O- and H2O-mole fractions at the evaporative sites, and $R_S$ is the D/H ratio of source water. In the steady-state, the proportion of D ($P_D$) of source water equals that of transpired water, and this gives (both denominators ($1+R_S$) and $g_D(d_i-d_a) + g_H(w_i-w_a)$ simplify):

$$R_S = \frac{g_D(d_i-d_a)}{g_H(w_i-w_a)}$$

This equation is identical to the Craig–Gordon model applied to leaf water (Flanagan et al. 1991). Re-arranging, that gives the D/H isotope ratio of water at the evaporative sites ($R_a$) as follows:

$$R_a = \alpha^+ (\alpha_0 R_S (1-h) + R_h h),$$

(A1)

where $R_a$ is the D/H ratio of atmosphere water, respectively. Here, $h$ is relative humidity and $\alpha^+$ (1.116) and $\alpha_0$ (1.040) are the isotope effects associated with the liquid–vapour equilibrium and diffusion in air, respectively. The mass-balance equation applied to heavy water gives:

$$u_e R_{in} w_e + s E R_E = (u_e + s(E + R_E E)) R_a w_a,$$

(A2)

where $u_e$ is the entering flow, $R_{in}$ the D/H isotope ratio of inlet water; $s$ is the leaf surface area and $E$ is H2O-transpiration. $w_e$ and $w_a$ are the water partial pressures of inlet air and outlet air, respectively. $R_E$ is the D/H isotope ratio of transpired water, which is equal, in the steady-state, to $R_a$. Eqn A1 may be re-arranged to:

$$R_a w_a = \frac{1 + \frac{1}{R_a} (u_e \times R_{in} w_e)}{1 + \frac{1}{R_a} (1 + \frac{w_a}{s E})}$$

In the present study, $R_E = R_S$ is 99.8/0.2, that is, 499, but inlet water is made of natural water (very small $R_{in}$). Here, $u_e/s E R_E$ is in the range 3.7 $10^{-4}$ mol s$^{-1}$/(10$^{-2}$ m$^2$ $\times$ 10$^{-4}$ mol m$^{-2}$ s$^{-1}$ $\times$ 499) = 0.76, so that the right term in the numerator can be neglected and $R_a w_a \sim 1/1.76 = 0.57$. With a value of $w_a = 0.01$ mol mol$^{-1}$, we find $R_a \sim 0.57$. Using Eqn A1 we then have $R_a = 166$, which is equivalent to 99.4% D2O. In other words, the dilution of heavy water in the leaf by inlet H2O is very small. This is consistent with the very small effect of heavy water on internal conductance (as discussed in the main text) is not caused by an artefactual depletion in deuterium in the leaf when H2O is used in the inlet.

Other steps possibly slowed down in heavy water

As already stated in the main text, heavy water has an effect on two main steps, namely, the Rubisco-catalysed reaction, and the CO2-HCO3$^{-}$ equilibrium, catalysed by carbonic anhydrase (Table 1). Other slowing effects caused by heavy water are negligible in the present study:

(i) there is no effect of heavy water on the $^{12}$C/$^{13}$C isotope effect associated with diffusion in water (Table 2), and

(ii) the effect of heavy water on the $^{12}$C/$^{13}$C isotope effect associated with dissolution in water is unknown, but one may assume it is small, as deuteration of water acts on dissolution through $(a)$, the partition function of CO2 in the liquid phase and $(b)$, the binding energy between solvent water molecules and CO2. The effect on binding is thought to be a negligible component (Vogel et al. 1970). Because the degrees of freedom and the motions of CO2 are similar, the partition function of CO2 in the liquid phase is likely not to be much influenced by deuteration of water, so that the change in the ratio of $^{13}$CO2 and $^{12}$CO2 solubility remains unchanged. This
would be in agreement with the very small effect of atomic substitution changes on solubility in water: for example, the H$_2$O/D$_2$O isotope effect on the solubility of CH$_4$ is 1.005 but that of (the much larger molecule) CF$_4$ is 1.017 (Cosgrove and Walkley 1981). If an effect of heavy water on the $^{12}$C/$^{13}$C isotope effect of dissolution were to occur, the overall impact would be very small, because of its order of magnitude (<1‰).

Thus, heavy water has roughly no effect on the carbon isotope fractionation by diffusion and dissolution (Table 2), and a change in the photosynthetic discrimination, if any, would have to be related to enzymatic effects.

Appendix II. Calculation of the conductance when inlet water is H$_2$O

The outlet D$_2$O vapour mole fraction, denoted as $d_a$, follows the mass-balance equation for heavy water, that is (as $R_m$ is small compared with 1):

$$u_e w_e R_m + \Phi s R_e s = d_a (u_e + \Phi s)$$

where $u_e$ is the entering air flow through the chamber, $\Phi$ is the water flux taken up by the leaf through the petiole, $s$ is the leaf surface area, and $R_s$ the D/H isotope ratio of source water. $R_m$ and $w_e$ are the D/H isotope ratio and the water mole fraction of inlet water, respectively. Because the ratio $R_m$ is very small when natural water is used for inlet air, we have:

$$d_a = R_e (1 + R_s) \frac{1 + u_e / \Phi s}{1 + u_e / \Phi s}$$

where $u_e$, that is close to $1 - d_a/(1 + R_s)$ because $R_m/(1 + R_s) = 0.998$

Although the leaf transpired heavy water, the natural vapour pressure (H$_2$O of inlet air) had to be taken into account because it may have entered the leaf in the steady-state (see also above). At ambient temperatures, the combination of both natural and heavy waters behave linearly with respect to their relative quantities (Abdulkadirova et al. 2002) so that the saturation vapour pressure of the mixture at the evaporating sites is $(1 - \varepsilon) d_i + \varepsilon w_i$, where $\varepsilon$ is the proportion of natural water at the evaporative sites, and $w_i$ and $d_i$ are the saturation mole fractions of H$_2$O and D$_2$O at leaf temperature [in other words, the actual H$_2$O vapour mole fraction in the leaf is $\varepsilon w_i$ and that of D$_2$O is $(1 - \varepsilon) d_i$]. Then the water exchange between the atmosphere and the leaf is made up of D$_2$O loss, that is, $g_{D, CO_2}$ $(1 - \varepsilon) d_i - d_a$ and H$_2$O uptake, $g_{D, H_2O} (w_i - \varepsilon w_i)$, where $w_i$ is the outlet mole fraction of H$_2$O. As the leaf compensated for the small diffusive influx of H$_2$O by consuming less D$_2$O through the petiole we have (assuming a mole-to-mole compensation):

$$g_{D, CO_2} (1 - \varepsilon) d_i - d_a - \alpha_d g_{D, CO_2} (w_a - \varepsilon w_i) = \Phi$$

where the diffusion ratio (H$_2$O/D$_2$O isotope effect) is denoted as $g_{H_2O/D, CO_2} = \alpha_d (-1.040)$ and the saturation mole fraction pressure $w_i/d_i$ denoted as $\alpha^+$, The net heavy water uptake by the leaf is denoted as $\Phi$. Rearranging, this gives:

$$g_{D, H_2O} = \frac{\Phi}{d_i (1 - \varepsilon + \varepsilon \alpha^+ \alpha_d) - (d_a + \alpha_d w_a)}$$

If $\varepsilon$ is small enough (see Appendix I), we define the ‘efficient’ heavy water mole fraction as:

$$d_{\text{eff}} = d_a + \alpha_d w_a$$

The D$_2$O saturation vapour pressure values (at leaf temperature), denoted as $d_{\text{sat}}$, were taken from data compiled for the NIST chemistry webbook (http://webbook.nist.gov/chemistry) and fromBesley and Bottomley (1973). Stomatal conductance to D$_2$O is then given by:

$$g_{D, H_2O} = \frac{\Phi (1 - d_{\text{sat}}/d_{\text{eff}})}{d_{\text{sat}} - d_{\text{eff}}}$$

The CO$_2$ conductance was then calculated with the ordinary equations (von Caemmerer and Farquhar 1981), with the ratio of diffusivity between CO$_2$ and D$_2$O of 1.5 (instead of 1.6 for H$_2$O) (Marrero and Mason 1973). It is noted that the diffusion coefficients of CO$_2$ in (heavy) water vapour and in air are not equal (the H$_2$O-to-D$_2$O ratio of CO$_2$ diffusivity is of around ~1.07), in contrast with the usual case (von Caemmerer and Farquhar 1981). However, the effect on the internal CO$_2$ concentration ($c_i$) is very small, typically of around 0.2–0.5 μmol mol$^{-1}$, and it was neglected in the present study.