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A new measurement technique reveals temporal variation in $\delta^{18}O$ of leaf-respired CO_2

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ABSTRACT

The oxygen isotope composition of CO_2 respired by *Ricinus communis* leaves ($\delta^{18}O_R$) was measured under non-steady-state conditions with a temporal resolution of 3 min using a tunable diode laser (*TDL*) absorption spectrometer coupled to a portable gas exchange system. The SD of $\delta^{18}O$ measurement by the *TDL* was ± 0.2‰ and close to that of traditional mass spectrometers. Further, $\delta^{18}O_R$ values at isotopic steady state were comparable to those obtained using traditional flask sampling and mass spectrometric techniques for *R. communis* grown and measured in similar environmental conditions. As well as higher temporal resolution, the online *TDL* method described here has a number of advantages over mass spectrometric techniques.

At isotopic steady state among plants grown at high light, the 'one-way flux' model was required to accurately predict $\delta^{18}O_R$. A comparison of measurements and the model suggests that plants grown under low-light conditions have either a lower proportion of chloroplast CO_2 that isotopically equilibrates with chloroplast water, or more enriched $\delta^{18}O$ of CO_2 in the chloroplast that has not equilibrated with local water. The high temporal resolution of isotopic measurements allowed the first measurements of $\delta^{18}O_R$ when stomatal conductance was rapidly changing. Under non-steady-state conditions, $\delta^{18}O_R$ varied between 50 and 220‰ for leaves of plants grown under different light and water environments, and varied by as much as 100‰ within 10 min for a single leaf. Stomatal conductance ranged from 0.001 to 1.586 mol m^{-2} s^{-1}, and had an important influence on $\delta^{18}O_R$ under non-steady-state conditions not only via effects on leaf water $H_2{}^{18}O$ enrichment, but also via effects on the rate of the one-way fluxes of CO_2 into and out of the leaf.

Key-words: leaf respiration; leaf water enrichment; oxygen isotope; tunable diode laser.

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INTRODUCTION

Interpretation of variation in the oxygen isotope composition of atmospheric CO_2 may provide a valuable method of verification of carbon cycle models at both global (Francey & Tans 1987; Farquhar *et al.* 1993; Ciais, Denning & Tans 1997) and ecosystem scales (Yakir & Wang 1996; Bowling *et al.* 2003a; Ogée *et al.* 2004; Ometto *et al.* 2005). Both respiration and photosynthesis affect the oxygen isotope composition of atmospheric CO_2, and under some conditions by an equally large amount (Cernusak *et al.* 2004; Seibt *et al.* 2006; Seibt, Wingate & Berry, in press). CO_2 respired by ecosystem components (e.g. leaves, stems and soil) reflects isotopic exchange between oxygen in CO_2 and water within components. Water within one ecosystem component may differ isotopically from water in another. For example, leaf water is often significantly more enriched in ^{18}O than soil water, so that CO_2 respired by leaves is expected to be more enriched than soil-respired CO_2 (Flanagan *et al.* 1997; Flanagan, Kubien & Ehleringer 1999). Measurements of the isotope composition of pools of water within ecosystems suggest that leaf water, in particular, is highly dynamic in time and space (Lai *et al.* 2006; Seibt *et al.* 2006). Even within relatively simple ecosystems, it is not possible to measure the oxygen isotope composition of leaf-respired CO_2 ($\delta^{18}O_R$) at relevant temporal and spatial resolutions, particularly when ecosystem measurements are made at half-hourly temporal resolution using new tunable diode laser (*TDL*) absorption spectrometric techniques (e.g. Griffis *et al.* 2005). Hence, accurate models of $\delta^{18}O_R$ are required to interpret ecosystem isoflux measurements.

A model that describes the environmental and physiological influences on $\delta^{18}O_R$, and accounts for the one-way fluxes of CO_2 into and out of the leaf, has recently been presented by Cernusak *et al.* (2004). The inclusion of the one-way fluxes creates a model analogous to the CO_2 invasion effect in soils described by Tans (1998; also see Miller *et al.* 1999; Stern, Amundson & Baisden 2001), and to the original treatment of isotopic effects during CO_2 assimilation (Farquhar *et al.* 1993). Cernusak *et al.* (2004) tested their model with leaves in controlled-environment gas exchange chambers under isotopic steady state and were

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able to demonstrate significant departures of $\delta^{18}O_R$ from values predicted by simpler models considering just the net respiratory flux (e.g. Flanagan *et al.* 1997, 1999; Bowling *et al.* 2003a). $\delta^{18}O_R$ predicted by the one-way flux model at isotopic steady state deviates from that predicted by a net flux model when the difference in isotopic composition of CO_2 between that in the chloroplast ($\delta^{18}O_c$) and that in the ambient air ($\delta^{18}O_a$) is large, and when stomatal conductance is high. Both these conditions were met in the model evaluation experiment conducted by Cernusak *et al.* (2004). However, it is not clear if the one-way flux model adequately predicts $\delta^{18}O_R$ when stomatal conductance is very low (but significantly greater than cuticular conductance alone; Donovan, Richards & Linton 2003; Barbour *et al.* 2005), as would be typical for most species at night in natural conditions.

We have three objectives in this paper. Firstly, we demonstrate the application of *TDL* absorption spectroscopy coupled to a portable gas exchange system for leaf-level measurements of $\delta^{18}O_R$ and compare with those reported by Cernusak *et al.* (2004) for plants under similar growth and measurements conditions but using traditional mass spectrometric techniques. Secondly, we quantify non-steady-state changes in $\delta^{18}O_R$ in the first 50 min after the plants were moved from the light into the dark. Finally, we tested theoretical models at isotopic steady state over a wide range in measured stomatal conductance, including very low values (0.001 mol m^{-2} s^{-1}).

THEORY

An objective of the current study was to test existing models of $\delta^{18}O_R$ over a very wide range in one of the key parameters, namely stomatal conductance. To aid interpretation of measurements, we present a summary of the recently developed theory. $\delta^{18}O$ of leaf-respired CO_2 ($\delta^{18}O_R$) may be described by (Cernusak *et al.* 2004):

$$\delta^{18}O_R = \frac{\theta[\delta^{18}O_e(1+\varepsilon_w)+\varepsilon_w]+(1-\theta)\delta^{18}O_{c0} - \frac{C_a}{C_c}(\delta^{18}O_a-\bar{a})-\bar{a}}{(1+\bar{a})\left(1-\frac{C_a}{C_c}\right)}, \quad (1)$$

where θ is the proportion of chloroplast CO_2 that is isotopically equilibrated with chloroplast water, $\delta^{18}O_e$, $\delta^{18}O_{c0}$ and $\delta^{18}O_a$ are the $\delta^{18}O$ of chloroplastic water, the $\delta^{18}O$ of CO_2 in the chloroplast that has not equilibrated with local water and the $\delta^{18}O$ of CO_2 in the atmosphere, respectively. C_a and C_c are the CO_2 mole fractions in the ambient atmosphere and in the chloroplasts (or mitochondria; Barbour *et al.* 2005), respectively, and ε_w is the equilibrium ^{18}O fractionation between CO_2 and water. The isotopic equilibrium between CO_2 and water is temperature dependent (Brenninkmeijer, Kraft & Mook 1983):

$$\varepsilon_w(‰) = \frac{17\,604}{T_l} - 17.93, \quad (2)$$

where T_l is leaf temperature in kelvin. In Eqn 1, $\bar{a}$ is the weighted mean discrimination against $C^{18}OO$ for diffusion from the chloroplast to the atmosphere, and is given by (Farquhar & Lloyd 1993)

$$\bar{a} = \frac{(C_c-C_i)a_w+(C_i-C_s)a+(C_s-C_a)a_b}{C_c-C_a}, \quad (3)$$

where a_w is the summed discriminations against $C^{18}OO$ during liquid phase diffusion and dissolution (0.8‰); a and a_b are the discriminations against $C^{18}OO$ during diffusion through the stomata and the boundary layer (8.8 and 5.8‰, respectively), and C_i and C_s are CO_2 mole fractions in the leaf intercellular spaces and at the leaf surface, respectively. Using the definition of oxygen isotope composition of CO_2 in the chloroplast of a respiring leaf ($\delta^{18}O_c$; Cernusak *et al.* 2004),

$$\delta^{18}O_c = \delta^{18}O_e\theta(1+\varepsilon_w)+\theta\varepsilon_w+\delta^{18}O_{c0}(1-\theta), \quad (4)$$

and ignoring second-order terms, Eqn 1 may be simplified to (Cernusak *et al.* 2004)

$$\delta^{18}O_R = \delta^{18}O_c - \bar{a} + (\delta^{18}O_c - \delta^{18}O_a)\left(\frac{C_a}{C_c-C_a}\right). \quad (5)$$

Eqn 5 may be compared to earlier 'net flux' models that describe $\delta^{18}O_R$ without consideration of the one-way fluxes (e.g. Flanagan *et al.* 1997, 1999; Bowling *et al.* 2003a):

$$\delta^{18}O_{Rn} = \delta^{18}O_e + \varepsilon_w - a, \quad (6)$$

where $\delta^{18}O_{Rn}$ refers to the isotopic composition of leaf-respired CO_2 predicted by the net flux model, to allow distinction from that predicted by the one-way flux model. Eqns 5 and 6 will predict very similar values of $\delta^{18}O_R$ when stomata are tightly closed (C_a/C_c approaches zero) and $\theta = 1$ (Barbour *et al.* 2005). Note that we apply the full derivation, including second-order terms (Eqn 1), for prediction of $\delta^{18}O_R$ in the one-way flux model.

The degree of evaporative enrichment of leaf water ($\delta^{18}O_e$) is a critical parameter in determining $\delta^{18}O_R$. The steady-state enrichment above source water of leaf water at the sites of evaporation [and, to a close approximation, the chloroplast and mitochondria (Barbour *et al.* 2005); Δ_{es}] has been modelled by Farquhar & Lloyd (1993), by modification of earlier models (Craig & Gordon 1965; Dongmann *et al.* 1974) as

$$\Delta^{18}O_{es} = \varepsilon^+ + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k)\frac{w_o}{w_l}, \quad (7)$$

where ε^+ is the equilibrium fractionation during the phase change from liquid to vapour, ε_k is the kinetic fractionation during diffusion through stomata and the leaf boundary layer, and $\Delta^{18}O_v$ is the ^{18}O enrichment above source water of atmospheric vapour. The oxygen isotopic composition of water at the evaporating sites in leaves and ambient water vapour are expressed relative to the source water ($\delta^{18}O_s$),

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such that $\Delta^{18}O_{es} = R_e/R_s - 1$ and $\Delta^{18}O_v = R_v/R_s - 1$, where R is the $^{18}O/^{16}O$ ratio, and subscripts e, v and s are water at the evaporating sites, water vapour and source water, respectively. $\delta^{18}O_{es}$ may be calculated from $\Delta^{18}O_{es}$ by

$$\delta^{18}O_{es} = \Delta^{18}O_{es}(1 + \delta^{18}O_s) + \delta^{18}O_s. \quad (8)$$

The equilibrium fractionation factor, ε^+, is dependent on temperature by (Bottinga & Craig 1969)

$$\varepsilon^+(‰) = 2.644 - 3.206\left(\frac{10^3}{T_l}\right) + 1.534\left(\frac{10^6}{T_l^2}\right), \quad (9)$$

where T_l is leaf temperature in kelvin. The total kinetic fractionation factor, ε_k, may be calculated from stomatal (g_{sw}) and boundary layer (g_{bw}) conductances by (Farquhar *et al.* 1989)

$$\varepsilon_k = \frac{32g_{sw}^{-1} + 21g_{bw}^{-1}}{g_{sw}^{-1} + g_{bw}^{-1}}. \quad (10)$$

The fractionation factor for diffusion through air (and stomata) was recently determined to be 32‰ by Cappa *et al.* (2003) and, assuming a two-thirds power effect according to Pohlhausen analysis (Kays 1966), fractionation during diffusion through the boundary layer will be 21‰ (Cernusak, Wong & Farquhar 2003).

Equation 7 applies at isotopic steady state. Leaves should be close to steady state when the turnover time of leaf water isotopes is rapid, such as when the one-way efflux of water through the stomata is high, and lamina water concentration (i.e. mol m^{-2}) is low (Farquhar & Cernusak 2005). Thus, thin leaves with high stomatal conductances are more likely to be at isotopic steady state than thick leaves or those with low stomatal conductances (Seibt *et al.* in press). The leaf water isotopic turnover time (τ) is calculated as a function of the one-way stomatal efflux (gw_i; stomatal and boundary layer conductances in series multiplied by the mole fraction of water vapour in the intercellular spaces) and the lamina water concentration (Farquhar & Cernusak 2005):

$$\tau = \frac{W\alpha_k\alpha^+}{gw_i} \quad (11)$$

where $\alpha_k = 1 + \varepsilon_k$ and $\alpha^+ = 1 - \varepsilon^+$. Note that this definition of τ differs from that presented by Farris and Strain (1978) by approximately $w_i/(w_i - w_a)$, where w_a is the mole fraction of water vapour in the atmosphere (Farquhar & Cernusak 2005). Time to isotopic steady state is estimated as 3τ (Förstel 1978).

MATERIALS AND METHODS

Growth conditions

Castor bean (*Ricinus communis* L.) plants were grown in 7 L pots with potting mix and a slow-release fertilizer in a glasshouse at Los Alamos National Laboratory, NM, USA, during April and May 2005. A shade cloth stretched across the roof of the glasshouse reduced the direct light within the glasshouse by about half. Average daytime air temperature within the glasshouse varied between 24.1 and 31.2 °C, and relative humidity between 19 and 35%, while average daytime air temperature over the experimental period was 27.1 ± 0.8 °C, and relative humidity 25 ± 2%. Average night temperature inside the glasshouse varied between 18.2 and 22.1 °C and relative humidity between 14 and 40%, while average night temperature over the experimental period was 20.6 ± 0.4 °C, and relative humidity 27 ± 2%.

The plants were grown either under high light (total daily PAR 6.3 – 22.6 mol m^{-2}, with an average 16.5 ± 1.6 mol m^{-2}) or beneath shade cloth to produce low light [total daily photosynthetically active radiation (PAR) 0.8 – 2.8 mol m^{-2}, with an average 2.1 ± 0.2 mol m^{-2}]. The plants were well watered every second day and measured on 6 May 2005, except two pots for which water was withheld for 6 d (0.5 L was added to the two droughted plants on the fourth day after watering ceased). Droughted plants were measured on the seventh day after watering ceased (12 May 2005), and were observed to be slightly wilted. The plants were moved into the dark under a large cardboard box 1–2 min prior to a leaf being sealed in the gas exchange chamber.

Gas exchange measurements

Measurements of leaf respiration rate (R) and stomatal conductance (g_s) were made in the dark on the youngest fully expanded leaf (leaf three to six, depending on the growth environment) using a portable photosynthesis system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) fitted with a custom-built leaf chamber. The chamber was milled from stainless steel to allow enclosure of up to 80 cm^2 leaf area, and was sealed with a closed cell foam gasket of 1 cm width. The chamber was leak tested after enclosing the leaf in the chamber by breathing around the seal. The large leaf area maximized the difference in concentration and isotopic composition between incoming and outgoing chamber air, reducing errors in calculated R and $\delta^{18}O_R$. A thermocouple was placed within the chamber to measure leaf temperature, and a 120 L buffer volume was used to stabilize the CO_2 concentration of air entering the leaf chamber. The relationship between leaf area and boundary layer conductance was measured for the chamber using wet filter paper. Leaf area within the chamber was measured from digitized images of the leaf and imaging software (Scion; Scion Corporation, Frederick, MD, USA), and R and g_s were recalculated with the corrected leaf area. Gas exchange measurements were recorded to coincide with isotopic measurements of incoming and outgoing chamber air (i.e. two measurements were recorded every 3 min).

The glasshouse air temperature was at most 2 °C lower than the chamber air temperature, so to check that no condensation occurred in the sample lines between the leaf chamber and the *TDL* water vapour trap (CO_2 could

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isotopically re-equilibrate with any condensed water in the lines), the dew point temperature was calculated for the glasshouse air and compared with the water vapour content of air leaving the chamber. The vapour pressure difference between actual and saturation vapour pressure in the lines was usually greater than 1.0 kPa, and always greater than 0.5 kPa, that is, there would have been no condensation at any time during the experiment.

Isotopic measurements

The mixing ratios of CO_2 entering and leaving the leaf chamber were determined using *TDL* absorption spectroscopy (TGA100A; Campbell Scientific Inc., Logan, UT, USA). This technique measures the mixing ratio of the individual isotopologues (Bowling *et al.* 2003b), but we recalculated the values as isotope ratios in the familiar delta notation. The oxygen ($\delta^{18}O_{VPDB}$) and carbon ($\delta^{13}C$) isotope ratios of CO_2, relative to the Vienna Pee Dee belemnite (VPDB) standard (VPDB for carbon, VPDB-CO_2 for oxygen), are given by

$$\delta^{18}O_{VPDB-CO2} = \frac{R_{so}}{R_{VPDBo}} - 1, \tag{12}$$

and

$$\delta^{13}C = \frac{R_{sc}}{R_{VPDBc}} - 1, \tag{13}$$

where R_{so} and R_{VPDBo} are the $^{18}O/^{16}O$ ratios of the sample and the VPDB-CO_2 standard (0.0020883; Allison, Francey & Meijer 1995), and R_{sc} and R_{VPDBc} are the $^{13}C/^{12}C$ ratios of the sample and the VPDB standard (0.011797; Zhang & Li 1990), respectively. Note that VPDB-CO_2 is the isotope ratio of CO_2 released by the VPDB carbonate, not the isotope ratio of the carbonate itself. R_{sc} is simply related to the isotopologue mixing ratio:

$$R_{sc} = \frac{[^{13}C^{16}O_2]}{[^{12}C^{16}O_2]}, \tag{14}$$

but the two atoms of oxygen in CO_2 mean that (Griffis *et al.* 2005)

$$R_{so} = 0.5\frac{[^{12}C^{18}O^{16}O]}{[^{12}C^{16}O_2]}. \tag{15}$$

Two primary calibration cylinders with total CO_2 mixing ratios ($[CO_2]_t$) of 352.02 and 566.65 μmol mol^{-1}, $\delta^{13}C$ of –8.44 and –17.06‰, and $\delta^{18}O_{VPDB}$ of –8.44 and –17.06‰, respectively, (measured by National Oceanic and Atmospheric Administration, Climate Monitoring and Diagnostic Laboratory) were used to calibrate the *TDL*. The mixing ratios of each isotopologue in the calibration cylinders were then calculated from $[CO_2]_t$, $\delta^{13}C$ and $\delta^{18}O$ as (in order from Eqns 16–18)

$$[^{12}C^{16}O_2] = \frac{[CO_2]_t(1 - f_{other})}{1 + R_{sc} + 2R_{so}}, \tag{16}$$

$$[^{13}C^{16}O_2] = [CO_2]_t(1 - f_{other}) - 2R_{so}[^{12}C^{16}O_2] - [^{12}C^{16}O_2], \tag{17}$$

$$[^{12}C^{16}O^{18}O] = [CO_2]_t(1 - f_{other}) - [^{13}C^{16}O_2] - [^{12}C^{16}O_2], \tag{18}$$

where f_{other} is the fraction of CO_2 containing all isotopologues other than $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{16}O^{18}O$, and is assumed to be 0.000821 (Eiler & Schauble 2004). The primary calibration cylinders were used to calibrate two working standards with mixing ratios of the three isotopologues of $[^{12}C^{16}O_2] = 334.587$, $[^{13}C^{16}O_2] = 3.626$, $[^{12}C^{16}O^{18}O] = 1.341$ and $[^{12}C^{16}O_2] = 550.3273$, $[^{13}C^{16}O_2] = 5.965$, $[^{12}C^{16}O^{18}O] = 2.208$ μmol mol^{-1}, respectively, and $\delta^{18}O_{VPDB-CO2}$ of –0.60 and 0.50‰, respectively. The working standards span the expected range in mixing ratios as required (Bowling *et al.* 2003b).

$[CO_2]_t$ for air streams from the LI-6400 chamber inlet and outlet lines (Li-Cor Inc.) was calculated from the mixing ratios of individual isotopologues by

$$[CO_2]_t = \frac{[^{12}C^{16}O_2] + [^{13}C^{16}O_2] + [^{12}C^{16}O^{18}O]}{1 - f_{other}}, \tag{19}$$

and $\delta^{18}O_{VPDB}$ values were then calculated using Eqns 12 and 15. We note that Griffis *et al.* (2005) calculate $[^{12}C^{16}O_2]$ and $[^{12}C^{18}O^{16}O]$ mixing ratios without using the $[^{13}C^{16}O_2]$ isotopologue and a value of f_{other} (0.01185) that corresponds to all isotopologues except $^{12}C^{16}O_2$ and $^{12}C^{18}O^{16}O$. The Griffis calculation results in slight (0.01‰) overestimation of $\delta^{18}O$, which is insignificant given typical precision of the *TDL* for $\delta^{18}O$ measurement (SD in $\delta^{18}O$ of approximately 1.2‰ for measurements at 10 Hz). However, the calculations we present here allow simultaneous measurement of both $\delta^{18}O$ and $\delta^{13}C$. For ease of comparison with $\delta^{18}O$ of H_2O liquid and vapour samples, we present $\delta^{18}O$ relative to Vienna standard mean oceanic water (VSMOW):

$$\delta^{18}O = \frac{R_{so}}{R_{VSMOW}} - 1, \tag{20}$$

where $R_{VSMOW} = 0.0020052$ (Gonfiantini 1984). Note that the VSMOW scale is offset from the VPDB-CO_2 scale by about 41‰. $\delta^{18}O$ is reported in parts per thousand (‰).

The *TDL* measures at 10 Hz, but for the current application, a manifold was used to switch between each of the two working standards and the leaf chamber inlet (reference) and outlet (sample) lines. Standard cylinders were sampled for 30 s, and sample and reference lines were sampled for 1 min each. We discarded all but the last 15 s of data to allow for flushing of the previous sample through the optical cell and plumbing and for pressure transients, allowing calculation of mean mixing ratios over 15 s for each working standard and the reference and sample air streams.

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Pressure within the optical cell was maintained at 2.0 mbar to minimize pressure broadening. A low-flow Nafion counterflow water trap (PD625 dual configurations, Campbell Scientific) was used to remove water vapour from sample air prior to measurement (Barbour *et al.* in press). The average SDs of each working standard calculated from repeated cylinder calibrations during our 2 week experiment were 0.07 μmol mol^{-1} for $[CO_2]_t$ and 0.2‰ for $\delta^{18}O$.

Calculating $\delta^{18}O_R$

The stable oxygen isotope ratio of CO_2 respired by the leaves ($\delta^{18}O_R$) was calculated by mass balance from the $\delta^{18}O$ and concentration of CO_2 entering ($\delta^{18}O_e$ and C_e, respectively) and leaving ($\delta^{18}O_o$ and C_o, respectively) the leaf chamber (Evans *et al.* 1986; Cernusak *et al.* 2004):

$$\delta^{18}O_R = \frac{\delta^{18}O_o - \delta^{18}O_e(1-p)}{p}, \tag{21}$$

where $p = (C_o - C_e)/C_o$. C_e and C_o represent $[CO_2]_t$ of dry air, as measured by the *TDL*. A single value of $\delta^{18}O_R$ was calculated every 3 min. A Monte Carlo analysis (following Barbour, Andrews & Farquhar 2001) was conducted to calculate the SD of individual estimates of $\delta^{18}O_R$. Variation in $[CO_2]_t$ and $\delta^{18}O$ (within the measured SDs of 0.07 μmol mol^{-1} and 0.2‰ for $[CO_2]_t$ and $\delta^{18}O$, respectively) was created using a random number generator for 10 000 individual calculations of Eqn 21, and the overall SD in $\delta^{18}O_R$ was determined.

Source water and water vapour sampling for isotopic analysis

Subsamples of irrigation water and vapour from glasshouse air (pumped at 20 L min^{-1} through a five-stage cascade cold-finger trap placed in an ethanol-dry ice bath) were taken on each measurement day (6 May for well-watered plants and 12 May for droughted plants). Water samples were analysed on an isotope ratio mass spectrometer (Isoprime; GV Instruments, Manchester, UK) using the CO_2 equilibration method of Socki, Karlsson & Gibson (1992). $\delta^{18}O$ of irrigation water and glasshouse water vapour were -10.4 ± 0.1 and -15.3 ± 0.1‰ for well-watered plants, respectively, and -10.1 ± 0.0 and -15.5 ± 0.2‰ for droughted plants, respectively (average ± SE of three samples in each case). The source water and vapour differed between well-watered and droughted plants because the measurements were made 6 d apart.

$\delta^{18}O_v$, the isotopic composition of water vapour in the leaf chamber, is estimated by assuming that (at isotopic steady state, for conservation of mass) $\delta^{18}O$ of leaf-transpired water vapour is equal to $\delta^{18}O$ of irrigation water ($\delta^{18}O_s$), so that

$$\delta^{18}O_v = p_t\delta^{18}O_s + (1-p_t)\delta^{18}O_{vg}, \tag{22}$$

where $\delta^{18}O_{vg}$ is the $\delta^{18}O$ of water vapour in glasshouse air (i.e. air entering the leaf chamber), and p_t is the proportional contribution to total chamber water vapour by leaf transpiration. p_t is calculated from the water vapour pressure of air entering (w_{in}) and leaving (w_a) the leaf chamber:

$$p_t = \frac{\dfrac{w_a - w_{in}(1-w_a)}{1-w_{in}}}{w_a}. \tag{23}$$

Leaf water content

Lamina water content [W mol m^{-2} = (g fresh weight – g dry weight) ÷ 18 g mol^{-1} ÷ disc area] was measured from leaf discs (9.62×10^{-4} m^2) cut from leaves of 10 well-watered plants (five grown under high light and five under low light, but not from leaves used for gas exchange and isotopic measurements) between 30 and 60 min after plants had been moved into the dark. There was no significant difference between high- and low-light plants ($P > 0.05$), with average $W = 6.85 \pm 0.10$ mol m^{-2}, and no evidence that W varied with time in the dark. Two or three leaf discs were cut for water content analysis from the leaves of the two droughted plants used for gas exchange and isotopic measurements (from the portion of the leaf not in the chamber). W of leaves from droughted plants tended to increase with time in the dark, from 6.41 to 6.86 mol m^{-2}.

RESULTS

Variability in respiration rate and stomatal conductance

Respiration rate was lowest for the leaves of plants grown under low light, at about 1 μmol m^{-2} s^{-1} (Fig. 1a–c). Leaves of plants grown under high light, whether droughted or well-watered, generally had higher respiration rates, between 1 and 2.25 μmol m^{-2} s^{-1}. Stomatal conductance to CO_2 (g_{sc}) varied between 0.001 and 1.584 mol m^{-2} s^{-1}, and was much lower for droughted than well-watered plants (Fig. 1f). g_{sc} for high-light and droughted plants declined after the plants were placed in the dark. Both low-light plants had fairly constant g_{sc} as time in the dark increased.

The time to isotopic steady state was estimated for each leaf using Eqn 11, measured W and the LI-6400 gas exchange measurements, and varied between 26 and 48 min for well-watered plants. This means that well-watered plants should have been close to isotopic steady state over the last 15 min of measurements. However, the extremely low one-way fluxes measured for droughted plants resulted in times to isotopic steady state between 8 and 94 h. Therefore, the two droughted plants were probably not at isotopic steady state, and the implications of assuming steady state and applying Eqns 22 and 23 are explored in the discussion.

Precision of $\delta^{18}O_R$ measurement

The large leaf chamber typically allowed a large difference in CO_2 mixing ratio (30 ± 7 μmol mol^{-1}) between air

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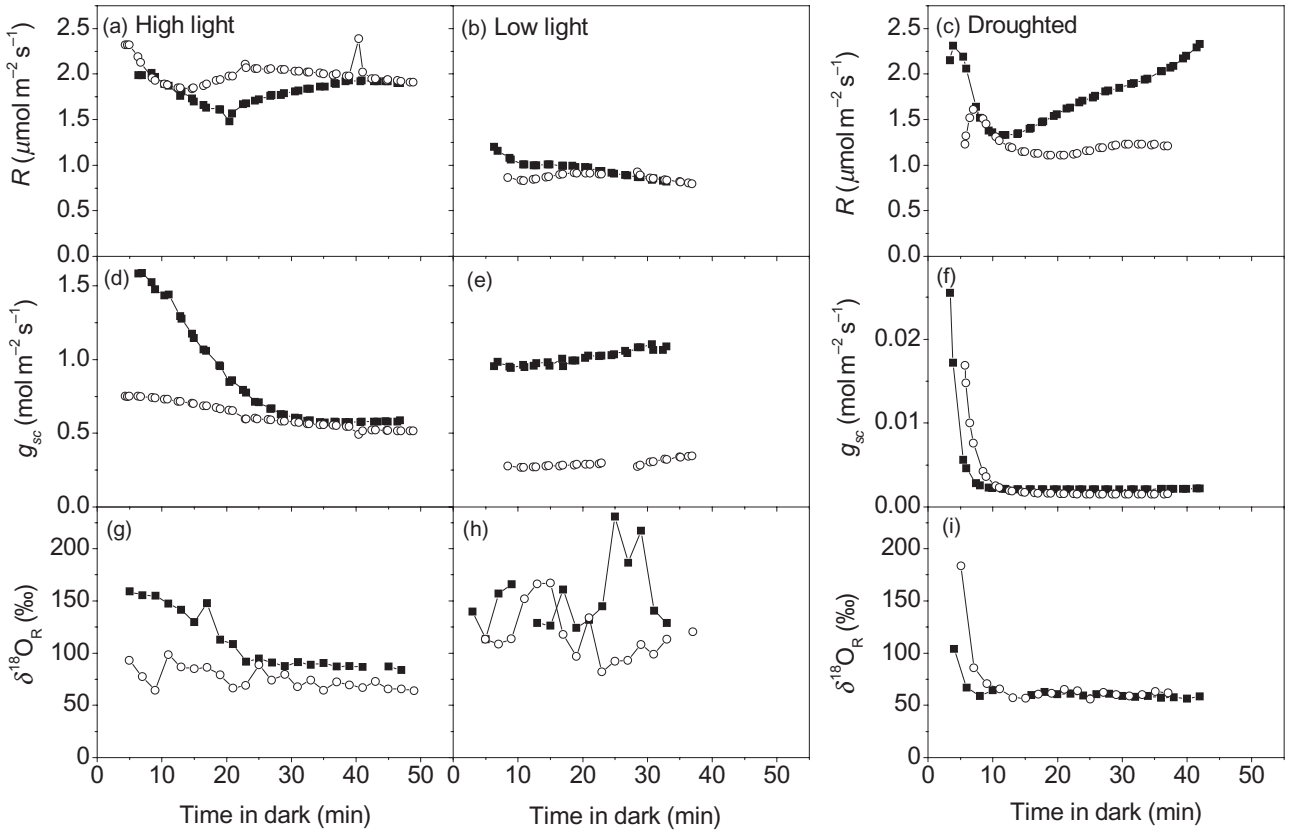

Figure 1. Temporal variation in leaf respiration rate (R), stomatal conductance to CO_2 (g_{sc}) and the oxygen isotope composition of leaf-respired CO_2 ($\delta^{18}O_R$) with time in the dark for each of two *Ricinus communis* plants grown under high light (a, d and g), low light (b, e and h) and droughted high-light conditions (c, f and i). Each leaf for a single growth environment is indicated with a different symbol.

entering and leaving the chamber. However, generally high stomatal conductance and transpiration rate set a lower limit to the maximum allowable flow rate without creating vapour pressure and condensation problems. A range in p (as defined in Eqn 21) between 0.020 and 0.141 was observed. A Monte Carlo analysis using Eqn 21 and the SDs of $[CO_2]_t$ and $\delta^{18}O$ (0.07 μmol mol^{-1} and 0.2‰, respectively) produced an SD of individual measurements of $\delta^{13}C_{Rl}$ between 1.1 and 5.1‰ (for $P = 0.141$ and $P = 0.020$, respectively).

A 120 L buffer volume was used to reduce fluctuations in C_e and $\delta^{18}O_e$, and was found to be effective. The SD of $\delta^{18}O_e$ over 15 min was typically 0.7‰, and C_e was less than 3 ppm. The exception was during measurements on the leaf of plant 2 grown at low light, for which the SDs over 15 min were 2.7‰ and 4.9 ppm. Both instrument noise and experimental variation were included in the calculated SE of the mean $\delta^{18}O_R$ over 15 min of measurements. SEs of the mean values varied between 0.4‰ (for the droughted plant 1) and 20.2‰ (for the low-light plant 2).

Temporal variability in measured $\delta^{18}O_R$

$\delta^{18}O_R$ calculated from *TDL* measurement of $\delta^{18}O$ of CO_2 entering and leaving the leaf chamber varied between 50 and 220‰. Leaves for which stomatal conductance declined with time (Plant 1 grown under high light, and both droughted plants) also showed a decline in $\delta^{18}O_R$ with time in the dark. $\delta^{18}O_R$ of the second high-light plant was rather constant at 50–80‰. $\delta^{18}O_R$ from leaves of both plants grown under low light were variable in time, between 70 and 220‰.

All leaves are assumed to be at isotopic steady state over the final 15 min of measurement, for comparison with modelled values and data presented by Cernusak *et al.* (2004). As discussed earlier, this assumption is likely to be valid for the four well-watered plants, but not for the droughted plants. Mean and SEs of $\delta^{18}O_R$ for each leaf are presented in Table 1, as are mean values for the well-watered, high-light-grown leaves measured by Cernusak *et al.* (2004).

Estimating θ and $\delta^{18}O_{c0}$

We utilized a regression technique originally presented by Cernusak *et al.* (2004) to predict θ and $\delta^{18}O_{c0}$ from a plot of $\delta^{18}O_e$ and $\delta^{18}O_c$. In the current experiment, variation in $\delta^{18}O_e$ resulted from variation in e_a/e_i due to differences in stomatal conductance and transpiration rate between growth environments. Equations 7–10 are used to estimate $\delta^{18}O_e$ for each leaf for the final 15 min of each experiment, using measured gas exchange parameters and isotopic

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Table 1. Gas exchange and isotopic characteristics of individual leaves between 30 and 50 min after plants had been moved to the dark, assuming isotope steady state

Parameter		High light 1	High light 2	Low light 1	Low light 2	Drought 1	Drought 2	Cernusak *et al.* (2004) Average	Cernusak *et al.* (2004) Range
T_l	(°C)	26.9	28.2	26.9	29.5	30.2	29.3	30.2	29.3–30.8
E	(mmol m^{-2} s^{-1})	1.85	2.38	1.62	1.04	0.09	0.06	2.7	1.3–4.0
g_{sc}	(mol m^{-2} s^{-1})	0.578	0.535	1.059	0.316	0.002	0.001	0.13	0.03–0.28
e_a/e_i	–	0.72	0.75	0.76	0.80	0.21	0.21	0.41	0.11–0.73
3τ	(min)	39	29	39	48	8–65 h	12–94 h		30–210
R_c	(μmol m^{-2} s^{-1})	1.91	1.99	0.88	0.85	2.05	1.20	1.4	1.0–2.0
C_a/C_c	–	0.98	0.97	0.99	0.99	0.32	0.35	0.96	0.90–0.98
δ_c	(‰)	5.3	4.4	3.7	3.1	19.9	19.2	20.5	10.8–29.8
δ_a	(‰)	45.0	44.4	46.3	46.4	44.3	43.5	43.5	37.7–51.2
$\bar{a}$	(‰)	4.89	4.83	4.06	5.47	8.58	8.64	5.9	3.7–8.3
θ		0.82	0.92	0.54	0.43	0.68	0.75	0.79	
$\delta^{18}O_R$	(‰)	86.4	66.9	180.7	107.0	57.7	60.8	277	233–324
Modelled $\delta^{18}O_R$	(‰)	86.2	67.2	181.1	106.8	57.7	60.9	291	149–476

Values are averages over the period of assumed isotopic steady state, and n is between 5 and 10. Modelled $\delta^{18}O_R$ values are those predicted using fitted θ. Listed also are averages and ranges in values from the Cernusak *et al.* (2004) experiment, in which well-watered plants grown at high-light intensity were measured at assumed isotopic steady state, and isotopic compositions were determined using traditional CO_2 trapping and mass spectrometric techniques.

T_l, leaf temperature; g_{sc}, stomatal conductance to CO_2; C_a/C_c, CO_2 mole fractions in the ambient atmosphere and in the chloroplast; θ, proportion of chloroplast CO_2 that is isotopically equilibrated with chloroplast water; $\delta^{18}O_R$, $\delta^{18}O$ of leaf-respired CO_2; $\bar{a}$, weighted mean discrimination against $C^{18}OO$ for diffusion from the chloroplast to the atmosphere.

compositions of source water ($\delta^{18}O_s$) and estimated $\delta^{18}O_v$. $\delta^{18}O_c$ is estimated from measurements of $\delta^{18}O_a$, $\delta^{18}O_R$ and gas exchange parameters using (Cernusak *et al.* 2004):

$$\delta^{18}O_c = \delta^{18}O_R(1+\bar{a})\left(1-\frac{C_a}{C_c}\right)+\frac{C_a}{C_c}(\delta^{18}O_a-\bar{a})+\bar{a}, \qquad (24)$$

where C_c is calculated from C_i and respiration rate assuming conductance from leaf intercellular air spaces to the chloroplasts is 0.3 mol m^{-2} s^{-1} (N.G. McDowell, D.T. Hanson and M.M. Barbour, unpublished data). According to Eqn 4, θ and $\delta^{18}O_{c0}$ may be estimated from plots of $\delta^{18}O_e$ versus $\delta^{18}O_c$. θ is estimated from the slope of the relationship [θ = slope/(1 + ε_w)], and $\delta^{18}O_{c0}$ from the intercept [$\delta^{18}O_{c0}$ = (intercept − $\theta\varepsilon_w$)/(1 − θ)], assuming θ and $\delta^{18}O_{c0}$ are constant between leaves (Cernusak *et al.* 2004). Figure 2 shows that data at assumed steady state from the current experiment are significantly offset from the original Cernusak *et al.* (2004) data, but have a similar slope, resulting in a similar estimate of θ (θ = 0.84), but higher $\delta^{18}O_{c0}$ (54.5‰). The Cernusak data fall below the relationship expected for full equilibration with chloroplast water, but the current data sit both above and below the θ = 1 line. The slope of the fitted regression, at 0.88, is significantly ($P < 0.05$) less than 1.04 (the slope when θ = 1), but we include modelled values for $\delta^{18}O_R$ assuming full equilibration in subsequent analysis for completeness.

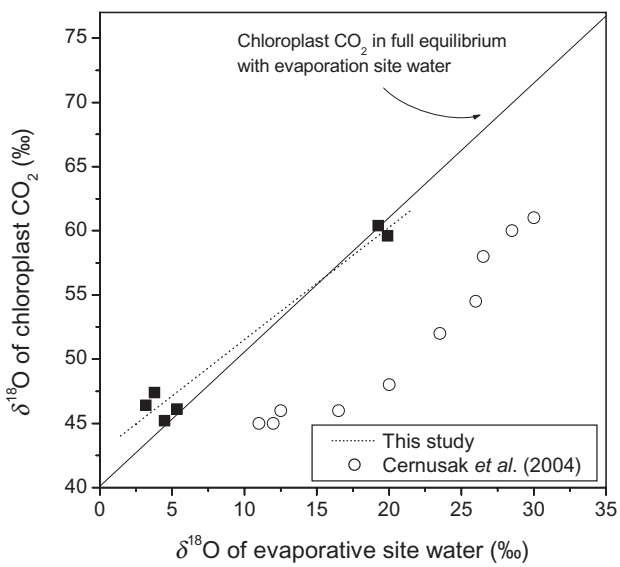

Figure 2. Relationship between $\delta^{18}O$ of chloroplast CO_2 ($\delta^{18}O_c$) and $\delta^{18}O$ of water at the evaporating sites in *Ricinus communis* leaves in the dark. The data shown are from the current experiment (filled squares) and from data published by Cernusak *et al.* (2004) (open circles). The solid line represents the relationship expected if chloroplast CO_2 was in isotopic equilibrium with evaporation site water, and the dotted line is a least square regression ($\delta^{18}O_c = 42.6 + 0.88\delta^{18}O_e$, $r^2 = 0.97$, $P = 0.0003$).

Modelling variation in $\delta^{18}O_R$

The simple net flux model (Eqn 6) significantly underestimated $\delta^{18}O_R$ of leaves for which stomata were open (i.e.

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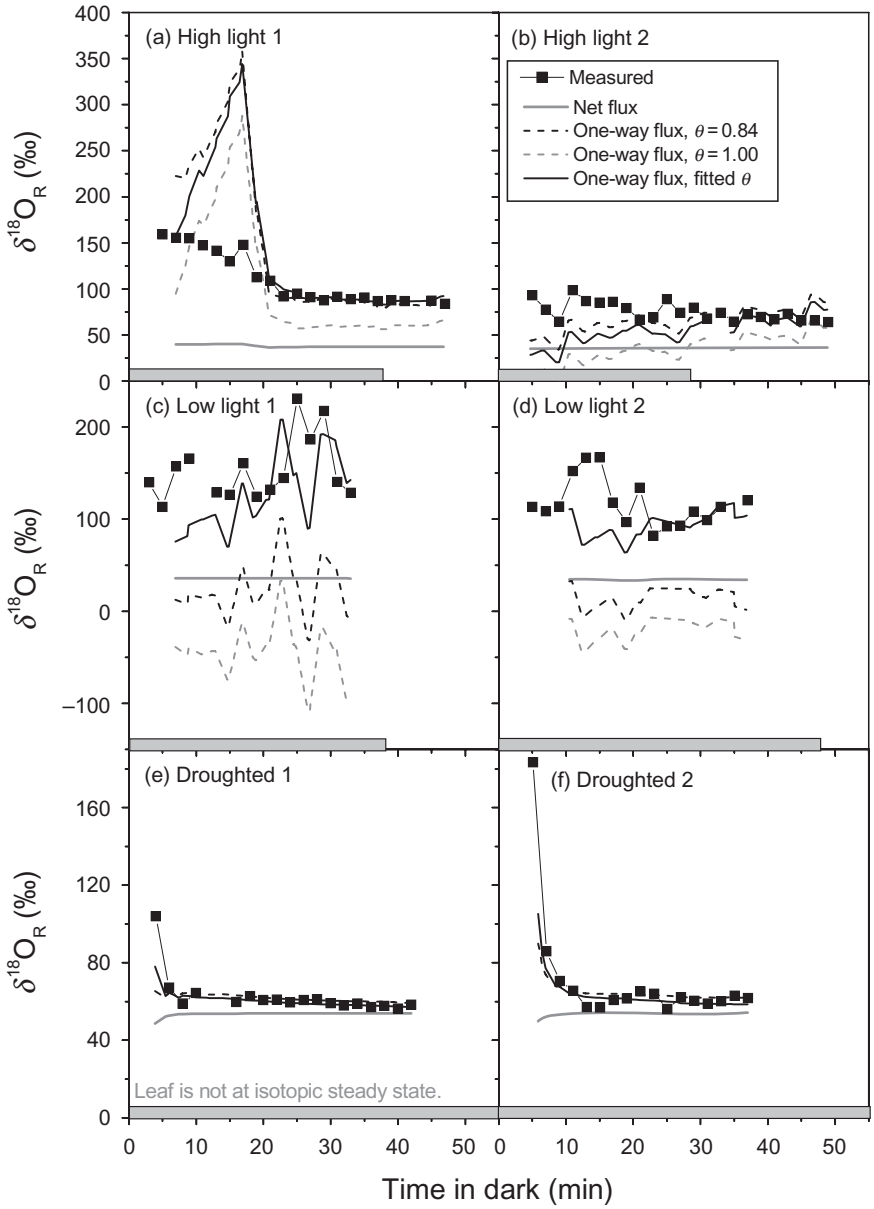

Figure 3. Modelled and measured temporal variation in $\delta^{18}O$ of leaf-respired CO_2 for *Ricinus communis* leaves grown under high light (a and b), low light (c and d), and droughted (e and f) conditions. The models applied all assume isotopic steady state, a valid assumption for well-watered high-light plants, but not for droughted plants. The time period for which each leaf is estimated to depart from isotopic steady state is shown as thick grey bars near the *x*-axis. The net flux modelled values are indicated by solid grey lines, using Eqns 6 and 7. The one-way flux modelled values, assuming $\theta = 1.00$, are indicated by dashed black lines, using Eqns 1 and 7. The one-way flux modelled values, assuming $\theta = 0.84$, are indicated by dashed grey lines using Eqns 1 and 7. The one-way flux model, with fitted θ, are indicated by solid black lines (Eqns 1 and 11). For the one-way flux model, θ was fitted to be 0.82, 0.92, 0.54, 0.43, 0.68 and 0.75 for a–f, respectively.

high- and low-light plants), but was within 6‰ of measured $\delta^{18}O_R$ for droughted plants with low stomatal conductance (Fig. 3). The one-way flux model with $\theta = 1$ also significantly underestimated $\delta^{18}O_R$ for well-watered plants. CO_2 was not in full equilibrium with chloroplast water for well-watered plants, even though $\delta^{18}O_c$ sits close to the expected relationship with $\delta^{18}O_e$ assuming full equilibration (see Fig. 2).

The one-way flux model with $\theta = 0.84$ and $\delta^{18}O_{c0} = 54.5‰$ adequately predicted $\delta^{18}O_R$ for leaves of high-light plants after the first 20 min (Fig. 3). During the first 20 min, the steady-state one-way flux models either over- or underestimated measured $\delta^{18}O_R$ for plants 1 and 2, respectively. While the net flux model adequately predicted $\delta^{18}O_R$ of droughted plants after 10 min in the dark, it failed to capture the rapid decline in $\delta^{18}O_R$ over the second 5 min, a decline that was partially modelled by the one-way flux model. However, both the net flux and one-way flux models (using $\theta = 0.84$ and $\delta^{18}O_{c0} = 54.5‰$) underestimated $\delta^{18}O_R$ for leaves of both low-light plants.

Assuming the one-way flux model to adequately model variation in $\delta^{18}O_R$ for plants from all growth environments, the model may be tuned to fit by varying either θ or $\delta^{18}O_{c0}$ while holding the other constant. To match observed $\delta^{18}O_R$ while assuming $\delta^{18}O_{c0} = 54.5‰$, θ was fitted to vary between 0.43 and 0.90 (Table 1; Fig. 4). The fitted values of θ tended to be lower for plants grown under low light, and higher for plants grown at high light (Table 1). Alternatively, if θ is assumed to be constant at 0.84, values for $\delta^{18}O_{c0}$ of between 52.6 and 82.0‰ were required to match modelled values of $\delta^{18}O_R$ to those measured.

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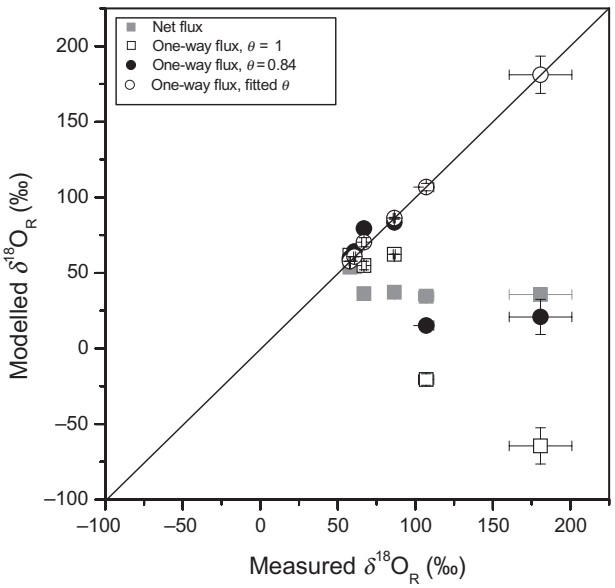

Figure 4. Modelled versus average measured variation in $\delta^{18}O$ of leaf-respired CO_2 of *Ricinus communis* leaves between 30 and 50 min after plants had been moved into the dark. Values are averages over the time period, and n is between 5 and 10. The models used are the net flux model (grey squares, Eqns 6 and 7), the one-way flux model with full equilibration (open squares, Eqns 1 and 7), the one-way flux model with $\theta = 0.84$ (black filled circles, Eqns 1 and 11) and the one-way flux model with fitted values for θ (open circles).

DISCUSSION

Utility and precision of the combined LI-6400-TDL system for $\delta^{18}O_R$ measurement

The SD of $\delta^{18}O$ measurements of CO_2 in air using the *TDL* (0.2‰) was higher than typically reported for traditional mass spectrometric analysis of pure trapped CO_2 (0.1‰; Cernusak *et al.* 2004) or using online mass spectrometric techniques (0.1‰, Cousins, Badger & von Caemmerer 2006b). The SD of individual $\delta^{18}O_R$ measurements calculated by Monte Carlo analysis varied between 1.1 and 5.1‰, depending on the difference in CO_2 mixing ratios between inlet and outlet air streams (as for estimates of $\delta^{13}C$ of leaf-respired CO_2; Barbour *et al.*, in press), but also on $\delta^{18}O_R$ itself (because $\delta^{18}O_R$ varied considerably). However, the high temporal resolution of measurements allowed multiple estimates of $\delta^{18}O_R$ thereby reducing uncertainty.

The *Ricinus communis* plants measured by Cernusak *et al.* (2004) were from the same seed source as those used in the current experiment, and were grown under similar glasshouse conditions as the well-watered and with high-light-grown plants in the current experiment, except the relative humidity in the current experiment was lower than the Cernusak experiment (25 compared to 40%, respectively). As in the Cernusak experiment, $\delta^{18}O_R$ was significantly more enriched than that predicted by the net flux model for the well-watered high-light plants (see Fig. 4). However, differences in the gas exchange measurement conditions between the two experiments resulted in generally lower air saturation deficits within the leaf chamber and hence lower stomatal conductances in the Cernusak experiment (Table 1). The lower stomatal conductances, combined with more enriched source water (–7.4‰ compared to –10‰ in the current experiment), resulted in more enriched water at the sites of evaporation within the leaf and hence much more enriched $\delta^{18}O_R$. Fitted values for θ were very similar between the Cernusak experiment and well-watered, high-light plants in the current experiment (Table 1), but fitted values for $\delta^{18}O_{c0}$ were higher for the current experiment than in the Cernusak *et al.* (2004) experiment (54.5 compared to 14.3‰, respectively). There is no clear explanation for this difference and further experimentation is required. However, despite differences in $\delta^{18}O_e$ and fitted $\delta^{18}O_{c0}$ between the experiments, overall, the data presented here suggest that the LI-6400 *TDL* measurement technique gives comparable isotope values to traditional mass spectrometric techniques.

We believe the combined LI-6400 *TDL* system has a number of advantages over mass spectrometric flask-based techniques, including high temporal resolution, real-time quality control of isotope data and ease of control of the chamber environment using the LI-6400. In addition, the system described here does not suffer from contamination of the isotopic signal by the interaction of O_2 and N_2 (producing NO_2) with a mass spectrometer source, as described by Cousins *et al.* (2006a). Hence, the LI-6400 *TDL* system allows experiments to be conducted at ambient O_2 partial pressures, unlike the online system described by Cousins *et al.* (2006b).

One improvement we recommend is concurrent measurement of $\delta^{18}O$ of water vapour entering and leaving the leaf chamber at the same temporal resolution as CO_2 measurement. This would allow parameterization of a non-steady-state leaf water enrichment model (e.g. Farquhar & Cernusak 2005). The low flow rate through the chamber excludes traditional cold-finger vapour trapping at relevant temporal resolutions, but *TDL*s capable of isotopic measurement of water vapour are available (Lee *et al.* 2005) and can be installed alongside the CO_2 laser in the *TDL*.

Applications of the technique in studies of leaf gas exchange

When combined with measurements of δ_v at appropriate temporal resolution, we foresee the high temporal resolution possible with the combined LI-6400 *TDL* technique having a number of potential applications in studies of leaf gas exchange. The first is in high temporal resolution studies of leaf internal conductance to CO_2 (g_i). Recent studies have pointed to previously unrecognized variability in g_i, and suggested that g_i may be quite dynamic as leaf environmental and biochemical conditions change (D.T. Hanson, unpublished data). Simultaneous measurement of the carbon and oxygen isotope compositions of CO_2 provides two estimates of g_i. Gillon & Yakir (2000a) found that the $^{18}O-g_i$ estimate was always lower than the $^{13}C-g_i$ estimate.

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They interpreted this difference in terms of the point at which CO_2 becomes equilibrated in ^{18}O with leaf water, which determines the $^{18}O-g_i$ estimate, compared to the average chloroplastic CO_2 concentration, which determines the $^{13}C - g_i$ estimate. Oxygen isotopic equilibration between CO_2 and leaf water is thought to occur at the evaporating sites within the mesophyll cell walls. Dual isotope measurements with the LI-6400 *TDL* would therefore allow partitioning of g_i into cell wall and chloroplast components at high temporal resolution in response to changing environmental conditions (e.g. ambient CO_2 concentration, light and temperature).

The second potential application of the LI-6400 *TDL* technique is for testing models of $\delta^{18}O_R$ under non-steady-state conditions. Leaf water in most terrestrial ecosystems is unlikely to be at isotopic steady state most of the time (Harwood *et al.* 1998; Lai *et al.* 2006), so accurate non-steady-state $\delta^{18}O_R$ models are required to interpret ecosystem $C^{18}O^{16}O$ flux data.

Finally, if the observation by Cernusak *et al.* (2004) that chloroplastic CO_2 is in full equilibrium with chloroplastic water during photosynthesis (i.e. θ is 1 in the light) is found to be generally the case for C_3 plants, measurements of changes in $\delta^{18}O$ of CO_2 moving across a photosynthesizing leaves may provide a simple way to monitor changes in Δ_e. Combined with measurements of Δ_L, the LI-6400 *TDL* system can be a powerful tool to test models of leaf water $H_2{}^{18}O$ enrichment models under non-steady-state conditions. If pathways of water movement through the leaf (i.e. through the apoplast versus aquaporins) influence the gradients in $H_2{}^{18}O$ within leaves, as suggested by Barbour & Farquhar (2003), then the system may also be useful in determining the importance of aquaporins to leaf hydraulics. Aquaporin transgenic plants would be particularly useful in this respect.

Modelling variability in $\delta^{18}O_R$

Temporal variability in $\delta^{18}O_R$ after plants were placed in the dark was as high as 100‰ within 10 min. To our knowledge, these are the first measurements of variation in $\delta^{18}O_R$ under non-steady-state conditions with a temporal resolution of minutes, and over a range in stomatal conductance of more than three orders of magnitude. Previously, Cernusak *et al.* (2004) measured $\delta^{18}O_R$ during *R. communis* leaf respiration at isotopic steady state and at moderate to high stomatal conductance, and Seibt *et al.* (2006; Seibt *et al.* in press) recently reported limited measurements of $\delta^{18}O_R$ over a number of nights under non-steady-state conditions for *Picea sitchensis* and *Fagas sylvatica* branches. As with these previous studies, the simple net flux model significantly underestimated $\delta^{18}O_R$ when stomata were open (Fig. 3).

When stomatal conductance was very low (i.e. < 0.005 mol m^{-2} s^{-1} for the two droughted plants), the net flux model predicted $\delta^{18}O_R$ to within 6‰. Such a small difference may be partly explained by the expectation that these leaves were not at isotopic steady state (so that Eqns 22 and 23 are not strictly valid), and will be explored. A 5‰ underestimation of δ_v [resulting from $\delta^{18}O$ of transpired water vapour (δ_E) being 26 and 33‰ more enriched than source water, for droughted plants 1 and 2, respectively] would result in only a 1‰ underestimation of δ_e. This error in δ_e results in the fitted values for θ being overestimated by 0.08 and 0.07 for droughted plants 1 and 2, respectively. If δ_E were actually lower than source water (by the same amount as previously described), δ_e would be underestimated by 1‰, and fitted values for θ would be 0.09 and 0.10 higher than calculated using the assumption of isotopic steady state. That is, errors in δ_E as large as 33‰ only result in errors of up to 1‰ in water at the evaporating sites in leaves with very low stomatal conductance. Even with such large errors in δ_E, the fitted values for θ are still significantly lower than unity, reinforcing the need to use the one-way flux model rather than the net flux model. However, the application of a combination of a one-way flux model and a non-steady-state leaf water enrichment model would be desirable.

For well-watered plants, the comparison between measured and modelled $\delta^{18}O_R$ showed that CO_2 was not in full equilibrium with chloroplast water, despite the relationship between calculated $\delta^{18}O$ of chloroplast CO_2 and estimated $\delta^{18}O$ of chloroplast water sitting close to that expected for full equilibration. The one-way flux model allowed more accurate prediction of variability in $\delta^{18}O_R$ than the net flux model for plants grown under high light, when time was allowed for leaf water to turn over and assumptions inherent in Eqns 22 and 23 to be valid (Fig. 3). However, measured $\delta^{18}O_R$ for the plants grown under low light did not match model predictions when single values for θ and $\delta^{18}O_{c0}$ were assumed. Assuming the one-way flux model to be adequate, the model may be tuned to fit observed values by fitting either a lower value for θ or a higher value for $\delta^{18}O_{c0}$ for plants grown under low light.

Variable θ or variable $\delta^{18}O_{c0}$?

The model predictions suggest that either θ or $\delta^{18}O_{c0}$ varied, with lower values of θ or higher values of $\delta^{18}O_{c0}$ for plants grown under low light. θ, the proportion of chloroplastic (or mitochondrial) CO_2 in isotopic equilibrium with water at the evaporating sites, depends on the number of hydration reactions achieved per CO_2 molecule ($k\tau$), which depends, in turn, on the ratio of carbonic anhydrase activity to the residence time of CO_2 in the leaf. Formally, Gillon & Yakir (2000a,b) defined θ as (after Mills & Urey 1940)

$$\theta = 1 - e^{-\frac{k\tau}{3}}, \tag{25}$$

and

$$k\tau = \frac{CA_{\text{leaf}}}{F}, \tag{26}$$

where CA_{leaf} is the rate of carbonic anhydrase activity *in vivo* (μmol m^{-2} s^{-1}) at T_l, and F is the gross flux of CO_2 into (in the case of net photosynthesis) or out of (in the case of

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net respiration) the leaf. For the current (respiring) leaves, F is given by the product of the CO_2 mole fraction at the chloroplasts or mitochondria, C_c, and the total conductance to CO_2 from the chloroplasts and mitochondria to the leaf chamber. Thus, F is low for droughted plants (2.8 and 1.2 μmol m^{-2} s^{-1} for plants 1 and 2, respectively) when stomatal conductance is low, and higher for well-watered plants (between 54.7 and 77.9 μmol m^{-2} s^{-1}). Using the fitted values of θ and calculated F for each leaf, CA_{leaf} was estimated to be around 30 μmol m^{-2} s^{-1} for droughted plants, 1400 μmol m^{-2} s^{-1} for high-light plants and 340 μmol m^{-2} s^{-1} for low-light plants. Among C_3 plants, CA_{leaf} is reported to vary between 45 μmol m^{-2} s^{-1} (*Phragmites australis*; Gillon & Yakir 2000b) and 4412 μmol m^{-2} s^{-1} (*Nerium oleander*; Gillon & Yakir 2001), and by as much as 70 μmol m^{-2} s^{-1} along a single C_4 leaf (*Zea mays*; Affek, Krisch & Yakir 2005). CA_{leaf} was not measured for the current leaves, but it seems possible that variation in CA_{leaf} between growth environments may partly explain the observed variation in fitted θ.

However, without measurements of CA_{leaf}, we are unable to distinguish variability in θ from variability in $\delta^{18}O_{c0}$. The one-way flux model also predicted $\delta^{18}O_R$ accurately if θ was held constant at 0.84, but $\delta^{18}O_{c0}$ was allowed to vary. Values for $\delta^{18}O_{c0}$ of between 52.6 and 59.7‰ for high-light plants, and 70.0 and 82.0‰ for low-light plants 1 and 2, respectively, were required to match modelled to measured $\delta^{18}O_R$. Gillon & Yakir (2000b) suggested that $\delta^{18}O_{c0}$ may be estimated by

$$\delta^{18}O_{c0} = \delta^{18}O_a - \bar{a}\left(1 - \frac{C_c}{C_a}\right), \tag{27}$$

or, for a respiring leaf,

$$\delta^{18}O_{c0} = \delta^{18}O_a - \bar{a}\left(1 - \frac{C_a}{C_c}\right). \tag{28}$$

Using measured $\delta^{18}O_a$ and C_a, and modelled $\bar{a}$ and C_c, $\delta^{18}O_{c0}$ may be calculated from Eqn 28 to be 76.3‰ for droughted plants, 44.6‰ for high-light plants and 46.3‰ for low-light plants, that is, less enriched than the estimate of $\delta^{18}O_{c0}$ from regression and fitting analysis. Further, $\delta^{18}O_{c0}$ for low-light plants is only 1.7‰ more enriched than high-light plants, rather than about 20‰ more enriched from the fitting analysis.

As Cernusak *et al.* (2004) point out, there are three possible sources for the oxygen in CO_2 evolved in the dark: atmospheric O_2, oxygen from leaf water and organic oxygen from respiratory substrates. Respired CO_2 with an oxygen atom from O_2 is expected to have an isotopic composition of between 0 and 5‰, and should not vary between leaves, assuming O_2 is 23.5‰, and discrimination against $^{18}O^{16}O$ during respiration is between 17 and 26‰ (Guy *et al.* 1992). Although not measured, leaf water enrichment is predicted to be slightly lower in low-light plants compared with high-light plants, that is, in the opposite direction from fitted variation in $\delta^{18}O_{c0}$. Organic oxygen from respiratory substrates should reflect leaf water enrichment during synthesis of the substrates, with a 27‰ enrichment (Cernusak *et al.* 2003). If there are differences in leaf water enrichment between high- and low-light plants during respiratory substrate synthesis, these are likely to be small and if anything, low-light plants should have slightly depleted leaf water than high-light plants because of lower leaf temperatures. So, none of the sources of mitochondrial oxygen can explain the higher fitted $\delta^{18}O_{c0}$ values at low light.

However, Cernusak *et al.* (2004) also point out that $\delta^{18}O_{c0}$ is dependent on $\delta^{18}O_a$. $\delta^{18}O_a$ was 2‰ more enriched, on average, for low-light plants than either droughted or well-watered high-light plants. By combining Eqns 4 and 24, we obtain

$$\delta^{18}O_{c0} = \frac{\left[\delta^{18}O_R(1+\bar{a})\left(1-\frac{C_a}{C_c}\right)+\frac{C_a}{C_c}(\delta^{18}O_a-\bar{a})+\bar{a}\right]-\delta^{18}O_e\theta(1+\varepsilon_w)-\theta\varepsilon_w}{1-\theta}, \tag{29}$$

from which the sensitivity of $\delta^{18}O_{c0}$ to $\delta^{18}O_a$ may be assessed. A 2‰ enrichment in $\delta^{18}O_a$ results in an 11‰ enrichment in calculated $\delta^{18}O_{c0}$ when θ is 0.84, and all other parameters are held constant at measured and calculated values for plant 1 under high light (note that for this sensitivity analysis, $\delta^{18}O_R$ is assumed constant, which is unrealistic). Therefore, it seems possible that $\delta^{18}O_{c0}$ varied between high- and low-light plants partly because of variation in $\delta^{18}O_a$.

In summary, the one-way flux modelled $\delta^{18}O_R$ to fit observed values for plants from all growth environments and either θ was lower or $\delta^{18}O_{c0}$ was higher for low-light plants than for those grown under high light. Unfortunately, without measurements of CA_{leaf}, we are unable to distinguish between the two. More measurements of $\delta^{18}O_R$ and CA_{leaf} from plants grown under a range of conditions are recommended.

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