

# Measurement and Interpretation of the Oxygen Isotope Composition of Carbon Dioxide Respired by Leaves in the Dark

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We measured the oxygen isotope composition ( $\delta^{18}\text{O}$ ) of  $\text{CO}_2$  respired by *Ricinus communis* leaves in the dark. Experiments were conducted at low  $\text{CO}_2$  partial pressure and at normal atmospheric  $\text{CO}_2$  partial pressure. Across both experiments, the  $\delta^{18}\text{O}$  of dark-respired  $\text{CO}_2$  ( $\delta_{\text{R}}$ ) ranged from 44‰ to 324‰ (Vienna Standard Mean Ocean Water scale). This seemingly implausible range of values reflects the large flux of  $\text{CO}_2$  that diffuses into leaves, equilibrates with leaf water via the catalytic activity of carbonic anhydrase, then diffuses out of the leaf, leaving the net  $\text{CO}_2$  efflux rate unaltered. The impact of this process on  $\delta_{\text{R}}$  is modulated by the  $\delta^{18}\text{O}$  difference between  $\text{CO}_2$  inside the leaf and in the air, and by variation in the  $\text{CO}_2$  partial pressure inside the leaf relative to that in the air. We developed theoretical equations to calculate  $\delta^{18}\text{O}$  of  $\text{CO}_2$  in leaf chloroplasts ( $\delta_{\text{c}}$ ), the assumed location of carbonic anhydrase activity, during dark respiration. Their application led to sensible estimates of  $\delta_{\text{c}}$ , suggesting that the theory adequately accounted for the labeling of  $\text{CO}_2$  by leaf water in excess of that expected from the net  $\text{CO}_2$  efflux. The  $\delta_{\text{c}}$  values were strongly correlated with  $\delta^{18}\text{O}$  of water at the evaporative sites within leaves. We estimated that approximately 80% of  $\text{CO}_2$  in chloroplasts had completely exchanged oxygen atoms with chloroplast water during dark respiration, whereas approximately 100% had exchanged during photosynthesis. Incorporation of the  $\delta^{18}\text{O}$  of leaf dark respiration into ecosystem and global scale models of  $\text{C}^{18}\text{OO}$  dynamics could affect model outputs and their interpretation.

Variations in the oxygen isotope composition ( $\delta^{18}\text{O}$ ) of  $\text{CO}_2$  in the atmosphere have the potential to reveal vital information about the global carbon cycle (Francey and Tans, 1987; Farquhar et al., 1993; Ciais et al., 1997). Furthermore, measurements of oxygen isotope composition of  $\text{CO}_2$  in canopy air may allow differentiation of  $\text{CO}_2$  fluxes into photosynthetic and respiratory components (Yakir and Wang, 1996). It was also recently suggested that nighttime measurements of  $\delta^{18}\text{O}$  in canopy air could be used to partition nocturnal ecosystem respiration between leaves and soil (Bowling et al., 2003a, 2003b). Leaf dark respiration is an important component of carbon cycling between vegetation and the atmosphere. An understanding of the factors controlling the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  respired by leaves in the dark could therefore be important for interpreting the  $\delta^{18}\text{O}$  of atmospheric  $\text{CO}_2$  at local, regional, and global scales.

The net rate of  $\text{CO}_2$  efflux from a leaf in the dark can be thought of as the difference between two one-way diffusional fluxes, one from the atmosphere to the leaf and the other from the leaf to the atmosphere. For example, if the net respiratory  $\text{CO}_2$  efflux ( $\mathfrak{R}_{\text{n}}$ ) is defined as  $\mathfrak{R}_{\text{n}} = g_{\text{c}}(c_{\text{i}} - c_{\text{a}})$ , where  $g_{\text{c}}$  is the leaf

conductance to  $\text{CO}_2$ , and  $c_{\text{i}}$  and  $c_{\text{a}}$  are  $\text{CO}_2$  mole fractions in the intercellular air spaces and atmosphere, respectively, the one-way flux from leaf to atmosphere becomes  $g_{\text{c}}c_{\text{i}}$  and that from atmosphere to leaf becomes  $g_{\text{c}}c_{\text{a}}$ . The difference between  $\mathfrak{R}_{\text{n}}$  and  $g_{\text{c}}c_{\text{i}}$  will depend on the magnitude of the  $\text{CO}_2$  concentration difference between  $c_{\text{i}}$  and  $c_{\text{a}}$ ; this difference will in turn depend on the leaf conductance to  $\text{CO}_2$  and the  $\text{CO}_2$  production rate inside the leaf. If the  $\text{CO}_2$  concentration difference between  $c_{\text{i}}$  and  $c_{\text{a}}$  is very large, then the magnitude of the net  $\text{CO}_2$  efflux will approach that of the one-way  $\text{CO}_2$  efflux from leaf to atmosphere. However, if the  $\text{CO}_2$  concentration inside the leaf is only a little larger than that in the atmosphere, the net  $\text{CO}_2$  efflux from the leaf will be much smaller than the one-way  $\text{CO}_2$  efflux from the leaf.

It has previously been recognized that one of the primary controls over the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  diffusing out of leaves in the dark should be the  $\delta^{18}\text{O}$  of leaf water (Flanagan et al., 1997, 1999). This is because gaseous  $\text{CO}_2$  exchanges oxygen atoms with water during interconversion between  $\text{CO}_2$  and bicarbonate. In plant tissues, this interconversion is catalyzed by the enzyme carbonic anhydrase. The rate constant for carbonic anhydrase is very fast, such that  $\text{CO}_2$  diffusing out of leaves is expected to reflect nearly complete oxygen isotope exchange with leaf water. There is an equilibrium fractionation that takes place during the exchange reaction, such that at 25°C, the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  will be enriched by approximately 41‰ compared to the  $\delta^{18}\text{O}$  of water with which it has equilibrated.

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In this article, we present measurements of the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  respired by *Ricinus communis* leaves in the dark. We theorized that it should be the one-way flux of  $\text{CO}_2$  out of a respiring leaf that is labeled with the leaf water  $\delta^{18}\text{O}$  signal, rather than the net  $\text{CO}_2$  efflux. This led us to hypothesize that the effect of a respiring leaf on the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  in air passing over the leaf could be much greater than predicted by considering the net  $\text{CO}_2$  efflux alone.

## THEORY

### Interpretation of the Oxygen Isotope Composition of Dark-Respired $\text{CO}_2$

Natural abundance oxygen isotope ratios are commonly expressed relative to the value of a standard:

$$\delta_X = \frac{R_X}{R_{\text{Std}}} - 1, \quad (1)$$

where  $\delta_X$  represents the proportional deviation of  $R_X$ , the  $^{18}\text{O}/^{16}\text{O}$  of material  $X$ , from  $R_{\text{Std}}$ , the  $^{18}\text{O}/^{16}\text{O}$  of a standard. Using  $\delta$  notation, we present the following equation for the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  respired by leaves in the dark ( $\delta_{\text{R}}$ ):

$$\delta_{\text{R}} = \frac{\theta[\delta_e(1 + \varepsilon_w) + \varepsilon_w] + (1 - \theta)\delta_{\text{c0}} - \frac{C_a}{C_c}(\delta_a - \bar{a}) - \bar{a}}{(1 + \bar{a})\left(1 - \frac{C_a}{C_c}\right)}, \quad (2)$$

where  $\theta$  is the proportion of  $\text{CO}_2$  in the chloroplast that has completely exchanged oxygen atoms with chloroplast water,  $\delta_e$  is the oxygen isotope composition of water at the evaporating sites within the leaf,  $\varepsilon_w$  is the equilibrium fractionation between water and  $\text{CO}_2$ ,  $\delta_{\text{c0}}$  is the oxygen isotope composition of  $\text{CO}_2$  in the chloroplast that has not exchanged oxygen atoms with chloroplast water,  $C_a$  is the ambient carbon dioxide partial pressure,  $C_c$  is the chloroplastic  $\text{CO}_2$  partial pressure,  $\delta_a$  is the oxygen isotope composition of ambient  $\text{CO}_2$ , and  $\bar{a}$  is the weighted mean isotopic discrimination against  $\text{C}^{18}\text{O}$  during diffusion from the chloroplast to the atmosphere. A summary of all symbols used in the text is given in Table I. A derivation of Equation 2 is presented (see "Derivation 1" in text). As described for photosynthesizing leaves by Gillon and Yakir (2000b), we make the assumption that  $\text{CO}_2$  inside the leaf comprises a mixture of  $\text{CO}_2$  completely equilibrated with leaf water (of proportion  $\theta$ ) and  $\text{CO}_2$  that has undergone no equilibration with leaf water (of proportion  $1 - \theta$ ). We further assume that chloroplasts are appressed against intercellular air spaces in the mesophyll cells (Evans and von Caemmerer, 1996), such that  $\text{CO}_2$  evolved from mitochondria interacts with chloroplasts during diffusion out of the cells. Because carbonic anhydrase resides primarily in chloroplasts in  $\text{C}_3$  leaves (Everson, 1970; Jacobson et al., 1975;

Tsuzuki et al., 1985), the chloroplastic  $\text{CO}_2$  concentration becomes the relevant parameter for modeling  $\delta_{\text{R}}$ .

The diffusional discrimination,  $\bar{a}$ , can be calculated as (Farquhar and 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  $C_i$  is the  $\text{CO}_2$  partial pressure in the intercellular air spaces, and  $C_s$  is that at the leaf surface. The term  $a_w$  describes the summed discrimination against  $\text{C}^{18}\text{O}$  during liquid-phase diffusion and dissolution (0.8‰);  $a$  is the discrimination during diffusion through the stomata (8.8‰); and  $a_b$  is the discrimination during diffusion through the leaf boundary layer (5.8‰). We note that Equation 3 is precisely the same as the equation given for  $\bar{a}$  by Farquhar and Lloyd (1993); we have simply multiplied both their numerator and denominator by  $-1$ . The equilibrium fractionation between water and  $\text{CO}_2$  can be calculated as (Brenninkmeijer et al., 1983)

$$\varepsilon_w(\text{‰}) = \frac{17604}{T} - 17.93, \quad (4)$$

where  $T$  is leaf temperature in K.

The oxygen isotope composition of  $\text{CO}_2$  in the chloroplast of a respiring leaf ( $\delta_c$ ) can be calculated from the following equation:

$$\delta_c = \delta_{\text{R}}(1 + \bar{a})\left(1 - \frac{C_a}{C_c}\right) + \frac{C_a}{C_c}(\delta_a - \bar{a}) + \bar{a}; \quad (5)$$

a derivation of equation 5 is presented (see "Derivation 1" in text). Equations 23 and 24 can be combined, and, after dividing through by  $R_{\text{Std}}$ , give

$$\delta_c = \delta_e\theta(1 + \varepsilon_w) + \theta\varepsilon_w + \delta_{\text{c0}}(1 - \theta). \quad (6)$$

For a series of measurements made at different values of  $\delta_e$ ,  $\delta_c$  can be calculated from Equation 5 and plotted against  $\delta_e$ . According to Equation 6, the slope of the relationship between  $\delta_c$  and  $\delta_e$  ( $m$ ) is then equal to  $\theta(1 + \varepsilon_w)$ , such that  $\theta$  can be calculated as  $\theta = m/(1 + \varepsilon_w)$ . The intercept of the relationship,  $I$ , is equal to  $\theta\varepsilon_w + \delta_{\text{c0}}(1 - \theta)$ , such that  $\delta_{\text{c0}}$  can be calculated as  $\delta_{\text{c0}} = (I - \theta\varepsilon_w)/(1 - \theta)$ . We note that such an analysis assumes that only  $\delta_e$  varies across the series of measurements; thus,  $\theta$ ,  $\varepsilon_w$  and  $\delta_{\text{c0}}$  are assumed invariant.

The oxygen isotope enrichment at the evaporative sites in leaves ( $\Delta_e$ ) can be calculated as (Craig and Gordon, 1965; Dongmann et al., 1974; Farquhar and Lloyd, 1993)

$$\Delta_e = \varepsilon^+ + \varepsilon_k + (\Delta_v - \varepsilon_k)\frac{e_a}{e_i}, \quad (7)$$

where  $\varepsilon^+$  is the equilibrium fractionation that occurs during the phase change from liquid to vapor,  $\varepsilon_k$  is the kinetic fractionation that occurs during diffusion of vapor from the leaf intercellular air space to the atmosphere,  $\Delta_v$  is the isotopic enrichment of vapor in

**Table 1.** Symbols used in text

$A$	Net photosynthesis rate
$\bar{a}$	Weighted mean discrimination against $C^{18}OO$ for diffusion from chloroplast to atmosphere
$a$	Discrimination against $C^{18}OO$ during diffusion through stomata
$a_b$	Discrimination against $C^{18}OO$ during diffusion through leaf boundary layer
$a_w$	Summed discriminations against $C^{18}OO$ during liquid phase diffusion and dissolution
$a^{13}$	Discrimination against $^{13}CO_2$ during diffusion through stomata
$a_b^{13}$	Discrimination against $^{13}CO_2$ during diffusion through leaf boundary layer
$a_w^{13}$	Summed discrimination against $^{13}CO_2$ during dissolution and liquid phase diffusion
$\alpha_w$	Equilibrium oxygen isotope effect between $CO_2$ and water
$b$	Discrimination against $^{13}CO_2$ by carboxylating enzymes
$b^{18}$	Discrimination against $C^{18}OO$ by Rubisco
$C$	Molar concentration of water
$C_a$	Partial pressure of $CO_2$ in atmosphere
$C_c$	Partial pressure of $CO_2$ in chloroplast
$C_{cs}$	Partial pressure of $CO_2$ at the chloroplast surface
$C_i$	Partial pressure of $CO_2$ in leaf intercellular air spaces
$C_{in}$	Partial pressure of $CO_2$ in dry air entering leaf chamber
$C_s$	Partial pressure of $CO_2$ at the leaf surface
$c_i$	Mole fraction of $CO_2$ in intercellular air spaces
$c_a$	Mole fraction of $CO_2$ in atmosphere
$D$	Diffusivity of $H_2^{18}O$ in water
$\Delta_A$	Discrimination against $^{13}C$ or $^{18}O$ during net $CO_2$ uptake by photosynthesis
$\Delta_{ca}$	$^{18}O$ enrichment of $CO_2$ in chloroplast compared to atmosphere
$\Delta_e$	$^{18}O$ enrichment at evaporative sites in leaves compared to source water
$\Delta_{ea}$	$^{18}O$ enrichment of $CO_2$ in chloroplast compared to atmosphere when chloroplast $CO_2$ is in full equilibrium with chloroplast water
$\Delta_i$	Discrimination against $^{13}CO_2$ that would occur if $g_i$ were infinite and photorespiration and day respiration did not discriminate
$\Delta_L$	$^{18}O$ enrichment of average lamina leaf water compared to source water
$\Delta_{obs}$	Observed discrimination against $^{13}CO_2$ during photosynthesis
$\Delta_v$	$^{18}O$ enrichment of vapor in atmosphere compared to source water
$\delta_A$	$\delta^{18}O$ of $CO_2$ taken up by net photosynthesis (VSMOW scale)
$\delta_a$	$\delta^{18}O$ of $CO_2$ in atmosphere (VSMOW scale)
$\delta_c$	$\delta^{18}O$ of $CO_2$ in chloroplast (VSMOW scale)
$\delta_{c0}$	$\delta^{18}O$ of $CO_2$ in chloroplast that has not equilibrated with chloroplast water (VSMOW scale)
$\delta_e$	$\delta^{18}O$ of water at evaporative sites in leaves (VSMOW scale)
$\delta_{in}$	$\delta^{18}O$ of $CO_2$ in air entering leaf chamber (VSMOW scale)
$\delta_L$	$\delta^{18}O$ of average lamina leaf water (VSMOW scale)
$\delta_R$	$\delta^{18}O$ of $CO_2$ efflux during dark respiration (VSMOW scale)
$\delta_s$	$\delta^{18}O$ of source water (VSMOW scale)
$E$	Transpiration rate
$e$	Discrimination against $^{13}C$ during day respiration
$e_a$	Vapor pressure in atmosphere
$e_i$	Vapor pressure in leaf intercellular air spaces
$\epsilon^+$	Equilibrium $^{18}O$ fractionation between liquid water and vapor
$\epsilon_k$	Kinetic fractionation during diffusion of $H_2^{18}O$ from leaf intercellular air spaces to atmosphere
$\epsilon_w$	Equilibrium $^{18}O$ fractionation between $CO_2$ and water
$f$	Discrimination against $^{13}C$ during photorespiration
$g_t$	Conductance to $H_2O$ from leaf intercellular air space to atmosphere
$g_i$	Conductance to $CO_2$ from leaf intercellular air spaces to sites of carboxylation
$g_{tc}$	Conductance to $CO_2$ from chloroplast to atmosphere
$\Gamma_*$	$CO_2$ compensation point in absence of day respiration
$k$	Carboxylation efficiency
$L$	Scaled effective path length for calculation of $\phi$
$\Lambda$	Area of leaf in leaf chamber

(Table continues on following page.)

**Table 1.** (Continued from previous page.)

$m^{13}$	Slope of the relationship between $\Delta_i - \Delta_{\text{obs}}$ and $A/C_a$
$m$	Slope of the relationship between $\delta_c$ and $\delta_e$
$n$	Number of measurements in each experiment
$\rho$	Péclet number
$\theta$	Proportion of chloroplast CO <sub>2</sub> isotopically equilibrated with chloroplast water
$P$	Atmospheric pressure
$R_A$	<sup>13</sup> C/ <sup>12</sup> C or <sup>18</sup> O/ <sup>16</sup> O of net CO <sub>2</sub> uptake by photosynthesis
$R_a$	<sup>13</sup> C/ <sup>12</sup> C or <sup>18</sup> O/ <sup>16</sup> O of CO <sub>2</sub> in atmosphere
$R_{ce}$	<sup>18</sup> O/ <sup>16</sup> O of CO <sub>2</sub> in equilibrium with chloroplast water
$R_{c0}$	<sup>18</sup> O/ <sup>16</sup> O of chloroplast CO <sub>2</sub> that has not equilibrated with chloroplast water
$R_e$	<sup>18</sup> O/ <sup>16</sup> O of water at evaporative sites in leaves
$R_R$	<sup>18</sup> O/ <sup>16</sup> O of net CO <sub>2</sub> efflux during dark respiration
$R_{\text{Std}}$	<sup>18</sup> O/ <sup>16</sup> O of VSMOW standard
$r_s$	Stomatal resistance to water vapor diffusion
$r_b$	Leaf boundary layer resistance to water vapor diffusion
$\mathfrak{R}_d$	Day respiration rate
$\mathfrak{R}_n$	Net CO <sub>2</sub> efflux rate during dark respiration
$\rho$	Ratio of rates of carboxylation and CO <sub>2</sub> hydration in chloroplast
$T_{\text{leaf}}$	Leaf temperature
$u$	Flow rate of air through leaf chamber
$w_i$	Mole fraction of water vapor in the leaf intercellular air spaces
$W$	Leaf lamina water concentration

the atmosphere, and  $e_a/e_i$  is the ratio of ambient to intercellular vapor pressures. The  $\Delta_e$  and  $\Delta_v$  are defined with respect to source water, such that  $\Delta_e = R_e/R_s - 1$  and  $\Delta_v = R_v/R_s - 1$ , where  $R_e$  is <sup>18</sup>O/<sup>16</sup>O of water at the evaporating sites,  $R_s$  is <sup>18</sup>O/<sup>16</sup>O of source water, and  $R_v$  is <sup>18</sup>O/<sup>16</sup>O of vapor in the atmosphere. The term  $\delta_e$  can be calculated from  $\Delta_e$  as

$$\delta_e = \Delta_e(1 + \delta_s) + \delta_s, \quad (8)$$

where  $\delta_s$  is the oxygen isotope composition of source water relative to a standard. The parameter  $\Delta_v$  in Equation 6 can be calculated from measurements of the oxygen isotope composition of vapor in the atmosphere ( $\delta_v$ ) and source water as  $\Delta_v = (\delta_v - \delta_s)/(1 + \delta_s)$ . The equilibrium fractionation between liquid and vapor,  $\varepsilon^+$ , can be calculated as (Bottinga and Craig, 1969)

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

where  $T$  is leaf temperature in K. The kinetic fractionation,  $\varepsilon_k$ , can be calculated as (Farquhar et al., 1989)

$$\varepsilon_k (\text{‰}) = \frac{32r_s + 21r_b}{r_s + r_b}, \quad (10)$$

where  $r_s$  and  $r_b$  are the stomatal and boundary layer resistances to water vapor diffusion ( $\text{m}^2 \text{s mol}^{-1}$ ), and 32 and 21 are associated fractionation factors scaled to per mil. These fractionation factors have been revised up from values of 28 and 19, respectively, based on recent measurements showing the isotope effect for diffusion of H<sub>2</sub><sup>18</sup>O in air to be 1.032 (Cappa et al., 2003), rather than 1.028 (Merlivat, 1978).

### Measurement of the Oxygen Isotope Composition of Dark-Respired CO<sub>2</sub>

For our first dark respiration experiment, in which air entering the leaf chamber was free of CO<sub>2</sub>, we calculated the oxygen isotope composition of respired CO<sub>2</sub>,  $\delta_R$ , simply as the oxygen isotope composition of CO<sub>2</sub> exiting the chamber,  $\delta_a$ . In our second dark respiration experiment, where air entering the leaf chamber had a CO<sub>2</sub> concentration sufficient to bring that inside the chamber close to that normally found in the atmosphere, we calculated  $\delta_R$  with a modified form of the equation presented previously by Evans et al. (1986):

$$\delta_R = \frac{C_a \delta_a - C_{\text{in}} \delta_{\text{in}}}{C_a - C_{\text{in}}}, \quad (11)$$

where  $C_a$  is the CO<sub>2</sub> partial pressure ( $\mu\text{bar}$ ) of air within the chamber when dried,  $\delta_a$  is  $\delta^{18}\text{O}$  of CO<sub>2</sub> within the chamber,  $C_{\text{in}}$  is the CO<sub>2</sub> partial pressure ( $\mu\text{bar}$ ) of dry air entering the chamber, and  $\delta_{\text{in}}$  is the  $\delta^{18}\text{O}$  of CO<sub>2</sub> entering the chamber. A derivation of Equation 11 is provided (see "Derivation 2" in text). The terms  $C_a$  and  $\delta_a$  are measured in gas exiting the leaf chamber, due to effective stirring of air within the chamber.

### Calculation of Photosynthetic Discrimination against <sup>13</sup>C and <sup>18</sup>O

For measurements in the light, we calculated carbon and oxygen isotope discrimination during photosynthesis as described by Evans et al. (1986):

$$\Delta_A = \frac{R_a}{R_A} - 1 = \frac{\xi(\delta_a - \delta_{\text{in}})}{1 + \delta_a - \xi(\delta_a - \delta_{\text{in}})}, \quad (12)$$

where  $R_a$  is  $^{13}\text{C}/^{12}\text{C}$  or  $^{18}\text{O}/^{16}\text{O}$  of  $\text{CO}_2$  within the leaf chamber,  $R_A$  is  $^{13}\text{C}/^{12}\text{C}$  or  $^{18}\text{O}/^{16}\text{O}$  of  $\text{CO}_2$  removed from the chamber by photosynthesis,  $\delta_a$  is  $\delta^{13}\text{C}$  or  $\delta^{18}\text{O}$  of  $\text{CO}_2$  within the leaf chamber,  $\delta_{\text{in}}$  is  $\delta^{13}\text{C}$  or  $\delta^{18}\text{O}$  of  $\text{CO}_2$  entering the chamber, and  $\xi$  is defined as  $C_{\text{in}}/(C_{\text{in}} - C_a)$ , where  $C_{\text{in}}$  and  $C_a$  refer to  $\text{CO}_2$  partial pressures in dry air. We calculated the oxygen isotope composition of chloroplast  $\text{CO}_2$  during photosynthesis by rearranging the  $\text{C}^{18}\text{OO}$  discrimination equation presented by Farquhar and Lloyd (1993):

$$\Delta_{\text{ca}} = \frac{\Delta_A - \bar{a}}{(1 + \Delta_A) \left( \frac{C_c}{C_a - C_c} \right)}, \quad (13)$$

where  $\Delta_A$  is discrimination against  $\text{C}^{18}\text{OO}$  during photosynthesis, as defined above, and  $\Delta_{\text{ca}}$  is defined as  $(R_c/R_a) - 1$ , where  $R_c$  is  $^{18}\text{O}/^{16}\text{O}$  of chloroplast  $\text{CO}_2$ . We then calculated  $\delta_c$  as  $\delta_c = \Delta_{\text{ca}}(1 + \delta_a) + \delta_a$ .

For the photosynthesis measurements that comprised our third experiment, we compared the regression approach to calculating  $\theta$ , as described above in the theory relating to dark respiration, to the method suggested by Gillon and Yakir (2000b), whereby  $\theta$  can be calculated separately for each individual photosynthesis measurement:

$$\theta = \frac{\Delta_{\text{ca}} + \bar{a} \left( 1 - \frac{C_c}{C_a} \right)}{\Delta_{\text{ea}} + \bar{a} \left( 1 - \frac{C_c}{C_a} \right)}, \quad (14)$$

where  $\Delta_{\text{ea}}$  is the value of  $\Delta_{\text{ca}}$  expected if chloroplastic  $\text{CO}_2$  were in full oxygen isotope equilibrium with  $\delta_e$ . The  $\Delta_{\text{ea}}$  was calculated as

$$\Delta_{\text{ea}} = \frac{\delta_e(1 + \varepsilon_w) + \varepsilon_w - \delta_a}{1 + \delta_a}. \quad (15)$$

Equation 14 incorporates an assumption that is not applied in the regression approach to calculating  $\theta$  that we described above for dark respiration. The assumption is that the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  in the chloroplast that has not equilibrated with leaf water can be calculated from the equation  $R_{\text{c0}} = R_a[1 - \bar{a}(1 - C_c/C_a)]$  (Gillon and Yakir, 2000b), which can be replaced, to a close approximation, by  $\delta_{\text{c0}} = \delta_a - \bar{a}(1 - C_c/C_a)$ . Defining  $\delta_{\text{c0}}$  in this way assumes no discrimination against  $\text{C}^{18}\text{OO}$  by Rubisco; it also ignores any possible effect of photorespiration or day respiration on  $\delta_{\text{c0}}$ .

#### Calculation of the Conductance from $C_i$ to $C_c$

The  $\text{CO}_2$  conductance from leaf intercellular air spaces to the sites of carboxylation in chloroplasts ( $g_i$ ) was calculated from  $^{13}\text{C}$  discrimination measurements during photosynthesis using the method of Evans et al. (1986):

$$\Delta_i - \Delta_{\text{obs}} = \frac{(b - a_w^{13})}{g_i} \left( \frac{A}{C_a} \right) - \frac{e\mathfrak{R}_d + f\Gamma_*}{C_a}, \quad (16)$$

where  $\Delta_{\text{obs}}$  is the observed  $^{13}\text{C}$  discrimination,  $b$  is the discrimination against  $^{13}\text{CO}_2$  during carboxylation (taken as 29‰),  $a_w^{13}$  is the sum of discriminations against  $^{13}\text{CO}_2$  during dissolution of  $\text{CO}_2$  and liquid phase diffusion (1.8‰),  $A$  is the net photosynthetic rate ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ),  $C_a$  is the ambient  $\text{CO}_2$  partial pressure ( $\mu\text{bar}$ ),  $\mathfrak{R}_d$  is day respiration ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ),  $e$  is the associated discrimination against  $^{13}\text{CO}_2$ ,  $k$  is the carboxylation efficiency ( $\text{mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ ),  $\Gamma_*$  is the  $\text{CO}_2$  compensation point in the absence of  $\mathfrak{R}_d$  ( $\mu\text{bar}$ ), and  $f$  is the discrimination against  $^{13}\text{CO}_2$  associated with photorespiration. The term  $\Delta_i$  represents the discrimination that would occur if  $g_i$  were infinite, and if photorespiration and day respiration did not discriminate (Farquhar et al., 1982):

$$\Delta_i = a_b^{13} \left( \frac{C_a - C_s}{C_a} \right) + a^{13} \left( \frac{C_s - C_i}{C_a} \right) + b \left( \frac{C_i}{C_a} \right), \quad (17)$$

where  $a_b^{13}$  is the discrimination against  $^{13}\text{CO}_2$  during diffusion through the boundary layer (2.8‰),  $C_s$  is the  $\text{CO}_2$  partial pressure at the leaf surface, and  $a^{13}$  is the discrimination against  $^{13}\text{CO}_2$  during diffusion through the stomata (4.4‰). The term  $(b - a_w^{13})/g_i$  was calculated from the slope,  $m^{13}$ , of a plot of  $\Delta_i - \Delta_{\text{obs}}$  against  $A/C_a$ . The term  $g_i$  was then calculated as  $(b - a_w^{13})/m^{13}$ . The value of  $m^{13}$  is independent of values assigned to  $f$  and  $e$  in Equation 16 because varying these parameters affects the intercept of the regression rather than the slope. Therefore, there is no need to assign values to  $f$  and  $e$  for calculation of  $g_i$ .

#### Calculation of the Oxygen Isotope Composition of Average Lamina Leaf Water

We estimated the average lamina leaf water  $^{18}\text{O}$  enrichment ( $\Delta_L$ ) of leaves during  $\text{CO}_2$  collections from a model relating  $\Delta_L$  to  $\Delta_e$  (Farquhar and Lloyd, 1993):

$$\Delta_L = \frac{\Delta_e(1 - e^{-\wp})}{\wp}, \quad (18)$$

where  $\Delta_e$  is as calculated in Equation 7, and  $\wp$  is a lamina radial Péclet number (Farquhar and Gan, 2003). The term  $\wp$  is defined as  $EL/(CD)$ , where  $E$  is transpiration rate ( $\text{mol m}^{-2} \text{ s}^{-1}$ ),  $L$  is a scaled effective path length (m),  $C$  is the molar concentration of water ( $5.55 \times 10^4 \text{ mol m}^{-3}$ ), and  $D$  is the diffusivity of  $\text{H}_2^{18}\text{O}$  in water ( $2.66 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ ). In a previous experiment, we found that the scaled effective path length for *R. communis*, grown and measured under the same conditions as in the present experiment, was  $15.0 \pm 3.5 \text{ mm}$  (mean  $\pm 1 \text{ SD}$ ;  $n = 5$ ; Cernusak et al., 2003). This mean value was used to calculate  $\Delta_L$ . The term  $\delta_L$  was calculated as  $\delta_L = \Delta_L(1 + \delta_s) + \delta_s$ . Cernusak et al. (2003) also found that the ethanol-dry ice traps on the bypass drying loop of the gas exchange system were not quite efficient enough to remove all of the water vapor from the air cycling back to the chamber. Due to fractionation during condensation of the vapor in the

traps, vapor in the air returning to the chamber was slightly enriched compared to that retained in the traps. As a result,  $\Delta_v$  for the air exiting the chamber was found to be  $1.2 \pm 0.5\%$  (mean  $\pm 1$  SE;  $n = 5$ ). This mean value was used in calculations of  $\Delta_e$ .

### Derivation 1: Equation for Predicting the $\delta^{18}\text{O}$ of Dark-Respired $\text{CO}_2$

We begin by writing an equation for the total  $\text{CO}_2$  flux from the leaf interior to the atmosphere in the dark in the steady state:

$$\mathfrak{R}_n = g_{tc} \left( \frac{C_c - C_a}{P} \right), \quad (19)$$

where  $\mathfrak{R}_n$  is the net  $\text{CO}_2$  efflux ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $g_{tc}$  is the total conductance to  $\text{CO}_2$  from chloroplast to atmosphere ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $C_c$  and  $C_a$  are the  $\text{CO}_2$  partial pressures in the chloroplast and atmosphere, respectively ( $\mu\text{bar}$ ); and  $P$  is atmospheric pressure (bar). We make the assumption that, in  $C_3$  plants, carbonic anhydrase resides primarily in the chloroplast (Everson, 1970; Jacobson et al., 1975; Tsuzuki et al., 1985) and that it is therefore the chloroplastic  $\text{CO}_2$  concentration that should be considered when calculating the  $\text{C}^{18}\text{OO}$  efflux from the leaf. We further assume that the chloroplasts in  $C_3$  plants are appressed against the intercellular air spaces in the leaf and that  $\text{CO}_2$  evolved in mitochondria interacts with chloroplasts during diffusion out of the leaf. These assumptions may need to be reassessed for application of the model to  $C_4$  plants. Equation 19 can be written for  $\text{C}^{18}\text{OO}$  as

$$\mathfrak{R}_n R_R = \frac{g_{tc}}{1 + \bar{a}} \left( \frac{C_c R_c - C_a R_a}{P} \right), \quad (20)$$

where  $R_R$  is the  $^{18}\text{O}/^{16}\text{O}$  of dark-respired  $\text{CO}_2$ ,  $\bar{a}$  is the weighted mean diffusional fractionation from chloroplast to atmosphere (calculated as described in Equation 3 above),  $R_c$  is  $^{18}\text{O}/^{16}\text{O}$  of chloroplastic  $\text{CO}_2$ , and  $R_a$  is  $^{18}\text{O}/^{16}\text{O}$  of ambient  $\text{CO}_2$ . Equations 19 and 20 can be combined to give

$$R_R(C_c - C_a) = \frac{1}{1 + \bar{a}} (C_c R_c - C_a R_a). \quad (21)$$

Dividing Equation 21 by the  $^{18}\text{O}/^{16}\text{O}$  of a standard,  $R_{\text{Std}}$ , and applying the relationship  $R_X/R_{\text{Std}} = \delta_X + 1$  leads to

$$(1 + \delta_R)(C_c - C_a) = \frac{1}{1 + \bar{a}} [C_c(1 + \delta_c) - C_a(1 + \delta_a)]. \quad (22)$$

Solving Equation 22 for  $\delta_c$  leads to Equation 5 above:

$$\delta_c = \delta_R(1 + \bar{a}) \left( 1 - \frac{C_a}{C_c} \right) + \frac{C_a}{C_c} (\delta_a - \bar{a}) + \bar{a}.$$

To write an expression for predicting  $\delta_R$ , we apply an assumption proposed by Gillon and Yakir (2000b), under which the  $\text{CO}_2$  within the chloroplast can be

divided into two pools: one pool, of proportion  $\theta$ , has completely exchanged oxygen atoms with chloroplast water and therefore has an  $^{18}\text{O}/^{16}\text{O}$  composition of  $R_{ce}$ ; the other pool, of proportion  $1 - \theta$ , has not exchanged oxygen atoms with chloroplast water and retains its initial  $^{18}\text{O}/^{16}\text{O}$  composition of  $R_{c0}$ . We note that the term  $R_{c0}$  could describe a mixture of mitochondrial  $\text{CO}_2$  and  $\text{CO}_2$  that has diffused into the leaf from the ambient air. Therefore, we do not define  $R_{c0}$  solely as a function of  $\text{CO}_2$  diffusing into the leaf from the atmosphere, as was done previously for photosynthesis (Gillon and Yakir, 2000b). The term  $R_c$  is then written as

$$R_c = \theta R_{ce} + (1 - \theta) R_{c0}. \quad (23)$$

The term  $R_{ce}$  can be calculated from the equilibrium fractionation between  $\text{CO}_2$  and water:

$$\alpha_w = \frac{R_{ce}}{R_e} = 1 + \varepsilon_w, \quad (24)$$

where  $R_e$  is  $^{18}\text{O}/^{16}\text{O}$  of chloroplast water, which we assume to be equal to  $^{18}\text{O}/^{16}\text{O}$  of water at the evaporative sites. Combining Equations 21, 23, and 24 leads to

$$R_R(C_c - C_a)(1 + \bar{a}) = C_c [R_e \alpha_w \theta + R_{c0}(1 - \theta)] - C_a R_a. \quad (25)$$

Dividing through by  $R_{\text{Std}}$ , and substituting  $1 + \varepsilon_w$  for  $\alpha_w$  gives

$$(1 + \delta_R)(C_c - C_a)(1 + \bar{a}) = C_c [(1 + \delta_e)(1 + \varepsilon_w)\theta + (1 + \delta_{c0})(1 - \theta)] - C_a(1 + \delta_a). \quad (26)$$

Solving Equation 26 for  $\delta_R$  leads to Equation 2 above, which is

$$\delta_R = \frac{\theta[\delta_e(1 + \varepsilon_w) + \varepsilon_w] + (1 - \theta)\delta_{c0} - \frac{C_a}{C_c}(\delta_a - \bar{a}) - \bar{a}}{(1 + \bar{a}) \left( 1 - \frac{C_a}{C_c} \right)}.$$

### Derivation 2: Calculating $\delta_R$ From Online Gas-Exchange Measurements

Under steady-state conditions, the increase in  $\text{CO}_2$  concentration in air flowing through a gas-exchange cuvette containing a respiring leaf can be described as

$$u \frac{C_a}{P} = u \frac{C_{in}}{P} + \Lambda \mathfrak{R}_n, \quad (27)$$

where  $u$  is the flow rate through the cuvette ( $\text{mol s}^{-1}$ ),  $\Lambda$  is the area of the leaf in the cuvette ( $\text{m}^2$ ),  $C_a$  and  $C_{in}$  are  $\text{CO}_2$  partial pressures of dry air exiting and entering the cuvette ( $\mu\text{bar}$ ),  $P$  is atmospheric pressure (bar), and  $\mathfrak{R}_n$  is the respiration rate of the

leaf ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ). The corresponding mass balance for  $\text{C}^{18}\text{O}$  can be written as

$$uR_a \frac{C_a}{P} = uR_{in} \frac{C_{in}}{P} + \Lambda R_R \mathcal{R}_n. \quad (28)$$

Combining Equations 27 and 28 gives

$$R_R = \frac{1}{C_a - C_{in}} (R_a C_a - R_{in} C_{in}). \quad (29)$$

Dividing through by the isotope ratio of a standard,  $R_{\text{Std}}$ , and substituting from the relationship  $R_X/R_{\text{Std}} = \delta_X + 1$  gives

$$\delta_R + 1 = \frac{1}{C_a - C_{in}} [(\delta_a + 1)C_a - (\delta_{in} + 1)C_{in}]. \quad (30)$$

Canceling common terms leads to Equation 11 above, which is

$$\delta_R = \frac{C_a \delta_a - C_{in} \delta_{in}}{C_a - C_{in}}.$$

We note that the equations derived in this and the previous section can also be applied in the light. Thus, for photosynthesis, the term  $\delta_R$  in Equations 2, 5, and 11 above can simply be replaced with the term  $\delta_A$ . The term  $\delta_A$  relates to  $\Delta_A$  by the relationship  $\Delta_A = (\delta_a - \delta_A)/(1 + \delta_A)$ .

## RESULTS

### Dark Respiration with $\text{CO}_2$ Free Air Entering the Leaf Chamber

In the first dark respiration experiment, air entering the leaf chamber was free of  $\text{CO}_2$ , and air exiting the

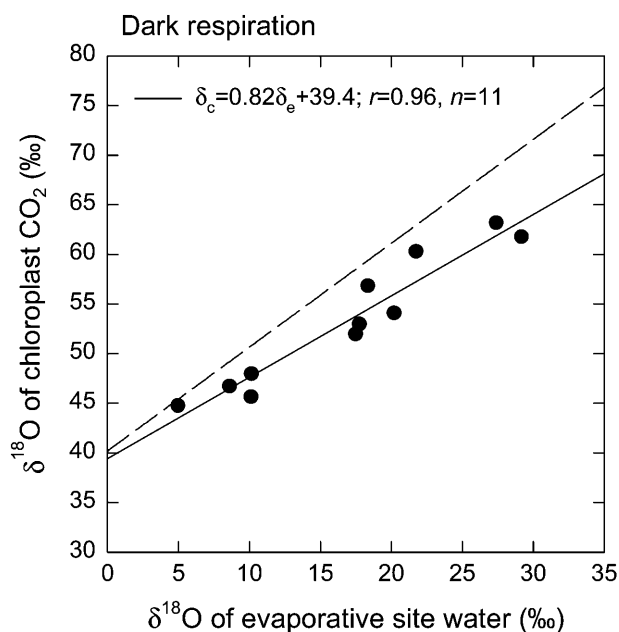
leaf chamber had a mean  $\text{CO}_2$  partial pressure of  $47 \mu\text{bar}$ . The  $\text{CO}_2$  exiting the leaf chamber was collected and analyzed for its isotopic composition. A summary of gas exchange parameters measured just prior to each  $\text{CO}_2$  collection is presented in Table II. The dark respiration rates of the leaves ranged from  $0.8$  to  $2.0 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  on a projected leaf area basis, with a mean value of  $1.5$ . The  $C_a/C_i$  values ranged from  $0.46$  to  $0.93$ , with a mean value of  $0.81$ .

Isotopic parameters derived by combining the results of the gas exchange measurements with results of analyses of the isotopic composition of  $\text{CO}_2$  exiting the leaf chamber, and of irrigation water fed to the plants, are given in Table II; these parameters are  $\delta_e$ ,  $\delta_L$ , and  $\delta_c$ . Results for  $\delta_a$ , the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  exiting the leaf chamber, are also given in Table II. The observed  $\delta_R$  values, which are equal to  $\delta_a$  in the first experiment, ranged from  $43.8\text{‰}$  to  $59.0\text{‰}$ , with a mean value of  $51.6\text{‰}$ . All  $\delta^{18}\text{O}$  values in this paper are reported relative to Vienna Standard Mean Ocean Water (VSMOW). The  $\delta_c$  values were significantly, positively correlated with corresponding values of  $\delta_e$  (Fig. 1); the Pearson correlation coefficient ( $r$ ) between the two was  $0.96$  ( $P < 0.0001$ ,  $n = 11$ ). The  $\delta_c$  values were also significantly correlated with values of  $\delta_L$  ( $r = 0.90$ ,  $P = 0.0001$ ,  $n = 11$ ), but the relationship was not as strong as that between  $\delta_c$  and  $\delta_e$ . The slope of the regression relating  $\delta_c$  to  $\delta_e$  was  $0.82$ , yielding an estimate for  $\theta$  of  $0.79$ . Thus, we estimated, by applying Equation 6, that  $79\%$  of the  $\text{CO}_2$  in the chloroplasts had equilibrated with chloroplast water during dark respiration in the first experiment. The intercept of the regression relating  $\delta_c$  to  $\delta_e$  was  $39.4\text{‰}$ ; this intercept yields an estimate for  $\delta_{c0}$  of  $36.2\text{‰}$ . This is the mean  $\delta^{18}\text{O}$  estimated for  $\text{CO}_2$  not equilibrated with chloroplast water.

**Table II.** Gas exchange and isotopic characteristics for *R. communis* leaves

Values are given as the mean, with the total range in parentheses, for the three experiments conducted. Symbols are as defined in Table I. Modeled  $\delta_R$  values were calculated using Equation 2 and the empirically determined coefficients for  $\theta$  and  $\delta_{c0}$  for experiments 1 and 2. Modeled  $\Delta_A$  values were calculated using Equation 13 and assuming  $\Delta_{ca} = \Delta_{ea}$ ; the term  $\Delta_{ea}$  was calculated as in Equation 15.

Parameter	Experiment 1: Dark Respiration at Low $\text{CO}_2$ Concentration	Experiment 2: Dark Respiration at Atmospheric $\text{CO}_2$ Concentration	Experiment 3: Photosynthesis at Atmospheric $\text{CO}_2$ Concentration
$n$	11	10	8
$g_s$ ( $\text{mol m}^{-2} \text{ s}^{-1}$ )	0.28 (0.04 to 0.55)	0.13 (0.03 to 0.28)	0.50 (0.18 to 0.77)
$E$ ( $\text{mmol m}^{-2} \text{ s}^{-1}$ )	4.4 (1.2 to 7.8)	2.7 (1.3 to 4.0)	8.8 (5.7 to 15.1)
$T_{\text{leaf}}$ ( $^{\circ}\text{C}$ )	29.3 (27.3 to 31.2)	30.2 (29.3 to 30.8)	27.2 (24.0 to 30.1)
$C_a$ ( $\mu\text{bar}$ )	47 (24 to 66)	347 (324 to 365)	363 (328 to 395)
$C_i$ ( $\mu\text{bar}$ )	63 (27 to 125)	357 (328 to 401)	285 (250 to 317)
$C_c$ ( $\mu\text{bar}$ )	66 (28 to 128)	360 (330 to 403)	249 (207 to 287)
$e_a/e_i$	0.53 (0.13 to 0.92)	0.41 (0.11 to 0.73)	0.51 (0.30 to 0.78)
$\bar{a}$ ( $\text{‰}$ )	6.6 (5.4 to 8.4)	5.9 (3.7 to 8.3)	6.0 (5.0 to 7.5)
$\delta_a$ ( $\text{‰}$ versus VSMOW)	51.6 (43.8 to 59.0)	43.5 (37.7 to 51.2)	42.3 (40.1 to 45.4)
$\delta_e$ ( $\text{‰}$ versus VSMOW)	16.9 (5.0 to 29.2)	20.5 (10.8 to 29.8)	17.5 (9.4 to 23.9)
$\delta_L$ ( $\text{‰}$ versus VSMOW)	12.4 (4.3 to 26.5)	17.2 (8.4 to 27.4)	9.5 (4.1 to 16.4)
$\delta_c$ ( $\text{‰}$ versus VSMOW)	53.3 (44.8 to 63.2)	51.4 (44.6 to 60.7)	59.6 (50.4 to 70.7)
$\delta_R$ ( $\text{‰}$ versus VSMOW)	51.6 (43.8 to 59.0)	277 (233 to 324)	
Modeled $\delta_R$	51.9 (34.0 to 64.4)	291 (149 to 476)	
$\Delta_A$ ( $\text{‰}$ versus VSMOW)			44.6 (32.4 to 78.5)
Modeled $\Delta_A$			43.4 (30.6 to 62.9)



**Figure 1.** The  $\delta^{18}\text{O}$  of chloroplast  $\text{CO}_2$  plotted against the  $\delta^{18}\text{O}$  of water at evaporative sites in *R. communis* leaves during dark respiration. In this experiment, air entering the leaf chamber was free of  $\text{CO}_2$ , and the  $\text{CO}_2$  partial pressure of air exiting the chamber averaged  $47 \mu\text{bar}$ . The  $\delta^{18}\text{O}$  of chloroplast  $\text{CO}_2$  was calculated from measurements of the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  exiting the chamber and gas exchange parameters, as described in the theory section of the main text. The broken line on the graph represents the relationship expected if chloroplast  $\text{CO}_2$  were in full oxygen isotope equilibrium with water at evaporative sites. The  $\delta^{18}\text{O}$  values are presented relative to VSMOW. The  $\delta^{18}\text{O}$  of irrigation water fed to the plants was  $-7.2\text{‰}$ .

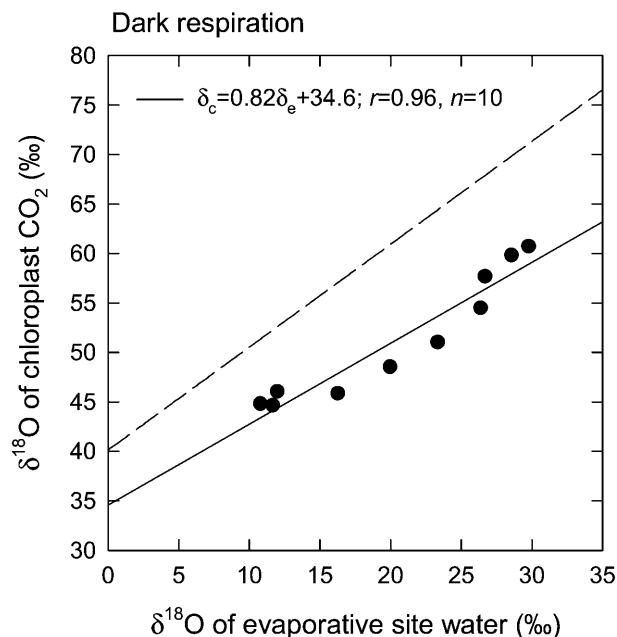
By applying the mean value of  $g_i$  derived from carbon isotope discrimination measurements during photosynthesis (see results below), we generated estimates of  $C_c$  and  $\bar{a}$ . These values are detailed in Table II. Estimates of  $C_a/C_c$  ranged from 0.45 to 0.88, with a mean value 0.78. When these values for  $C_a/C_c$  and  $\bar{a}$  were inserted into Equation 2, along with the values of  $\theta$  and  $\delta_{c0}$  described above, a mean *modeled*  $\delta_R$  of  $51.9\text{‰}$  was predicted, in good agreement with the mean observed  $\delta_R$  of  $51.6\text{‰}$ . The range of *modeled*  $\delta_R$  can be compared with the range of observed  $\delta_R$  in Table II.

#### Dark Respiration at Atmospheric $\text{CO}_2$ Concentration

In the second dark respiration experiment, the partial pressure of  $\text{CO}_2$  in the air entering the leaf chamber was adjusted such that the air exiting the chamber had a partial pressure of approximately  $350 \mu\text{bar}$ . Under these conditions, leaf dark respiration rates were similar to those observed in the first experiment, ranging from  $1.0$  to  $2.0 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , with a mean value of  $1.4$ . Stomatal conductance was lower than in the first experiment, having a mean value less than half that observed in the first experiment (Table II). This presumably reflects a response to

the increased  $\text{CO}_2$  partial pressure within the leaf chamber. Although stomatal conductance was lower,  $C_a/C_i$  values were higher than in the first experiment due to the increase in  $C_a$ ; values ranged from 0.91 to 0.99, with a mean of 0.97. The  $\delta^{18}\text{O}$  of  $\text{CO}_2$  in air entering the leaf chamber was  $19.1 \pm 0.1\text{‰}$  (mean  $\pm 1$  SE;  $n = 5$ ). The mean  $\delta^{18}\text{O}$  of  $\text{CO}_2$  exiting the chamber was  $43.5\text{‰}$ .

The most striking difference between the first and second dark respiration experiments was the difference in observed  $\delta_R$ . The mean observed  $\delta_R$  in the second experiment was  $277\text{‰}$ , which can be compared with  $52\text{‰}$  for the first experiment (Table II). Mean values for  $\delta_e$ ,  $\delta_L$ , and  $\delta_c$  were similar between the two experiments (Table II). Differences between  $\delta_e$  and  $\delta_L$  in the second experiment were slightly less than in the first experiment, reflecting the lower transpiration rates (Table II). As in the first experiment, variation in  $\delta_c$  was significantly correlated with variation in  $\delta_e$  (Fig. 2), showing an  $r$  value of  $0.95$  ( $P < 0.0001$ ,  $n = 10$ ). It was also correlated with  $\delta_L$ , with a slightly lower correlation coefficient ( $r = 0.94$ ,  $P < 0.0001$ ,  $n = 10$ ). The regression slope of the relationship between  $\delta_c$  and  $\delta_e$  was  $0.82$ , resulting in an estimate for  $\theta$  of  $0.79$ , suggesting that  $79\%$  of the  $\text{CO}_2$  in chloroplasts had equilibrated with chloroplast water during dark



**Figure 2.** The  $\delta^{18}\text{O}$  of chloroplast  $\text{CO}_2$  plotted against the  $\delta^{18}\text{O}$  of water at evaporative sites in *R. communis* leaves during dark respiration. In this experiment, air entering the leaf chamber had an average  $\text{CO}_2$  partial pressure of  $314 \mu\text{bar}$ , and air exiting the chamber had an average  $\text{CO}_2$  partial pressure of  $347 \mu\text{bar}$ . The broken line on the graph represents the relationship expected if chloroplast  $\text{CO}_2$  were in full oxygen isotope equilibrium with water at the evaporative sites. The  $\delta^{18}\text{O}$  values are presented relative to VSMOW. The  $\delta^{18}\text{O}$  of irrigation water fed to the plants was  $-7.2\text{‰}$ .



respiration in the second experiment. This  $\theta$  value is the same as the value of 0.79 estimated in the first experiment. The value of the intercept of the regression of  $\delta_c$  on  $\delta_e$  was 34.6‰, yielding an estimate for  $\delta_{c0}$  of 14.3‰; this value is lower than the  $\delta_{c0}$  of 36.2‰ estimated in the first experiment.

Values of  $C_a/C_c$  in the second experiment did not differ from values for  $C_a/C_i$  when calculated to two decimal places; the range was from 0.91 to 0.99, with a mean of 0.97. This mean of 0.97 is considerably higher than the mean  $C_a/C_c$  of 0.78 observed in the first experiment. Mean estimates for  $\bar{a}$  were similar between the two experiments (Table II). When the empirically determined coefficients for  $\theta$  and  $\delta_{c0}$  for the second experiment were inserted into Equation 2, along with the other relevant parameters, the mean value of modeled  $\delta_R$  was 291‰, which compares reasonably well with the mean observed  $\delta_R$  of 277‰. The relatively small difference between the two presumably reflects variation around the regression line in Figure 2, which was used to estimate  $\theta$  and  $\delta_{c0}$ .

A comparison of modeled  $\delta_R$  values across both experiments with observed  $\delta_R$  showed that modeled  $\delta_R$  accounted for 80% of variation in observed  $\delta_R$ . The regression line relating the two was  $\delta_R(\text{observed}) = 0.72\delta_R(\text{modeled}) + 39.5$  ( $R^2 = 0.80$ ,  $P < 0.0001$ ,  $n = 21$ ).

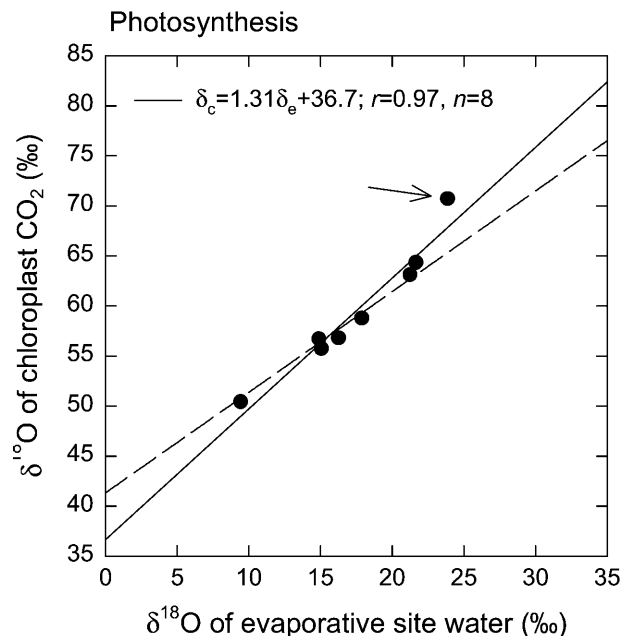
### Carbon and Oxygen Isotope Discrimination during Photosynthesis

In the third experiment, *R. communis* leaves were placed in the leaf chamber in the light, and gas exchange and isotopic analyses were conducted. Photosynthesis rates ranged from 8.5 to 30.9  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , with a mean value of 20.4. The  $\text{CO}_2$  partial pressure of air exiting the chamber ranged from 328 to 395  $\mu\text{bar}$ , whereas the  $\text{CO}_2$  partial pressure of incoming air ranged from 533 to 967  $\mu\text{bar}$ ; this gave rise to  $\xi$  values ranging from 1.5 to 3.0. Stomatal conductance was approximately 4-fold larger in the light than in the dark at similar  $\text{CO}_2$  partial pressure (Table II). The  $C_i/C_a$  ranged from 0.66 to 0.90, with a mean of 0.79. The  $\delta^{18}\text{O}$  of  $\text{CO}_2$  entering the leaf chamber was  $19.1 \pm 0.1\text{‰}$  (mean  $\pm 1$  SE;  $n = 5$ ); the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  entering the leaf chamber was  $-33.1 \pm 0.2\text{‰}$  (mean  $\pm 1$  SE;  $n = 5$ ). The  $\delta^{18}\text{O}$  of  $\text{CO}_2$  exiting the leaf chamber ranged from 40.1‰ to 45.4‰; the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  exiting the chamber ranged from  $-25.3\text{‰}$  to  $-19.3\text{‰}$ .

The mean observed oxygen isotope discrimination during photosynthesis ( $\Delta_A$ ) was 44.6‰; the range is given in Table II. The  $\delta_c$  values for the photosynthesis experiment were somewhat higher than for the dark respiration experiment at similar  $\text{CO}_2$  concentration, presumably reflecting a higher proportion of chloroplast  $\text{CO}_2$  equilibrated with chloroplast water (i.e. higher  $\theta$ ). Differences between  $\delta_e$  and  $\delta_L$  were larger in the photosynthesis experiment than in the dark respiration experiments, reflecting the higher transpiration rates (Table II). Variation in  $\delta_c$  was significantly correlated with variation in  $\delta_e$  ( $r = 0.97$ ,  $P < 0.0001$ ,  $n =$

8), as shown in Figure 3. The  $\delta_c$  was also correlated with  $\delta_L$  ( $r = 0.91$ ,  $P = 0.001$ ,  $n = 8$ ), but the correlation was not as strong as with  $\delta_e$ . The slope of the relationship between  $\delta_c$  and  $\delta_e$  was 1.31; using Equation 6, this indicates a value for  $\theta$  of 1.25. However, this slope estimate was strongly influenced by one outlying data point; this datum is identified by an arrow in Figure 3. If this outlying datum is excluded from the analysis, the slope of the relationship between  $\delta_c$  and  $\delta_e$  becomes 1.11, yielding an estimate for  $\theta$  of 1.06. The individual  $\theta$  values calculated according to the method of Gillon and Yakir (2000b) ranged from 0.93 to 1.24, with a mean value of 1.02. If the outlying data point identified with the arrow in Figure 3 is excluded, these individual  $\theta$  estimates ranged from 0.93 to 1.06, with a mean of 0.99. Because the  $\theta$  values were very close to 1.0, we did not estimate a  $\delta_{c0}$  value for the photosynthesis experiment.

Observed carbon isotope discrimination values,  $\Delta_{\text{obs}}$ , ranged from 19.4‰ to 25.2‰, whereas values predicted for infinite  $g_i$  and no discrimination by photorespiration or day respiration,  $\Delta_i$ , ranged from 20.6‰ to 26.5‰. The slope of the relationship between



**Figure 3.** The  $\delta^{18}\text{O}$  of chloroplast  $\text{CO}_2$  plotted against the  $\delta^{18}\text{O}$  of water at evaporative sites in *R. communis* leaves during photosynthesis. In this experiment, air entering the leaf chamber had an average  $\text{CO}_2$  partial pressure of 833  $\mu\text{bar}$ , and air exiting the chamber had an average  $\text{CO}_2$  partial pressure of 363  $\mu\text{bar}$ . Irradiance varied from 300 to 800  $\mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ , and chamber air temperature varied between 25 and 30°C. The  $\delta^{18}\text{O}$  of chloroplast  $\text{CO}_2$  was calculated as described in the theory section of the main text. The broken line on the graph represents the relationship expected if chloroplast  $\text{CO}_2$  were in full oxygen isotope equilibrium with water at the evaporative sites. The  $\delta^{18}\text{O}$  values are presented relative to VSMOW. The  $\delta^{18}\text{O}$  of irrigation water fed to the plants was  $-7.2\text{‰}$ . The arrow on the graph indicates an outlying datum that strongly influenced the slope of the regression between  $\delta_c$  and  $\delta_e$ . Excluding this datum resulted in a slope between  $\delta_c$  and  $\delta_e$  of 1.10.



$(1 + \bar{a})$  equals unity introduces a very small bias into calculations with this equation; however, this bias is less than 1% and is therefore negligible. Thus, Equation 31, in combination with Equation 6, can be used in place of Equation 2, if so desired.

Photosynthesis enriches the atmosphere in  $C^{18}OO$  due to exchange of  $CO_2$  with evaporatively enriched leaf water in the chloroplast, whereas soil respiration is generally thought of as depleting the atmosphere in  $C^{18}OO$ , because soil  $CO_2$  exchanges with water in soil that has generally not been enriched by evaporation (Flanagan and Ehleringer, 1998). In this study, we have observed that leaf dark respiration is capable of enriching air passing over a leaf in  $C^{18}OO$  to as great an extent as photosynthesis. The mean  $\delta^{18}O$  value of  $CO_2$  exiting the leaf chamber in the respiration measurements at atmospheric  $CO_2$  partial pressure was 43.5‰; the mean value for photosynthesis measurements at similar  $C_a$  was 42.3‰. The  $\delta^{18}O$  of incoming  $CO_2$  in both experiments was 19.1‰, and flow rates through the chamber were similar between the two experiments. Thus, dark respiration had as marked an effect as photosynthesis on the  $\delta^{18}O$  of  $CO_2$  passing over the leaves, even though the net exchange of  $CO_2$  between the leaf and ambient air is roughly an order of magnitude less, and in the opposite direction, during dark respiration.

The effect of both photosynthesis and respiration on  $\delta^{18}O$  of  $CO_2$  in canopy air is partly controlled by the isotopic composition of leaf water. In natural systems, nighttime leaf water  $\delta^{18}O$  is typically intermediate between daytime leaf water  $\delta^{18}O$  and the  $\delta^{18}O$  of source water (Dongmann et al., 1974; Förstel, 1978; Zundel et al., 1978; Förstel and Hütten, 1983; Flanagan and Ehleringer, 1991; Flanagan et al., 1993, 1999; Cernusak et al., 2002; Mortazavi and Chanton, 2002). We therefore expect nighttime leaf respiration to impart a  $C^{18}OO$  signal on the atmosphere that is intermediate between the soil respiration signal and the photosynthesis signal.

Accurate prediction of the oxygen isotope composition of leaf water is important for interpreting vegetation effects on  $\delta^{18}O$  of atmospheric  $CO_2$ . Equation 7 can be used to calculate  $\delta_e$  under steady state conditions. However, leaf water  $\delta^{18}O$  is unlikely to be at steady state at night (Flanagan and Ehleringer, 1991; Harwood et al., 1998; Cernusak et al., 2002). Cernusak et al. (2002) applied a non-steady state equation for  $\delta^{18}O$  in leaf water, derived by G.D. Farquhar and L.A. Cernusak (unpublished theory), and found good agreement between predicted and observed nighttime values. The combination of the non-steady state leaf water equation and the model that we have provided here for  $\delta_R$  should allow reasonable predictions to be made of the impact of leaf dark respiration on  $\delta^{18}O$  of atmospheric  $CO_2$ .

Stomatal conductance will be an important parameter in the prediction of both  $\delta_e$  and  $\delta_R$  during the night. However, little attention has been paid historically to nighttime stomatal conductance. Snyder et al.

(2003) recently observed nighttime stomatal conductance to water vapor ranging from 10 to 150  $mmol\ m^{-2}\ s^{-1}$  for 17 plant species in the western United States. However, a mechanistic framework for interpreting such variation does not currently exist. Further investigation into the patterns and processes controlling nighttime stomatal conductance will lead to more accurate prediction of nighttime  $\delta_e$  and  $\delta_R$ . We note that the mean stomatal conductance that we observed in the dark for *R. communis* at normal atmospheric  $CO_2$  concentration was 130  $mmol\ m^{-2}\ s^{-1}$  (Table II), near the high end of values observed by Snyder et al. (2003) at night in the field. Our measurements were made during the day, and it is likely that stomatal conductance was influenced by circadian rhythms, causing it to be higher than it would be in the dark at night.

The mean value of  $\theta$  for the photosynthesis experiment calculated by the method described by Gillon and Yakir (2000b) was very close to 1.0. If the outlying data point, indicated by an arrow in Figure 3, was excluded from the analysis, the regression method resulted in a similar estimate of 1.06. Thus, both calculations suggested  $\theta$  values close to unity for photosynthesizing *R. communis* leaves. A quick examination of Figure 3 shows that observed  $\delta_c$  estimates lie very close to those expected for full equilibrium, with the exception of the one outlier, which is several per mil above the value expected for full equilibrium. We are unable to find a satisfactory explanation for why this particular datum should differ so markedly from the others. Results have been reported for a number of other  $C_3$  species in which the  $CO_2$  diffusing out of photosynthesizing leaves appeared to be very close to full equilibrium with  $\delta_e$  (Farquhar et al., 1993; Gillon and Yakir, 2001). Interestingly, the  $\theta$  values that we observed during dark respiration in *R. communis* were lower than those observed during photosynthesis, having values close to 0.80. Further research is necessary to determine the cause of this apparent discrepancy between  $\theta$  in the light and in the dark.

Gillon and Yakir (2000b) suggested that during photosynthesis  $\delta_{c0}$ , the  $\delta^{18}O$  of  $CO_2$  in the chloroplast not equilibrated with chloroplast water, can be calculated, to a close approximation, as  $\delta_{c0} = \delta_a - \bar{a}(1 - C_c/C_a)$ . This definition assumes no discrimination against  $C^{18}OO$  by Rubisco during photosynthesis, and neglects any influence of photorespiration or day respiration on  $\delta_{c0}$ . The latter statement is tantamount to saying that  $CO_2$  evolved from the mitochondria in the light has the same oxygen isotope composition as  $CO_2$  in the chloroplast. In that case, any addition of mitochondrial  $CO_2$  will have no impact upon the  $\delta^{18}O$  of chloroplast  $CO_2$ . The photosynthesis data set that we collected for *R. communis* did not allow us to test these assumptions because  $\theta$  was very close to 1.0; thus, the  $\delta_{c0}$  signal was completely washed out by the activity of carbonic anhydrase.

However, this was not the case for dark respiration, during which  $\theta$  was approximately 0.80. The method of Gillon and Yakir (2000b) leads to mean  $\delta_{c0}$  values for

the first and second dark respiration experiments of 55.2 and 43.7‰, respectively. These values can be compared to the mean  $\delta_{c0}$  values generated by the regression method of 30.8 and 14.3‰, respectively. Although the regression method makes no a priori assumptions about the controls on  $\delta_{c0}$ , we caution against over-interpretation of these latter values for the following reason: the regression analysis, as summarized in Equation 6, assumes no variation in  $\theta$  and  $\delta_{c0}$  among individual measurements in each experiment. The  $\delta_a$  values varied among measurements according to how the leaf was modifying the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  in the leaf chamber. Therefore, to the extent that  $\delta_{c0}$  is controlled by  $\delta_a$ ,  $\delta_{c0}$  could also have varied among individual measurements.

Nonetheless, the large variation between  $\delta_{c0}$  calculated as suggested by Gillon and Yakir (2000b) and the apparent  $\delta_{c0}$  values observed in the dark respiration experiments warrants some discussion. There are three possible sources for the oxygen in  $\text{CO}_2$  evolved in mitochondria during either dark respiration or photosynthesis: atmospheric  $\text{O}_2$ , organic oxygen from respiratory substrates, and oxygen from leaf water. Atmospheric  $\text{O}_2$  has a  $\delta^{18}\text{O}$  near 23.5‰ (VSMOW scale), and discrimination against  $^{18}\text{O}$  during respiration in plant tissues ranges from about 17‰ to 26‰ (Guy et al., 1992). We would therefore expect the  $\delta^{18}\text{O}$  of respiratory  $\text{CO}_2$  deriving its oxygen atoms from  $\text{O}_2$  to be in the range of 0‰ to 5‰. Assuming the  $\text{O}_2$  tank used in our experiments had a  $\delta^{18}\text{O}$  similar to atmospheric  $\text{O}_2$ , this range of values would apply. Organic oxygen in phloem sap sugars of the *R. communis* plants that we studied had a mean  $\delta^{18}\text{O}$  of  $27.5 \pm 0.6$ ‰ (mean  $\pm 1$  SD;  $n = 10$ ). Generally, this oxygen pool is expected to have a  $\delta^{18}\text{O}$  enriched by 27‰ compared to  $\delta_L$  at the time of photosynthesis (Cernusak et al., 2003). Oxygen atoms derived from water during respiratory reactions would also be expected to be enriched by 27‰ compared to the  $\delta^{18}\text{O}$  of the water source. The difference between the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  derived from any of these three sources and that of  $\text{CO}_2$  diffusing into the leaf from the atmosphere, prior to equilibration with leaf water, would depend on  $\delta_a$  and, in the case of organic oxygen and oxygen from water,  $\delta_L$ . However, it seems likely that under most circumstances the effect of incomplete equilibration between  $\text{CO}_2$  evolved from mitochondria and leaf water would be to decrease  $\delta_{c0}$  below the value predicted by the formulation given by Gillon and Yakir (2000b). More experiments like those conducted by Yakir et al. (1994) would be helpful for resolving this issue.

Farquhar and Lloyd (1993) discussed the departure of  $\delta_c$  from that predicted for equilibrium with  $\delta_e$  during photosynthesis in terms of the ratio of the rate of carboxylation by Rubisco to the rate of  $\text{CO}_2$  hydration by carbonic anhydrase. This ratio was termed  $\rho$ . A simplified non-equilibrium equation for discrimination against  $^{18}\text{O}$  during photosynthesis, neglecting the possible effects of photorespiration and day respiration, was presented as (Farquhar and Lloyd, 1993)

$$\Delta_A = \frac{\bar{a}(1 + 3\rho) + \left(\frac{C_c}{C_a - C_c}\right)(\Delta_{ea} + 3\rho b^{18})}{1 - \left(\frac{C_c}{C_a - C_c}\right)\Delta_{ea} + 3\rho\left(\frac{C_a}{C_a - C_c}\right)}, \quad (32)$$

where  $b^{18}$  is discrimination against  $^{18}\text{O}$  by Rubisco. Using this equation, and assuming  $b^{18} = 0$ , we calculated a mean  $\rho$  value for our photosynthesis measurements of  $-0.002 \pm 0.009$  (mean  $\pm 1$  SD;  $n = 8$ ); if the outlier in Figure 3 is excluded, the mean  $\rho$  value becomes  $0.001 \pm 0.006$  (mean  $\pm 1$  SD;  $n = 7$ ). These values can be compared to a mean  $\rho$  value calculated for *Phaseolus vulgaris* of 0.025 (Flanagan et al., 1994). Thus, the  $\rho$  values that we observed for *R. communis* were somewhat smaller than those observed previously for *P. vulgaris*. These values can be compared to a theoretical prediction for  $\rho$  of approximately 0.05 (Cowan, 1986).

In our calculations we have assumed that the  $\delta^{18}\text{O}$  of chloroplast water is equivalent to  $\delta_e$ . One might expect chloroplast water to be slightly less enriched than  $\delta_e$  due to the Péclet effect (Farquhar and Lloyd, 1993), which describes the interplay between advection of water toward the evaporative sites and diffusion of heavy isotopes away from the evaporative sites. We found that correlations between  $\delta_c$  and  $\delta_e$  were generally stronger than between  $\delta_c$  and  $\delta_L$ . This agrees with previous results (Flanagan et al., 1994), and suggests that  $\delta_e$  is a more relevant parameter for predicting  $\delta^{18}\text{O}$  of  $\text{CO}_2$  diffusing out of leaves than  $\delta_L$ .

Gillon and Yakir (2000a) suggested that the  $\text{CO}_2$  partial pressure at the chloroplast surface ( $C_{cs}$ ) is a more appropriate parameter for predicting discrimination against  $^{18}\text{O}$  during photosynthesis than that at the sites of carboxylation by Rubisco ( $C_c$ ). They reconstructed  $C_{cs}$  by combining measurements of  $^{18}\text{O}$  discrimination and carbonic anhydrase activity. We did not measure carbonic anhydrase activity directly, and so could not modify our calculations to take into account  $C_{cs}$ . In cases where the total resistance from the chloroplast to the atmosphere in the dark is dominated by the stomatal resistance, use of  $C_{cs}$  in place of  $C_c$  will likely not alter predictions of  $\delta_R$  to a very large extent. However, if stomata are relatively open and  $(\delta_c - \delta_a)$  is large, such that the invasion term in Equation 31 is large, a variation between  $C_c$  and  $C_{cs}$  of as little as 2  $\mu\text{bar}$  could have a significant effect on predicted  $\delta_R$ . In such cases it may prove helpful to use  $C_{cs}$  in place of  $C_c$ , if possible.

Farquhar et al. (1993) found that a globally averaged leaf water  $\delta^{18}\text{O}$  of 4.4‰ satisfactorily balanced the global budget for  $\delta^{18}\text{O}$  of atmospheric  $\text{CO}_2$ . In the most recent study of the global budget for  $\delta^{18}\text{O}$  of atmospheric  $\text{O}_2$ , a globally averaged leaf water  $\delta^{18}\text{O}$  of between 6.1 and 6.8‰ was estimated (Hoffmann et al., 2004). Gillon and Yakir (2001) suggested that the globally averaged leaf water  $\delta^{18}\text{O}$  could be as much as 3‰ more than the estimate of Farquhar et al. (1993), in agreement with the requirement for balancing the Dole

effect (global  $^{18}\text{O}$  budget); the global  $\text{C}^{18}\text{O}$  budget could then be maintained by incomplete equilibration of chloroplast  $\text{CO}_2$  with chloroplast water (i.e.  $\theta < 1$ ). They estimated a globally averaged  $\theta$  of 0.80. The results presented in this study provide an additional reason that the apparent leaf water signals required to balance the global  $\text{C}^{18}\text{O}$  and  $^{18}\text{O}$  budgets should not be expected to resolve into a single value. The apparent leaf water signal relevant to the global  $\delta^{18}\text{O}$  budget for  $\text{O}_2$  is the average daytime leaf water  $\delta^{18}\text{O}$ , weighted by diurnal (daytime) variation in photosynthetic oxygen evolution rates. In contrast, the apparent leaf water signal relevant to the global  $\delta^{18}\text{O}$  budget for  $\text{CO}_2$  is the 24-h average leaf water  $\delta^{18}\text{O}$ , weighted by diel (day and night) variation in  $g_{\text{tc}}C_c/P$ . Thus, the apparent leaf water  $\delta^{18}\text{O}$  signals relevant to the global  $\text{C}^{18}\text{O}$  and  $^{18}\text{O}$  budgets are fundamentally different.

## CONCLUSION

We observed a very large variation in the  $\delta^{18}\text{O}$  of  $\text{CO}_2$  respired by leaves in the dark, with observed values ranging from 44‰ to as high as 324‰. We have shown that this large range of  $\delta_{\text{R}}$  values can be satisfactorily explained by taking into account the flux of  $\text{CO}_2$  that enters the leaf, equilibrates with leaf water, and diffuses out of the leaf without affecting the net  $\text{CO}_2$  efflux. Incorporation of the correct expression for  $\delta^{18}\text{O}$  of leaf dark respiration into ecosystem and global scales models of  $\text{C}^{18}\text{O}$  dynamics could affect model outputs and their interpretation.

## MATERIALS AND METHODS

### Plant Material and Gas Exchange Measurements

*Ricinus communis* plants were grown from seeds in 10-L pots for 8 to 12 weeks in a temperature and humidity controlled glasshouse. Growth conditions were essentially the same as those described by Cernusak et al. (2003). Daytime temperature and humidity were  $27^\circ\text{C} \pm 2^\circ\text{C}$  and  $40\% \pm 10\%$ , respectively. Nighttime temperature was  $20^\circ\text{C}$ , with the same humidity as during the day. Measurements were made on fully expanded leaves of plants that were approximately 1 m tall. Projected areas of measured leaves ranged from approximately 400 to 800  $\text{cm}^2$ . The configuration of the gas exchange system was recently described (Cernusak et al., 2003). The through-flow rate of air in the leaf chamber was approximately  $3 \text{ L min}^{-1}$ . Chamber air cycled continuously through a bypass drying loop to remove water vapor. The flow rate through the bypass drying loop was varied between 5 and 45  $\text{L min}^{-1}$  to achieve different vapor pressures within the chamber, and therefore different values of  $e_a/e_v$ , and consequently of  $\delta_e$ . Air entering the leaf chamber was generated by mixing 79% dry nitrogen with 21% dry oxygen using two mass flow controllers. Carbon dioxide was added to this air stream from a cylinder of 10%  $\text{CO}_2$  in air. Leaf temperature was measured with eight thermocouples arrayed across the underside of the leaf, and the average of these measurements used in gas-exchange and isotopic calculations. Gas-exchange calculations were performed according to the equations of Caemmerer and Farquhar (1981).

After gas exchange conditions in the leaf chamber stabilized for a time period judged long enough for leaf water to reach isotopic steady state,  $\text{CO}_2$  was cryogenically trapped from air exiting the chamber, as described previously (Evans et al., 1986; Caemmerer and Evans, 1991). Trapping continued until approximately 50  $\mu\text{mol}$  of  $\text{CO}_2$  was obtained. The time period sufficient for leaf water to reach isotopic steady state was assumed to be three times the residence time of lamina leaf water (Förstel, 1978). The residence time of lamina leaf water was calculated as  $W/g_s w_v$ , where  $W$  is the lamina water concentration ( $\text{mol m}^{-2}$ ),  $g_s$  is the total conductance of boundary layer plus stomata to water vapor ( $\text{mol m}^{-2} \text{ s}^{-1}$ ), and  $w_v$  is the mole fraction of water

vapor in the leaf intercellular air spaces ( $\text{mol mol}^{-1}$ ). The term  $W$  was determined to be  $6.3 \pm 0.4 \text{ mol m}^{-2}$  (mean  $\pm 1 \text{ SD}$ ) from measurements of the difference between fresh weight and dry weight for one leaf from each of five plants. This mean value of  $W$  was assumed for all leaves in the experiment;  $g_s$  and  $w_v$  were calculated continuously for each leaf being measured. Time periods calculated in this way for leaf water to reach isotopic steady state after a step change in humidity ranged from approximately 0.5 to 3.5 h.

Three experiments were conducted, two in the dark and one in the light. In the first dark experiment, air entering the leaf chamber was free of  $\text{CO}_2$ . All  $\text{CO}_2$  in the air exiting the chamber was therefore derived from the leaf. Measurements were conducted on one leaf from each of five plants. Each leaf was subject to two or three different chamber vapor pressures, and  $\text{CO}_2$  collected after gas exchange had stabilized for the requisite amount of time at each vapor pressure. Chamber air temperature was maintained at approximately  $30^\circ\text{C}$ . The second dark experiment was similar to the first, but differed in that  $\text{CO}_2$  was added to the air entering the chamber, such that the partial pressure within the chamber was approximately 350  $\mu\text{bar}$ . The third experiment was in the light. Irradiance varied between 300 and 800  $\mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ , and chamber air temperature varied between  $25^\circ\text{C}$  and  $30^\circ\text{C}$ . The  $\text{CO}_2$  partial pressure within the chamber was approximately 350  $\mu\text{bar}$ .

### Isotope Measurements

The carbon and oxygen isotope composition of  $\text{CO}_2$  exiting the leaf chamber was determined on an Isoprime mass spectrometer (Micromass, Manchester, UK) operating in dual inlet mode. Repeated analyses of the same gas sample generally showed a precision of better than 0.1‰ (1 SD,  $n = 10$ ) for  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ . The carbon and oxygen isotopic composition of the gas used as a reference for the dual inlet measurements was calibrated against standard gases supplied by the International Atomic Energy Agency (Vienna). Oxygen isotope ratios in this paper are presented relative to VSMOW; carbon isotope ratios are presented relative to the Vienna Pee Dee Belemnite standard (VPDB). The oxygen isotope composition of irrigation water fed to the plants was determined with an Isochrom mass spectrometer (Micromass) operating in continuous flow mode (Farquhar et al., 1997). The water samples were pyrolyzed in a custom-built furnace at  $1,300^\circ\text{C}$  prior to entering the mass spectrometer. Precision of analyses, based on repeated measurements of a laboratory standard water sample, was 0.3‰ (1 SD,  $n = 10$ ). The  $\delta^{18}\text{O}$  of the irrigation water was found to be  $-7.2 \pm 0.2\text{‰}$  (mean  $\pm 1 \text{ SE}$ ;  $n = 6$ ).

We assumed that the only source of  $\text{N}_2\text{O}$  in the leaf chamber was the compressed air that the  $\text{CO}_2$  was mixed into, and that the concentration of  $\text{N}_2\text{O}$  in this air was 300  $\text{nmol mol}^{-1}$ . The  $\text{CO}_2$  concentration was 10%, giving a ratio of  $\text{N}_2\text{O}$  to  $\text{CO}_2$  of  $3 \times 10^{-6}$ . This ratio could have been doubled during photosynthesis measurements, when the  $\text{CO}_2$  concentration exiting the chamber was as little as one-half that entering it, giving a ratio of  $6 \times 10^{-6}$ . Using the empirical equations of Mook and van der Hoek (1983), this ratio of  $\text{N}_2\text{O}$  to  $\text{CO}_2$  would result in measurement biases of 0.002‰ for both  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ . This bias was considered negligible, and no attempt was made to account for contamination of  $\text{CO}_2$  samples by  $\text{N}_2\text{O}$ .

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