Carbon Isotope Fractionation during Photorespiration and Carboxylation in Senecio

Gary J. Lanigan², Nicholas Betson³, Howard Griffiths, and Ulli Seibt⁴*

Physiological Ecology Group, Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom

The magnitude of fractionation during photorespiration and the effect on net photosynthetic $^{13}$C discrimination ($\Delta$) were investigated for three Senecio species, S. squilidus, S. cineraria, and S. greyii. We determined the contributions of different processes during photosynthesis to $\Delta$ by comparing observations ($\Delta_{ob}$) with discrimination predicted from gas-exchange measurements ($\Delta_{pred}$). Photorespiration rates were manipulated by altering the O$_2$ partial pressure (pO$_2$) in the air surrounding the leaves. Contributions from $^{13}$C-depleted photorespiratory CO$_2$ were largest at high pO$_2$. The parameters for photorespiratory fractionation ($f$), net fractionation during carboxylation by Rubisco and phosphoenoxypruvate carboxylase ($b$), and mesophyll conductance ($g_i$) were determined simultaneously for all measurements. Instead of using $\Delta_{ob}$ data to obtain $g_i$ and $f$ successively, which requires that $b$ is known, we treated $b$, $f$, and $g_i$ as unknowns. We propose this as an alternative approach to analyze measurements under field conditions when $b$ and $g_i$ are not known or cannot be determined in separate experiments. Good agreement between modeled and observed $\Delta$ was achieved with $f$ = 11.6$_{\text{obs}} $$\pm$ 1.5$_{\text{obs}}$, $b$ = 26.0$_{\text{obs}} $$\pm$ 0.3$_{\text{obs}}$, and $g_i$ of 0.27 $\pm$ 0.01, 0.25 $\pm$ 0.01, and 0.22 $\pm$ 0.01 mol m$^{-2}$ s$^{-1}$ for S. squilidus, S. cineraria, and S. greyii, respectively. We estimate that photorespiratory fractionation decreases $\Delta$ by about 1.2$_{\text{obs}}$ on average under field conditions. In addition, diurnal changes in $\Delta$ are likely to reflect variations in photorespiration even at the canopy level. Our results emphasize that the effects of photorespiration must be taken into account when partitioning net CO$_2$ exchange of ecosystems into gross fluxes of photosynthesis and respiration.

Development of the theory linking the $\delta^{13}$C signatures of plant CO$_2$ fluxes or organic material to leaf gas exchange (Farquhar et al., 1982) has led to a wide range of applications for crops and natural vegetation. For example, $\delta^{13}$C data are used to study plant water use efficiency (Hobbie and Colpaert, 2004; Cernusak et al., 2008; Seibt et al., 2008) and respiration and secondary fractionation processes (Ghashghaie et al., 2003; Wingate et al., 2007; Bathellier et al., 2008) and to partition net ecosystem CO$_2$ fluxes between photosynthesis and respiration (Bowling et al., 2001; Ogée et al., 2003; Zobitz et al., 2007). These applications require robust estimates of net $^{13}$C discrimination ($\Delta$) during photosynthesis. In C3 species, leaf level $\Delta$ during photosynthetic gas exchange primarily reflects the balance between CO$_2$ supply by diffusion through stomata and CO$_2$ demand by biochemical reactions in chloroplasts, most importantly catalysis by Rubisco (Farquhar et al., 1982). Both processes discriminate against the heavier isotope, but the fractionation during CO$_2$ diffusion through stomata ($a$ ~ 4.4$_{\text{dry}}$, Craig, 1953). Measurements of $\Delta$ can thus offer insights into the interplay between stomatal conductance and carbon assimilation of leaves.

But additional processes also affect net $\Delta$ values: leaf boundary layer diffusion, internal (mesophyll) diffusion, photorespiration, and day respiration. Integrating all contributions, net $^{13}$C discrimination can be calculated (Farquhar et al., 1982; Wingate et al., 2007) as:

$$ \Delta = a_b \frac{C_a - C_i}{C_s} + a_c \frac{C_a - C_i}{C_a} + a_m \frac{C_i - C_c}{C_a} + b \frac{C_i - f \Gamma^* \left(\frac{R_d}{C_a - \Gamma^* (A + R_d)}\right)}{C_s} $$

(1)

where $C_a$, $C_s$, $C_i$, and $C_c$ are the CO$_2$ mole fractions in ambient air, at the leaf surface, in the intercellular air space, and at the sites of carboxylation, respectively; $\Gamma^*$ is the compensation point in the absence of dark respiration, and $R_d$ is the rate of dark respiration. In addition to stomatal diffusion ($a$) and carboxylation ($b$), there are fractionations associated with CO$_2$ diffusion through...
the leaf boundary layer ($a_b \sim 2.9\%$) and mesophyll ($a_{mv}$ consisting of CO$_2$ dissolution [1.1\%; Vogel, 1980] and liquid phase diffusion [0.7\%; O’Leary, 1984]) into the chloroplasts, as well as photorespiration ($f$) and day respiration ($e$). A full list of symbols and abbreviations is given in Supplemental Table S1.

Several of these processes are still major sources of uncertainty for estimating $\Delta$. For example, there is no consensus on the fractionation factors during photorespiration ($f$) and day respiration ($e$), which can amplify diurnal patterns in $\Delta$ (Wingate et al., 2007). The net fractionation during carboxylation ($b$) may be lower than that of Rubisco ($b_1 \sim 29\%$) due to contributions from phosphoenolpyruvate carboxylase (PEPc; $b_1 \sim -5.7\%$; O’Leary et al., 1991). In addition, the mesophyll conductance ($g_l$) of leaves needs to be determined for the CO$_2$ mole fraction at the sites of carboxylation [$A = g_l(C_i - C_a)$]. For photorespiration, $f$ values of $7\%$ (Rooney, 1988), $8\%$ (Gillon, 1997), and $10\%$ to $14\%$ (Igamberdiev et al., 2004) have been reported from a limited number of in vivo experiments on intact leaves, with $11\%$ expected from theory (Tcherkez, 2006).

Here, we present new in vivo estimates of the fractionation factor associated with photorespiration ($f$) and the net fractionation during carboxylation ($b$), determined from leaf level $\Delta$ measurements for three species in the genus Senecio, with contrasting leaf morphology, photosynthetic rates, and stomatal sensitivities. Photorespiration rates were manipulated by varying the O$_2$ partial pressure (pO$_2$) during the experiments. The parameters $f$, $b$, and $g_l$ were treated as unknowns and determined simultaneously for all measurements. We propose this as an alternative approach to analyze measurements under field conditions when $b$ and $g_l$ are not known or cannot be determined in separate experiments.

RESULTS

Leaf Physiology and Gas-Exchange Characteristics

Patterns in gas-exchange characteristics common to all species (Table I) included higher maximum rates of photosynthesis, higher stomatal conductance, and lower $I^*$ (compensation point in the absence of dark respiration, derived from $A/C_i$ curves) at low pO$_2$ due to reduced rates of oxygenation. Day respiration rates ($R_d$) were generally low and showed little effect of pO$_2$.

In addition, gas-exchange and leaf variables exhibited systematic differences between the species. S. squalidus had the lowest specific leaf mass, $I^*$, and $R_d$ and the highest photosynthetic capacity and stomatal conductance, whereas S. greyii had the highest specific leaf mass, $I^*$, and the lowest photosynthetic capacity.

<table>
<thead>
<tr>
<th>Species</th>
<th>Specific Leaf Mass</th>
<th>PEPc Activity</th>
<th>pO$_2$</th>
<th>$I^*$</th>
<th>$A_{max}$</th>
<th>$g_l$</th>
<th>$b$</th>
<th>$R_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. squalidus</td>
<td>37.8 g m$^{-2}$</td>
<td>0.30 ± 0.05</td>
<td>20</td>
<td>2.7</td>
<td>20.6</td>
<td>0.57</td>
<td>0.27</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>210</td>
<td>38</td>
<td>14.8</td>
<td>0.44</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>60</td>
<td>9.9</td>
<td>0.40</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>S. cineraria</td>
<td>58.7 g m$^{-2}$</td>
<td>0.16 ± 0.05</td>
<td>20</td>
<td>4.0</td>
<td>16.8</td>
<td>0.46</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>210</td>
<td>41</td>
<td>12.4</td>
<td>0.38</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>70</td>
<td>6.7</td>
<td>0.31</td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>S. greyii</td>
<td>74.0 g m$^{-2}$</td>
<td>0.60 ± 0.20</td>
<td>20</td>
<td>4.1</td>
<td>11.0</td>
<td>0.19</td>
<td>0.22</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>210</td>
<td>45</td>
<td>8.4</td>
<td>0.17</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>77</td>
<td>5.1</td>
<td>0.10</td>
<td></td>
<td>0.39</td>
</tr>
</tbody>
</table>

Net Photosynthetic $^{13}$C Discrimination and Photorespiratory Fractionation

At all pO$_2$ levels, S. squalidus and S. cineraria had higher $\Delta_{obs}$ values (calculated using Eq. 5 below) than S. greyii (Fig. 1). In a qualitative comparison at similar $C_i/C_a$ ratios, $\Delta_{obs}$ measured under nonphotorespiratory conditions (20 mbar pO$_2$) was $1.6\%$ to $2.0\%$ higher than at typical atmospheric oxygen concentrations (210 mbar pO$_2$), illustrating the decrease in $\Delta_{obs}$ due to isotopically depleted CO$_2$ released during photorespiration. Except for S. greyii, this offset increased further at 300 mbar pO$_2$.

For all $\Delta_{obs}$ measurements, $\Delta_{pred}$ was calculated from gas-exchange data using Equation 1. Mesophyll conductance ($g_l$) and the fractionation factors $b$ and $f$ were treated as unknowns. A range of values was tested for these parameters: 0.1 to 0.3 $\mu$mol m$^{-2}$ s$^{-1}$ for $g_l$, 20 $\mu$mol to 30 $\mu$mol for $b$, and 0 $\%$, to 20 $\%$ for $f$. In addition, all calculations were repeated for values of $-6\%$, $0\%$, and $+6\%$ for $e$. For each parameter combination, $\Delta_{pred}$ was calculated for all data points, and a least absolute deviations regression was performed for $\Delta_{pred}$ versus $\Delta_{obs}$. The resulting regression parameters (slope, intercept, and mean absolute deviation) are presented in Figure 2 for a range of combinations of $b$ and $f$, using $e = 0\%$, and the best fit $g_l$ values (see below). Figure 2 illustrates that varying $b$ mainly affects the slope of the $\Delta_{pred}$ versus $\Delta_{obs}$ regression, whereas $f$ mainly affects the intercept.

We then determined the combinations that led to the best agreement between $\Delta_{pred}$ and $\Delta_{obs}$ for all pO$_2$ conditions (i.e. the parameter set [b, f, and $g_l$] that produced a regression with a slope of 1 and an intercept of 0; Fig. 3). This was achieved for $f = 11.6\%$, $b = 26.0\%$. 

Table I. Characteristics of three Senecio species measured at different pO$_2$ levels


Downloaded from on January 16, 2018 - Published by www.plantphysiol.org
Copyright © 2008 American Society of Plant Biologists. All rights reserved.
and \( g_i \) values of 0.27, 0.25, and 0.22 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for \( S. \) squilidus, \( S. \) cineraria, and \( S. \) greyii, respectively (Fig. 3), yielding a robust correlation \( (r^2 = 0.91) \) and small absolute deviation (0.72) between \( \Delta_{\text{pred}} \) and \( \Delta_{\text{obs}} \) values for all species and conditions combined.

For leaves assimilating carbon at a temperature of 21.4°C (Helliker and Richter, 2008), neglecting \( f \) would lead to overestimation of \( \Delta \) by 1.2\% compared with our best fit estimate of \( f = 11.6\% \). Applying the commonly used value of \( f = 8\% \) would result in a small but detectable overestimation by 0.4\% compared with our estimate of \( f \).

DISCUSSION

This article attempts to quantify the contributions from different processes on net \(^{13}\)C discrimination during photosynthesis. In particular, leaf level measurements of gas exchange and \( \Delta \) were used to determine the fractionation factor \( f \) for photorespiration in vivo under controlled laboratory conditions. Keeping everything else constant, different rates of photorespiration were achieved in our experiments by varying the oxygen partial pressure in the air surrounding the leaves. At low \( \text{pO}_2 \), the decreased oxygenase activity (as indicated by smaller \( \text{G}^* \); Table I) was manifested in 1\% to 2\% higher \( \Delta_{\text{obs}} \) at similar \( C_i/C_a \) compared with ambient or elevated \( \text{pO}_2 \) conditions (Fig. 1).

The simultaneous effects of different processes on \( \Delta \) cannot be separated easily, because \( \Delta \) contains several unknown parameters: the fractionation factors \( b, f \), and \( e \) and mesophyll conductance, \( g_i \). This problem is often addressed successively: \( g_i \) is derived using a prescribed value of \( b \) (usually 29\%\), and the residual is then assumed to reflect the contribution from photorespiration. A commonly used method to derive \( g_i \) is based on a regression of \( \Delta_{\text{obs}} - \Delta_{\text{pred}} \) versus \( A/C_a \) (Evans et al., 1986), where \( \Delta_i \) is the predicted value assuming \( C_c = C_i \) (i.e., no resistance to \( \text{CO}_2 \) transfer during internal [mesophyll] diffusion to the sites of carboxylation):

\[
\Delta_i = a \frac{C_c - C_i}{C_a} + b' \frac{C_i}{C_a}
\]

Assuming that Equation 1 reflects \( \Delta_{\text{obs}} \) (neglecting boundary layer effects for simplicity) and using \( A = g_i \), \( (C_i - C_c) \), combining Equations 1 and 2 yields:
The parameters (assumed in Equation 2 (1991)) denote the 1:1 correlation. However, this approach requires that the value of \( b \) is known and that the contributions from respiratory terms do not change in a systematic way (with \( A \)). Otherwise, any errors in the estimate of \( b \) are propagated into errors in \( g \), and affect subsequent calculations, such as the solution for \( f \). Alternatively, the difference between the actual value of \( b \) and that assumed in Equation 2 (\( b' \)) can be estimated from the \( y \) intercept of \( (\Delta_i - \Delta_{obs})C_a/C_i \) against \( A/C_i \) (von Caemmerer and Evans, 1991):

\[
(\Delta_i - \Delta_{obs}) \frac{C_a}{C_i} = \frac{b - a_m - e}{g_i} \frac{R_d}{(A + R_d)} \frac{A}{C_a} + f \frac{\Gamma^*}{C_i} + e \frac{R_d}{(A + R_d)} \frac{C_c - \Gamma^*}{C_a}
\]

Combining data from all experiments, we determined a photosynthetic fractionation factor \( f \) of 11.6\(^{go} \pm 1.5\%^{go} \). (Note that a preliminary version of this data set was presented in Table II and Fig. 6 of Ghoshal et al. [2003], with \( f \) reported as 9\(^{go} \) and 11\(^{go} \).) Our new value is larger than previous in vivo estimates (Table II) on intact leaves of 6.2\(^{go} \pm 0.5\%^{go}, 7.4\%^{go} \pm 0.3\%^{go} \) (Rooney, 1988), and 8\(^{go} \) (Gillon, 1997; revised from 0.5\(^{go} \) and 3.3\(^{go} \) [Gillon and Griffiths, 1997]). The experiments of Rooney (1988) were carried out at the CO\(_2\) compensation point (\( \Gamma \)), where photosynthetic CO\(_2\) uptake is balanced by respiratory CO\(_2\) releases. If the isotopic fluxes are at steady state, all diffusional fractionations cancel. An additional assumption was that there is no day respiration (i.e. \( \Gamma = \Gamma^* \)), so that \( C_a = C_c = C_i = C_i = \Gamma^* \), and Equation 1 can be simplified to \( \Delta = b - f \). In two experiments, \( \Delta \) was determined as 22.6\(^{go} \) and 21.4\(^{go} \) from the isotopic composition of chamber air and leaf material, yielding \( f = 6.4^{go} \) and 7.6\(^{go} \) for \( b = 29^{go} \), with \( f \) again depending on the choice of \( b \). If day respiration is included (\( \Gamma > \Gamma^* \)), then \( C_a = C_c = C_i = C_i = \Gamma \), and the above equation changes to \( \Delta = b - f \Gamma^*/\Gamma - e(1 - \Gamma^*/\Gamma) \). The

### Table II. Estimates of the fractionation factors during photorespiration (\( f \)) and Gly decarboxylation (\( g \))

<table>
<thead>
<tr>
<th>Species</th>
<th>( g ) (%)</th>
<th>( f ) (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Theoretical</strong></td>
<td>15 to 20</td>
<td>7.5 to 10</td>
<td>Abell and O’Leary (1988)</td>
</tr>
<tr>
<td>Pismium sativum, Spinacia</td>
<td>9.8, 11.4</td>
<td>8</td>
<td>Ileve et al. (1996)</td>
</tr>
<tr>
<td><em>oleracea</em></td>
<td>-16 to +8</td>
<td>11</td>
<td>Tcherkez (2006)</td>
</tr>
<tr>
<td>In vitro (mitochondria)</td>
<td>6.2, 7.4</td>
<td>8</td>
<td>Rooney (1988)</td>
</tr>
<tr>
<td>Glycine max</td>
<td>9.8, 11.4</td>
<td>8.3</td>
<td>Rooney (1988; recalculated</td>
</tr>
<tr>
<td>Triticum aestivum, Phaseolus</td>
<td>8</td>
<td>8</td>
<td>Gillon (1997)</td>
</tr>
<tr>
<td>vulgaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hordeum vulgare, Arabidopsis</td>
<td>9.8 to 13.7</td>
<td>8</td>
<td>Igamberdiev et al. (2004)</td>
</tr>
<tr>
<td>thaliana, Solanum tuberosum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. squallus, S. cineraria,</td>
<td>11.6 ( \pm ) 1.5</td>
<td>8</td>
<td>This study</td>
</tr>
<tr>
<td>S. greyii</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}f = g/2\) (Rooney, 1988; Tcherkez, 2006). \(^{b}\)Revised from Gillon and Griffiths (1997).
equation now reflects the relative contributions from photorespiration and day respiration to total respiratory fluxes, even if there is no fractionation during day respiration (e = 0). With a rough estimate of 0.73 for \( \Gamma^a/\Gamma \) (calculated from Farquhar et al. [1980], using \( V_{\text{max}} = 50 \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) and \( R_d = 1.7 \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \), with \( R_d/R_n \sim 0.3 \) at 140 \( \mu\text{L} \text{CO}_2 \text{L}^{-1} \text{m}^{-2} \text{s}^{-1} \), where \( R_n [-1 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}] \) is the nighttime respiration rate), the resulting \( f \) for the experiments of Rooney (1988) would be larger: 8.7\% for \( e = 0 \) and 10.9\% for \( e = -6 \% \), very close to our estimate of 11.6\% ± 1.5\%. Our estimate is also similar to recent results of 10\% to 14\% (Igamberdiev et al., 2004). In these experiments, photorespiration was manipulated through imposed stress (e.g. drought) and photorespiratory mutants, which may have affected other metabolic processes and complicated the identification of \( f \) itself.

Glycine decarboxylase (GDC), the enzyme responsible for \( \text{CO}_2 \) release during photorespiration, discriminates against $^{13}\text{C}$, with the resultant photorespired \( \text{CO}_2 \) depleted in $^{13}\text{C}$. GDC is a multienzyme complex consisting of four enzymes and requires pyridoxal phosphate as a cofactor (Walker and Oliver, 1986; Rooney, 1988). Because of similar reaction mechanisms, the fractionation during Gly decarboxylation (\( g \)) was expected to be in the same range as other pyridoxal phosphate-dependent decarboxylases, 15\% to 20\% (Abell and O'Leary, 1988; Rooney, 1988). As half of the substrate of GDC is converted to product (\( \text{CO}_2 \)), \( f \) should be 7.5\% to 10\% at a steady-state flux of carbon through the pathway if Gly has the same isotopic composition as current photoassimilates. Recent theoretical estimates for \( g \) were 22\% (yielding \( f \) on the order of 11\%) (Tcherkez et al., 2005; Tcherkez, 2006), very close to the value of \( f \) observed in our study. Theory predicts interactions between \( f \) and \( e \), but these are minor across the range of plausible \( f \) values (Tcherkez, 2006). The in vitro estimates of \( g \) for different \( \text{C}_4 \) species (Table II) span a large range of -16\% to +8\% (Ivlev et al., 1996; Igamberdiev et al., 2001; Ivlev, 2001). However, these results cannot easily be related to \( f \). The measurements were performed on purified enzymes or isolated mitochondria at a range of pH values and with various cofactors (e.g. NAD$^+$ and ADP) added to the reaction. As it is not known which of these experimental setups best reflects the conditions in a living cell, the in vitro estimates can only give a range of possible \( g \) values, not the most likely value for \( f \) in actively photosynthesizing cells.

We obtained a value of 26\% for \( b \), the net fractionation during carboxylation, lower than previous estimates of 27\% to 32\% (von Caemmerer and Evans, 1991). In vitro determinations of Rubisco fractionation (\( b_t \)) have yielded values of 27\% to 31\% (Roeske and O'Leary, 1984; Guy et al., 1993; McNevin et al., 2006), but the net value of \( b \) can be lower due to contributions from PEPc carboxylation (\( b_d \)). It is also possible that \( b_d \) itself is smaller in some species. For our experiments on the three \textit{Senecio} species, differences in net \( b \) values were not evident in their \( \Delta_{\text{obs}} \) data. Based on in vitro estimates of enzyme activity, \textit{S. greyii} had the highest extractable PEPc activity (Table I) and the lowest maximum photosynthetic rate (\( A_{\text{max}} \)), reflecting Rubisco activity. As PEPc discrimination has the opposite sign from that of Rubisco, \textit{S. greyii}, with the highest PEPc:\( A_{\text{max}} \), could have a lower \( b \) than \textit{S. squalidus} (PEPc: \( A_{\text{max}} \) smaller by a factor of 4). However, the \( \Delta_{\text{obs}} \) data of \textit{S. greyii} and \textit{S. squalidus} had almost identical slopes (Fig. 1), and \( \Delta_{\text{pred}} \) versus \( \Delta_{\text{obs}} \) fits well with a single \( b \) value (Fig. 3). Thus, extractable PEPc activity assayed in vitro does not appear to be a reliable indicator of the in vivo PEPc metabolic flux and its influence on \( b \), the net discrimination during carboxylation.

Because many parameter combinations gave a 1:1 slope and an intercept of 0 for \( \Delta_{\text{pred}} \) versus \( \Delta_{\text{obs}} \), the mean absolute deviation was an important criterion in determining the best fit parameters. For example, assuming \( b = 29\% \), a 1:1 fit could be achieved for \( f = 5\% \) and \( g = 0.14, 0.13, \) and 0.11 mol m$^{-2}$ s$^{-1}$ (for \textit{S. squalidus}, \textit{S. cineraria}, and \textit{S. greyii}, respectively), but the mean absolute deviation of 1.8\% was more than twice that of the best fit parameters (0.7\%). Nevertheless, a range of parameter combinations gave almost equally good agreement between \( \Delta_{\text{pred}} \) and \( \Delta_{\text{obs}} \). Specifically, all \( g \) values had a range of ±0.01 mol m$^{-2}$ s$^{-1}$ with similar regression parameters as the best fit parameters reported above. In most cases, the two herbaceous species, \textit{S. squalidus} and \textit{S. cineraria}, had higher \( g \) than the woody species with the thickest leaves, \textit{S. greyii}, as expected (Warren and Adams, 2006). Within the 0.01 range, the value obtained for \( b \) was not sensitive to changes in \( g \), but the resulting value of \( f \) changed by up to 1.5\% depending on the \( g \) used. To take this uncertainty into account, we report the value of \( f \) for our study as 11.6\% ± 1.5\%. Our analysis method is particularly useful for field measurements, in which experimental conditions cannot be as carefully controlled as in the laboratory. On the other hand, we propose that the contributions from different processes to net \( \Delta \) can be identified best by combining \( \Delta_{\text{obs}} \) data with independent measurements of \( b \) and \( g \) (through \( \Delta_{\text{obs}} \) at low \( \text{O}_2 \) and fluorescence) in controlled laboratory studies.

Our results were not sensitive to the value chosen for \( e \), the fractionation during day respiration. Repeating our analysis for \( e \) values of +6\% and -6\% (Duranceau et al., 1999; Ghashghaie et al., 2003) did not have an effect on the resulting \( b \) value and changed the resulting \( f \) by less than 0.5\%. The value of \( e \) for use in Equation 1 has not been established yet. Respiratory \( \text{CO}_2 \) signals reflect the partitioning between starch and sugars (Nogués et al., 2004; Tcherkez and Farquhar, 2005). Additional minor complications can arise from variations in \( g \) (for review, see Flexas et al., 2008) or changes in the substrate used for day respiration, for example, due to mitochondrial respiration mobilizing older carbon pools (Ghashghaie et al., 2001, 2003; Tcherkez et al., 2003, 2005; Gessler et al., 2008),
particular at low assimilation rates (Wingate et al., 2007).

Neglecting photorespiratory fractionation would lead to an overestimation by 1.2% in the mean assimilation weighted $\Delta$ for leaves at temperatures of 21.4°C (Helliker and Richter, 2008), but this deviation can be larger in arid and tropical ecosystems or during periods of higher temperatures in any ecosystem. Diurnal changes in $\Delta$ are likely to reflect variations in photorespiration even at the canopy level. For example, photorespiratory contributions can have a large increase between the typically lower morning temperatures (0.9% at 15°C) and higher midday temperatures (2% at 35°C). Thus, reliable $f$ values are required to derive accurate estimates of $\Delta$ at the canopy scale and reduce the uncertainty associated with isotopic partitioning of net CO$_2$ fluxes. At the same time, the amount of structural carbon laid down at times of enhanced photorespiration (drought, high temperature, or salinity; for review, see Wingler et al., 2000) should be minimal. However, relative rates of photorespiration would have been generally higher during periods of low atmospheric CO$_2$ such as the last glacial maximum or even in the preindustrial atmosphere compared with today’s atmosphere. For example, the contribution of photorespiration to net $\Delta$ would increase to 1.6% at 280 μmol mol$^{-1}$ and to 2.5% at 180 μmol mol$^{-1}$ atmospheric CO$_2$ mole fraction, suggesting small but detectable effects of changing photorespiratory contributions on δ$_{\text{13C}}$plant (Seibt et al., 2008).

**CONCLUSION**

We have demonstrated the effects of fractionation during photorespiration on net $\Delta$ at the leaf level. Photorespiratory fractionation was observed as a decrease in $\Delta$ at high pH$_{CO_2}$ resulting from the release of isotopically lighter CO$_2$ during the Gly decarboxylase reaction. From concurrent measurements of $\Delta$ and gas exchange, we determined the in vivo value of $f$, the photorespiratory fractionation factor, as 11.6% higher than previous estimates (Rooney, 1988; Gillon, 1997) but similar to theoretical predictions (Tcherkez, 2006). Mesophyll conductance ($g_i$) and fractionation factors ($b$ and $f$) were determined simultaneously to avoid propagating errors in $b$ or $g_i$ estimates into subsequent calculations. Our results confirm that photorespiration is an important component of the net photosynthetic discrimination of C3 plants. Photorespiratory effects on $\Delta$ should be taken into account to partition net ecosystem exchange into gross CO$_2$ fluxes at the canopy scale.

**MATERIALS AND METHODS**

**Plant Material**

Three species of the genus *Senecio* were studied: (1) *S. squallidus* (Oxford ragwort), a fast-growing, short-lived annual herb; (2) *S. cineraria*, a slower-growing, annual/biennial herb with thick hairy leaves; and (3) *S. greyii*, a slow-growing shrub with thick hairy leaves. *S. squallidus* was grown from seeds collected from specimens grown in the Botanic Gardens at the University of Cambridge and soaked overnight. Postgermination, plants were transplanted into 8-cm pots containing John Innes No. 2 compost and grown in an air-conditioned, naturally lit greenhouse for 3 weeks prior to experimentation. *S. cineraria* and *S. greyii* plants were purchased at 2 weeks and 6 months age, respectively (from Assells Nurseries). They were transplanted into 8-cm and 30-cm pots containing John Innes No. 2 compost and grown in the same greenhouse as the *S. squallidus* specimens for 2 months prior to experimentation.

**Gas-Exchange Measurements**

Gas-exchange measurements were made on the youngest fully expanded leaves using an infrared gas analyzer (CIRAS-1; PP Systems) with a 10-cm$^2$ Parkinson leaf chamber illuminated by a Walz fiber-optic lighting unit (Fiber Illuminator FL-440 and Special Fibreoptics 400-F; Walz). Compressed air ($\delta^{13}$C = 1.0%o) containing O$_2$ at partial pressures of 30, 210, and 300 mbar (BOC Special Gases) and CO$_2$ at 370 and 410 μbar were used to supply air to the chamber, bubbled through a saturated solution of NaCl at 25°C to achieve relative humidity of approximately 80%. Light response curves were performed to estimate the maximum photosynthetic rate ($A_{\text{max}}$). CO$_2$ response ($A/C$) curves were carried out on four plants at each pO$_2$ and light levels of 100, 300, and 1,000 μmol m$^{-2}$ s$^{-1}$ to obtain the compensation point in the absence of dark respiration ($P_d$) and day respiration ($P_g$) (Brooks and Farquhar, 1985).

**Measurements of $^{13}$C Discrimination**

Attached leaves were placed in the leaf chamber and acclimated to the chamber conditions for 20 min. Flow rates were maintained at 250 mL min$^{-1}$ to obtain large CO$_2$ depletions across the chamber. A range of $C_i/C_a$ values was achieved by measuring each leaf at high and low light (900 and 250 μmol m$^{-2}$ s$^{-1}$). Four plants were sampled for each species, and measurements at all three pO$_2$ levels were performed on the same leaf. The CO$_2$ in the air exiting the chamber was trapped cryogenically (for detailed description, see Broadmeadow et al., 1992). Briefly, air from downstream of the cuvette (analysis gas) was passed at positive pressure to a glass collection line at a rate of 150 mL min$^{-1}$ via a needle valve. The CO$_2$ was trapped by passing the air through a cold trap submerged in liquid N$_2$ at a pressure of less than 0.6 kPa. The line was then isolated and evacuated to 10$^{-1}$ kPa, after which the CO$_2$ was liberated from the cold trap in acetone at 80°C to retain water vapor. The CO$_2$ was then drawn into a removable vial, which was sealed with a butane gas torch. Gas-exchange parameters were recorded on the infrared gas analyzer before and after CO$_2$ collection, with the mean of both readings used in the analyses. Reference gas was collected at the start of each experimentation day and after every fourth sample collection. The samples of analysis and reference gas were purified via two further cryodistillations (Borland et al., 1993) and analyzed on a VG-903 dual-inlet triple collector mass spectrometer (modified by Provac Services). The $^{13}$C/$^{12}$C ratios of the samples were determined against those of $\delta^{13}$C = 42.5%o, BDH) and reported with respect to the PeeDee Belemnite standard. Defining net photosynthetic discrimination as $\Delta = \delta_{\text{13C}}/\delta_{\text{12C}} - 1$, where $\delta_{\text{13C}}$ and $\delta_{\text{12C}}$ are the isotope ratios of atmospheric CO$_2$ and the product (e.g. photoassimilates), the observed $^{13}$C discrimination in a leaf cuvette during photosynthesis ($\Delta_{\text{obs}}$) was determined following Evans et al. (1986) from:

$$\Delta_{\text{obs}} = \frac{\xi(\delta^{13}C_i - \delta^{13}C_a)}{1 + \delta^{13}C_i - \delta^{13}C_a}$$

where $\xi = C_i/C_a - C_i$, and $C_i$ and $C_a$, $\delta^{13}C_i$, and $\delta^{13}C_a$ refer to the mole fractions and isotope ratios of CO$_2$ in air entering and exiting the leaf cuvette, respectively.

**Determination of PEPc Activity**

Frozen leaf tissue (200 mg) was extracted at 4°C in 2 mL of buffer containing 200 mM Tris base (pH 8), 2 mM EDTA, 2% polyethylene glycol 20,000, 1 mM dithiothreitol, 1 mM benzamidine, 10 mM malate, and 350 mM NaHCO$_3$. Samples were centrifuged at 12,000g for 3 min, and the supernatant was desalted on a Sephadex G-25 column. PEPc activity was measured as the oxidation of NADH in the presence of PEP, malate dehydrogenase, and total leaf protein (Chu et al., 1990). Briefly, NADH consumption was measured over
6 min at 340 nm using a UV-300 spectrophotometer (Spectronic Unicam), with the reaction initiated by the addition of 100 μL of extract to 850 μL of assay cocktail (65 mM Tris base [pH 7.8], 0.2 mM NADH, 10 mM NaHCO3, and 5 mM MgCl) with 2 mM PEP. Total soluble protein content of extracts was obtained by mixing 10 μL of protein extract with 90 μL of water and 4 mL of Bradford reagent (Bradford, 1976). The sample was precipitated for 15 min and the absorbance at 595 nm was measured. Protein content was calibrated across a range of 0 to 100 mg using a 1-ng stock of bovine serum albumin in water.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. List of abbreviations and symbols used in the text.

ACKNOWLEDGMENTS

We thank Barney Davies for his help with the PEPc protocol. We are grateful to Glynn Jones for technical assistance with isoatitude ratio mass spectrometry. We thank Guillaume Tcherkez, Jaleh Ghashghaie, and Graham Farquhar for valuable discussions and the anonymous reviewers for their helpful comments on this and an earlier version of the manuscript.

Received September 20, 2008; accepted October 12, 2008; published October 15, 2008.

LITERATURE CITED


Tcherkez G, Farquhar GD (2005) Carbon isotope effect predictions for...
enzymes involved in the primary carbon metabolism of plant leaves.


