REVIEW PAPER

The efficiency of C₄ photosynthesis under low light conditions: assumptions and calculations with CO₂ isotope discrimination

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Abstract

Leakiness (f), the proportion of carbon fixed by phosphoenolpyruvate carboxylation that leaks out of the bundle-sheath cells, determines C₄ photosynthetic efficiency. Large increases in f have been described at low irradiance. The underlying mechanisms for this increase remain uncertain, but changes in photorespiration or the energy partitioning between the C₄ and C₃ cycles have been suggested. Additionally, values of f at low light could be magnified from assumptions made when comparing measured photosynthetic discrimination against ¹³C (Δ) with the theoretical formulation for Δ. For example, several simplifications are often made when modelling Δ to predict f including: (i) negligible fractionation during photorespiration and dark respiration; (ii) infinite mesophyll conductance; and (iii) CO₂ inside bundle-sheath cells (Cₛ) is much larger than values in mesophyll cells (Cₘ). Theoretical models for C₄ photosynthesis and C₄ Δ were combined to evaluate how these simplifications affect calculations of Δ and f at different light intensities. It was demonstrated that the effects of photorespiratory fractionations and mesophyll conductance were negligible at low light. Respiratory fractionation was relevant only when the magnitude of the fractionation factor was artificially increased during measurements. The largest error in estimating f occurred when assuming Cₛ was much larger than Cₘ at low light levels, when bundle-sheath conductance was large (gₛ), or at low O₂ concentrations. Under these conditions, the simplified equation for Δ overestimated f, and compromised comparisons between species with different gₛ, and comparisons across O₂ concentrations.

Key words: Bundle-sheath conductance, carbon isotope discrimination, CO₂ leakiness, C₄ photosynthesis, Flaveria bidentis, gas exchange, low light, mesophyll conductance, photorespiration.

Introduction

The conversion of CO₂ into plant biomass is mediated by the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). However, Rubisco catalyses both the oxygenation and carboxylation of ribulose-1,5-bisphosphate (RuBP); therefore, under current atmospheric conditions (380 µmol mol⁻¹ CO₂ and 210 mmol mol⁻¹ O₂) the high O₂ concentration inhibits the carboxylation reaction. To reduce the oxygenation reaction (i.e. photorespiration) some plants have evolved a mechanism to concentrate CO₂ around Rubisco. This CO₂-concentrating mechanism, known as C₄ photosynthesis, initially fixes bicarbonate (HCO₃⁻) and phosphoenolpyruvate (PEP) in the mesophyll cells via phosphoenolpyruvate carboxylase (PEPc) into four-carbon acids (Hatch et al., 1967). In Kranz-type C₄ plants, the C₄ acids are subsequently decarboxylated, releasing CO₂ around Rubisco compartmentalized within the bundle-sheath cells (Kanai and Edwards, 1999). The [CO₂] in the bundle-sheath cells (Cₛ) is ~10-fold higher than air concentrations (Furbank and Hatch, 1987), reducing rates of photorespiration to 2–3% of photosynthesis (Jenkins et al., 1989b). However, the improved efficiency of Rubisco carboxylation and inhibition of photorespiration comes with an energetic cost of ATP (Hatch, 1987; Edwards et al., 2000). In general, the extra...
energy needed in the C₄ pathway is required for (i) regenerating PEP [two ATP per CO₂ fixed in NADP-malic enzyme (NADP-ME) and NAD-ME plants] (Hatch, 1987; Kanai and Edwards, 1999) and (ii) overcycling CO₂ to compensate for the leakage of CO₂ out of the bundle-sheath cells.

Leakiness (φ) has been defined as the proportion of carbon fixed by PEP carboxylation, \( V_p \), which subsequently leaks out of the bundle-sheath cells, where \( L \) is the leak rate and \( φ = L/V_p \) (Farquhar, 1983; Hatch et al., 1995). The amount of φ determines the additional energetic cost of the CO₂-concentrating mechanism. Despite φ being a fundamental parameter describing C₄ photosynthetic efficiency, it is difficult to quantify and cannot be directly measured. Leakiness has been estimated using \(^{14}\)C labelling (Hatch et al., 1995), mathematical modelling (Farquhar, 1983; Jenkins et al., 1989b; He and Edwards, 1996), and coupling measurements of photosynthetic discrimination against \(^{13}\)C (Δ) and gas exchange (Evans et al., 1986; von Caemmerer et al., 1997), with values ranging from 0.08 to 0.5 across C₄ subtypes.

The isotope method derives φ by comparing measured Δ with a theoretical model where φ is assumed to be the only unknown. In this situation Δ during C₄ photosynthesis is typically described by using a simplification of a more complete model presented by Farquhar (Eqn 11, 1983)

\[
\Delta_i = \frac{a(C_a - C_i)}{C_a} + \left[b_i + φ(b_i - s)\right] \frac{C_i}{C_a}
\]

where \( C_a \) and \( C_i \) are the CO₂ mol fractions in the atmosphere and the leaf intercellular spaces, respectively, and \( a \) (4.4\%o), \( s \) (1.8\%o), \( b_i \) (30\%o), and \( b_i' \) (–5.7\%o) are fractionations during CO₂ diffusion in air (Craig, 1953), leakage of CO₂ out of the bundle-sheath cells (Henderson et al., 1992), fractionation by Rubisco (Roeske and O’Leary, 1984), and net effect of CO₂ dissolution, hydration, and PEPC activity (Farquhar, 1983). This equation is a simplification of a more complex model, that assumes (i) there is no fractionation associated with photorespiration and dark respiration; (ii) CO₂ is in equilibrium with HCO₃⁻ in the mesophyll cytoplasm; (iii) mesophyll conductance is infinite; and (iv) CO₂ inside the bundle-sheath cells (\( C_i \)) is much larger than in the mesophyll cells. With this approach, changes in Δ for a given \( C_i/C_a \) are attributed entirely to φ. However, if the simplifying assumptions are violated, then changes in Δ cannot be directly attributed to φ.

Using this type of analysis φ has been shown to be relatively constant over a wide range of CO₂ concentrations, temperatures, and C₄ subtypes (Henderson et al., 1992; Cousins et al., 2008); however, increases in φ have been shown at low irradiance levels. This change in φ in response to light has been shown in several species including Flaveria bidentis (Cousins et al., 2006; Pengelly et al., 2010), Amaranthus cruentus (Tazoe et al., 2006, 2008), Amaranthus edulis (Henderson et al., 1992), Zea mays (Henderson et al., 1992; Kromdijk et al., 2010), and Miscanthus×giganteus (Kromdijk et al., 2008). Alternatively, no changes in Δ or φ were seen at low light in Sorghum bicolor (Henderson et al., 1992), and in Sorghum nigrum and Boerhavia coccinea (Kubasek et al., 2007).

The underlying mechanisms explaining the increase in φ at low irradiance remain uncertain. Potentially, at low irradiance small fluxes through the C₄ and C₃ cycles (Leegood et al., 1989) result in low \( C_i \) values (Furbank and Hatch, 1987) and probably high rates of photorespiration (Henderson et al., 1992; Kromdijk et al., 2010). Under these photorespiratory conditions, an increase in φ is expected due to the low ratio of [CO₂] to [O₂] in the bundle-sheath cells. This hypothesis was supported theoretically by the C₄ model of Laisk and Edwards (2000) and experimentally by Kromdijk et al. (2010), who found that Δ and φ increased with increasing O₂ partial pressure. However, Kromdijk et al. (2010) also concluded that photorespiration alone could not explain all the changes in φ at low light. Alternatively, at very low light intensities (<100 μmol quanta m⁻² s⁻¹), a significant portion of the CO₂ contained in the bundle-sheath cells originated from mitochondrial respiration. This impact of respiration on \(^{13}\)CO₂ exchange may also influence the estimations of φ using the predicted and measured values of Δ.

Additionally, increased φ at low irradiance could be explained by changes in the energy distribution between the C₄ and C₃ cycles. C₄ photosynthesis requires a precise coordination of both cycles to minimize φ and excess energy consumption. Of the five ATP required per CO₂ assimilated during C₄ photosynthesis, two are for the C₄ cycle to regenerate PEP. If PEP regeneration is compromised, then not enough CO₂ can be pumped inside the bundle-sheat cells, and photorespiration increases. Alternatively, if the C₃ cycle is energy limited, φ will increase if the C₄ cycle is not also limited. Changes of energy distribution could explain temporary changes in φ, but over time a regulation between both cycles could be expected. There are feedbacks between C₄ and C₃ cycles (Furbank et al., 2000), such as the ability to share reduction of phosphoglycerate (PGA) to triose phosphate between bundle-sheath cells and mesophyll chloroplast. For example, interconversion of PGA and PEP was shown to occur in maize (Furbank and Leegood, 1984). Additionally, light regulates the activity of key enzymes, probably to ensure the conservation of metabolite levels (Furbank et al., 1997). Tazoe et al. (2008) found that concurrently with high φ at low light, Rubisco activation state, pyruvate orthophosphate dikinase (PPDK) initial activity, and the phosphorylation state of PEPC decreased, which indicated that the carboxylation in C₃ and C₃ cycles simultaneously decreased.

Finally, larger φ at low light could originate from the nature of the calculations. Several simplifications are generally made in the equations for Δ calculations, which may not be applicable under low light environments. Initial calculations by Henderson et al. (1992) showed that fractionations during respiration and photorespiration might have a small effect on Δ calculations and subsequent φ derivation. These authors showed that by altering the value of \( b_i' \) by 3%o, leakiness estimations were changed only by 0.03. The same authors illustrated that accounting for a [CO₂] drop between the leaf intercellular spaces and the
mesophyll cells of 60 μbar, modified φ by <0.04. However, the effect of ignoring $C_n$ in the calculations has not yet been investigated. This might be the more critical simplification for $φ$ calculations at low light, where $C_n$ is expected to be low (Furbank and Hatch, 1987). Light response studies in $Z$. mays have shown increases in observed $Δ$ from high light to low light conditions of $~1.5\%$ (Henderson et al., 1992) and $~6\%$ (Kromdijk et al., 2010). Using the simplified Equation 1, variation in observed $Δ$ is attributed only to changes in $φ$, suggesting that the change in $φ$ is different in these two studies. However, both Henderson (1992) and Kromdijk (2010) concluded that $φ$ doubled from high ($~0.2$) to low light ($~0.4$). This discrepancy is attributed to Kromdijk et al. (2010) calculating $φ$ by comparing observed $Δ$ with the theoretical value taking into account $C_n$ as well as respiratory and photorespiratory fractionations, whereas Henderson et al. (1992) used Equation 1, which ignores these terms. This example illustrates the large impact that different formulas for calculating $Δ$ can have on estimates of $φ$.

To our knowledge, there has not been an assessment on how calculations for $φ$ vary when different formulations of $Δ$ are used in response to changing light levels. Because $φ$ is derived by comparing observed and theoretical $Δ$ values, the choice of equation is critical. Here, using a modelling approach that combines theoretical models for $C_4$ photosynthesis (von Caemmerer, 2000) and $C_4$ photosynthetic discrimination against $^{13}C$ (Farquhar, 1983) it is explained how $C_n$ values, fractionations during respiration and photorespiration, and mesophyll conductance affect calculations of $Δ$ and $φ$ at different light intensities. As an example, measurements of $Δ$ in Flaveria bidentis at different light intensities and $O_2$ concentrations are used to illustrate the impact of different formulations of $Δ$ in calculations of $φ$.

**Model for light-limited $C_4$ photosynthesis**

The photosynthetic electron transport chain generates the energy required for the $C_4$ and $C_3$ cycles. Total electron flux ($J_t$) can be calculated from a theoretical relationship with absorbed irradiance as (in the following equations the first number in brackets identifies the equation in this manuscript and the second number refers to numeration in von Caemmerer (2000)):

$$0J_t^2 - J_t(I_2 + J_{max}) + I_2J_{max} = 0 \quad (2, 4.36)$$

where $θ$ (see Table 1 for variable definitions and units) is an empirical curvature factor, $J_{max}$ is the maximum electron transport rate, and $I_2$ is the useful light absorbed by photosystem II (PSII) and is calculated as:

$$I_2 = \frac{I \times \text{abs}(1-F)}{2} \quad (3, 2.14)$$

where $I$ is the incident irradiance, $\text{abs}$ is the leaf absorbance, and $F$ is a coefficient to correct for spectral quality of light. Equation 3 assumes that 50% of the absorbed light is partitioned to PSII. However, this proportion might be lower and variable among species. Edwards and Baker (1993) suggested that for $Z$. mays, the energy partitioned to PSII was 40% of the absorbed light. This value was assumed to be 50% in the present modelling exercise for simplicity, generality, and because it is a conservative approach.

From Equation 2 and solving for $J_t$:

$$J_t = \frac{I_2 + J_{max} - \sqrt{(I_2 + J_{max})^2 - 4I_2J_{max}}}{2} \quad (4, 2.15)$$

Equation 4 allows for the calculation of $J_t$ for a given absorbed irradiance as a function of $θ$ and $J_{max}$. As previously noted, an allocation of 50% of the absorbed irradiance to PSII was assumed. A lower value (40%) would have slightly reduced values for $J_t$ (by $\leq 10 \mu$ mol electrons m$^{-2}$ s$^{-1}$ in the present modelling exercise). $J_t$ can be partitioned between the $C_4$ cycle ($J_m$) and the $C_3$ cycle ($J_s$), where $x$ is the portion of ATP required for $J_m$ and $1-\gamma$ is the requirement for $J_s$. The theoretical minimum cost of the $C_4$ photosynthetic pathway is five ATP, three for the $C_3$ cycle and two required to regenerate PEP from pyruvate in the mesophyll cells, so it is generally assumed that $x=0.4$ ($=2/5$). von Caemmerer (2000) calculated $x$ values following an optimization procedure and found that $x=0.404$ for a wide range of irradiances. Accordingly, for the present calculations $x=0.4$ was used. However, $x$ may vary among species, growing conditions, or light environments (Kromdijk et al., 2010). For example, von Caemmerer (2000) analysis concluded that the optimal partitioning was 0.417 if there was a significant O$_2$ evolution in the bundle-sheath cells and 0.415 for large bundle-sheath conductance.

Assuming that photophosphorylation operates with Q-cycle activity, the energetic costs of the $C_4$ and $C_3$ cycles can be described respectively as:

$$J_m = 2V_p \quad (5, 4.31)$$

$$J_s = 3 \times \left(1 + \frac{τpO_s}{3C_s}\right) \times V_c \quad (6, 4.33)$$

where $V_p$ and $V_c$ are PEP and Rubisco carboxylation rates respectively, $τp$ is half of the reciprocal of Rubisco specificity, $O_s$ and $C_s$ are the $O_2$ and $CO_2$ mol fraction in the bundle-sheath cells, respectively.

This energetic requirement can be incorporated into a general equation for photosynthesis ($A$):

$$A = (1 - \frac{Γ^*}{C})V_c - R_d \quad (7, 2.19)$$

by solving Equation 6 for $V_c$ and substituting it in Equation 7 with $J_s=(1-x)J_t$ and $Γ^*=Γ^*O_s$ resulting in:

$$A = \frac{(1 - \frac{τpO_s}{C_s})(1-x)J_t}{3 \times \left(1 + \frac{τpO_s}{3C_s}\right)} - R_d \quad (8, 4.38)$$

where variables are previously defined and $R_d$ is the leaf mitochondrial respiration rate. In Equation 8, the variables...
Table 1. Variable definitions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Values/units</th>
</tr>
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<tr>
<td>$A$</td>
<td>Leaf photosynthetic rate</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$a$</td>
<td>$^{13}$C fractionation due to diffusion of CO$_2$ in air (Craig, 1953)</td>
<td>$4.4%_a$</td>
</tr>
<tr>
<td>$a_{d}$</td>
<td>$^{13}$C fractionation due to diffusion of CO$_2$ through water (O’Leary, 1984)</td>
<td>$0.7%_o$</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Fraction of PSI activity in the bundle sheath</td>
<td>$0 &lt; \alpha &lt; 1$, 0.2 in this study</td>
</tr>
<tr>
<td>$b_3$</td>
<td>$^{13}$C fractionation due to carboxylation by Rubisco including respiration and photorespiration fractionations, Equation 25 (Farquhar, 1983)</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$b’_3$</td>
<td>$^{13}$C fractionation due to carboxylation by Rubisco (Roeseke and O’Leary, 1984)</td>
<td>$30%_o$</td>
</tr>
<tr>
<td>$b_4$</td>
<td>Net fractionation by CO$_2$ dissolution, hydration, and PEPc including respiratory fractionation, Equation 26 (Farquhar, 1983)</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$b_{LC}$</td>
<td>Net fractionation by CO$_2$ dissolution, hydration, and PEPc including respiratory fractionation and CA, Equation 27 (Farquhar, 1983)</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$b’_4$</td>
<td>Net fractionation by CO$_2$ dissolution, hydration, and PEPc activity (Farquhar, 1983)</td>
<td>$-5.7%_o$ at 25 °C, but variable with temperature</td>
</tr>
<tr>
<td>$C_a$</td>
<td>CO$_2$ mol fraction in the atmosphere</td>
<td>$380 \mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_i$</td>
<td>CO$_2$ mol fraction in the leaf intercellular spaces</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_m$</td>
<td>CO$_2$ mol fraction in the mesophyll cells</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_s$</td>
<td>CO$_2$ mol fraction in the bundle-sheath cells, Equations 15 and 16</td>
<td>$\mu$mol mol$^{-1}$</td>
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<tr>
<td>$\Delta$</td>
<td>Photosynthetic discrimination against $^{13}$C</td>
<td>$\mu$mol$^{-1}$</td>
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<tr>
<td>$\Delta_4$</td>
<td>Photosynthetic discrimination against $^{13}$C excluding boundary layer, mesophyll conductance, and $C_{s}$, Equation 31 (Farquhar, 1983)</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$\Delta_{bc}$</td>
<td>Photosynthetic discrimination against $^{13}$C excluding boundary layer and mesophyll conductance, Equation 29</td>
<td>$%_o$</td>
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<tr>
<td>$\Delta_{bc}$</td>
<td>Photosynthetic discrimination against $^{13}$C excluding boundary layer, Equation 28</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$b_{bc}$</td>
<td>Observed $^{13}$C photosynthetic discrimination</td>
<td>$%_o$</td>
</tr>
<tr>
<td>$s^{13}$C$_a$</td>
<td>Carbon isotope composition ($R_a/R_{mol}=1$) x 1000</td>
<td>$%_o$</td>
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<tr>
<td>$e$</td>
<td>$^{13}$C fractionation during decarboxylation (Ghashghaie et al., 2001; Hymus et al., 2005; Barbour et al., 2007)</td>
<td>$0%_o$ to $-10%_o$</td>
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<tr>
<td>$e’$</td>
<td>$^{13}$C fractionation during decarboxylation including measurements artefacts (depleted reference tank) (Wingate et al., 2007), Equation 34</td>
<td>$%_o$</td>
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<tr>
<td>$e_s$</td>
<td>$^{13}$C fractionation during internal CO$_2$ dissolution (Mock et al., 1974; Vogel, 1980)</td>
<td>$1.1%_o$</td>
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<tr>
<td>$f$</td>
<td>$^{13}$C fractionation during photorespiration (Rooney, 1988; von Caemmerer and Evans, 1991; Gillon and Griffiths, 1997; Scartazzia et al., 1998; Lanigan et al., 2008)</td>
<td>$0$–$11.6%_o$</td>
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<td>$F$</td>
<td>Coefficient to correct for spectral quality of light (Evans, 1987)</td>
<td>$0.15$</td>
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<tr>
<td>$\phi$</td>
<td>Leakiness, proportion of carbon fixed by PEPc, which leaks out of the bundle-sheath cells, Equation 23 (Farquhar, 1983; Hatch et al., 1995)</td>
<td>Unitless</td>
</tr>
<tr>
<td>$\phi_a$</td>
<td>Leakiness derived from $\Delta_a$, Equation 33 (Evans, 1983)</td>
<td>Unitless</td>
</tr>
<tr>
<td>$\phi_m$</td>
<td>Leakiness derived from $\Delta_{bc}$, Equation 32 (Evans, 1983)</td>
<td>Unitless</td>
</tr>
<tr>
<td>$g_m$</td>
<td>Mesophyll (transfer or internal) conductance to CO$_2$</td>
<td>mol m$^{-2}$ s$^{-1}$ bar$^{-1}$</td>
</tr>
<tr>
<td>$g_s$</td>
<td>Bundle-sheath conductance to CO$_2$</td>
<td>mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Half of the reciprocal of Rubisco specificity (von Caemmerer et al., 1994)</td>
<td>$0.000193$</td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>CO$_2$ compensation point in the absence of dark respiration ($=^{13}$O$_2$) (von Caemmerer, 2000)</td>
<td>$1.1%_o$</td>
</tr>
<tr>
<td>$h$</td>
<td>Catalysed fractionation during CO$_2$ hydration (Cousins et al., 2006)</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$I_i$</td>
<td>Incident radiation</td>
<td>$\mu$mol PFD mol$^{-2}$ s$^{-1}$</td>
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<tr>
<td>$I_L$</td>
<td>Useless light absorbed by PSII, Equation 3 (Evans, 1983)</td>
<td>$\mu$mol PFD mol$^{-2}$ s$^{-1}$</td>
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<td>$J_i$</td>
<td>Part of $J_i$ allocated to the C$_4$ cycle, Equation 5 (Evans, 1983)</td>
<td>$\mu$mol electrons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$J_{i\max}$</td>
<td>Maximum electron transport rate</td>
<td>$\mu$mol electrons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$J_i$</td>
<td>Total electron transport rate, Equation 4 ($J_i=J_m+J_k$)</td>
<td>$\mu$mol electrons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$J_m$</td>
<td>Part of $J_i$ allocated to the C$_3$ cycle, Equation 6 (Evans, 1983)</td>
<td>$\mu$mol electrons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$L$</td>
<td>Rate of CO$_2$ leakage from the bundle sheath to the mesophyll, Equations 21 and 22</td>
<td>$\mu$mol mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$O_2$</td>
<td>O$_2$ mol fraction in the mesophyll cells</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$O_{s}$</td>
<td>O$_2$ mol fraction in the bundle-sheath cells</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$\theta$</td>
<td>Empirical curvature factor (Evans, 1989)</td>
<td>$0.1$ in this study but variable</td>
</tr>
<tr>
<td>$R_a$</td>
<td>Leaf mitochondrial respiration rate</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$R_m$</td>
<td>Mesophyll mitochondrial respiration rate ($R_m=0.5R_a$) (von Caemmerer, 2000)</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$s$</td>
<td>Fractionation during leakage of CO$_2$ out of the bundle-sheath cells (Henderson et al., 1992)</td>
<td>$1.8%_o$</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Rubisco carboxylation rate, Equations 18 and 19 (Evans, 1983)</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$V_i$</td>
<td>CO$_2$ hydration rate</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$V_o$</td>
<td>Oxygenation rate, Equation 20 (Evans, 1983)</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$V_G$</td>
<td>PEP carboxylation rate, Equation 21 (Evans, 1983)</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$x$</td>
<td>Fraction of $J_i$ allocated to the C$_4$ cycle</td>
<td>$0.4$ in this study</td>
</tr>
</tbody>
</table>
\( \gamma^* \) and \( x \) are constants, \( J_i \) is calculated with Equation 4, and \( O_s \) is calculated as:

\[
O_s = \frac{xA}{0.047g_s} + O_m \tag{9, 4.16}
\]

where \( g_s \) is the bundle-sheath conductance to \( \text{CO}_2 \), \( O_m \) is the \( \text{O}_2 \) mol fraction in the mesophyll cells, and \( \alpha (0 < \alpha < 1) \) is the fraction of PSII activity in the bundle-sheath. von Caemmerer (2000) suggested that for species such as sorghum and maize, \( \alpha = 0 \), but \( \alpha \) could approach 0.5 in other species. An intermediate value of \( \alpha = 0.2 \) was chosen here.

Equation 8 cannot be directly solved for \( A \) because \( C_s \) is unknown. Therefore, another expression is used to calculate \( A \):

\[
A = V_p - L - R_m = \frac{xJ_i}{2} - g_s(C_s - C_m) - R_m \tag{10, 4.3 and 4.37}
\]

where \( R_m \) is the mesophyll mitochondrial respiration rate. By combining Equations 8–10 a quadratic expression for \( C_s \) is derived whose solution is given by:

\[
A = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \tag{11, 4.41}
\]

where

\[
a = 1 - \frac{7\gamma^* \times \alpha}{3 \times 0.047} \tag{12, 4.42}
\]

\[
b = - \left\{ \left( \frac{xJ_i}{2} - R_m + g_s C_m \right) + \left( \frac{1 - \alpha} {3} \right) J_i - R_d \right\}
+ \left( \frac{7\gamma^* \times O_m}{3} \right) + \frac{\gamma^*}{0.047} \left( \frac{(1 - \alpha) J_i}{3} + \frac{7R_d}{3} \right) \tag{13, 4.43}
\]

\[
c = \left[ \left( \frac{xJ_i}{2} - R_m + g_s C_m \right) \left( \frac{(1 - \alpha) J_i}{3} - R_d \right) \right]
- g_s \gamma^* O_m \left( \frac{(1 - \alpha) J_i}{3} + \frac{7R_d}{3} \right) \tag{14, 4.44}
\]

Equation 11 describes \( A \) as a function of energy requirements for both the \( C_4 \) and \( C_3 \) cycles. This equation calculates \( A \) with only a few parameters including respiration rates \( (R_d \text{ and } R_m) \), bundle-sheath conductance to \( \text{CO}_2 \) \( (g_s) \), \( \text{CO}_2 \) partial pressure in the mesophyll cells \( (C_m) \), and total electron flux \( (J_i) \). \( R_d \) can be estimated using gas exchange measurements, and \( R_m \) is commonly assumed to be half of \( R_d \) (von Caemmerer, 2000). The variable \( g_s \) cannot be measured directly but is estimated to range between 0.001 mol m\(^{-2}\) s\(^{-1}\) and 0.01 mol m\(^{-2}\) s\(^{-1}\) (von Caemmerer and Furbank, 2003). \( C_m \) is often assumed to be equal to \( C_o \), but it can also be calculated for different values of mesophyll conductance. With the modelled relationships between light, \( A \), and \( J_i \), several variables that describe the \( C_4 \) photosynthesis including \( C_s \), \( V_p \), \( V_c \), \( V_o \), \( L \), and \( \phi \) can be calculated (see Table 1 for variable definitions and Table 2 for the derived equations; Equations 15–23).

Model for \( C_4 \) photosynthetic discrimination

The original formulation for \( C_4 \) discrimination presented in Farquhar (1983, Eqn 7b, substituting \( L = \phi \times V_p \) and ignoring the boundary layer) is:

\[
A_{\text{com}} = \frac{a(C_o - C_i)}{C_o} + (e_s + a_t) \frac{C_t - C_m}{C_o} + \frac{b_3}{1 + \frac{g_s C_m}{C_o}} \times \left( \frac{C_m}{C_o} \right) \tag{24}
\]

where terms are as previously defined (Table 1), and \( a (4.4\%) \), \( e_s (1.1\%) \), \( a_t (0.7\%) \), and \( s (1.8\%) \) are fractionations during \( \text{CO}_2 \) diffusion in air (Craig, 1953), dissolution in water (Mook et al., 1974), diffusion of aqueous \( \text{CO}_2 \) (O’Leary, 1984), and leakage of \( \text{CO}_2 \) out of the bundle-sheath cells (Henderson et al., 1992). The terms \( b_3 \) and \( b_4 \) are defined as (Farquhar, 1983):

\[
b_3 = b_3' - \frac{eR_d}{V_c} - \frac{fV_d}{V_c} \tag{25}
\]

\[
b_4 = b_4' - \frac{eR_m}{V_p} \tag{26}
\]

### Table 2. Equations to derive \( C_4 \) photosynthesis parameters

The first number in parentheses identifies the equation in this manuscript. The second number refers to numeration used in von Caemmerer (2000)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_o )</td>
<td>( C_o = \frac{1 - \gamma^* \times \alpha}{\gamma^* \times \alpha} ) ( (15, 4.39) ) ( C_s = \frac{1 - \gamma^* \times \alpha}{\gamma^* \times \alpha} + C_m ) ( (16, 4.12) )</td>
</tr>
<tr>
<td>( V_p )</td>
<td>( V_p = \frac{L}{L - R_m} ) ( (17, \text{ solved from } 4.31) )</td>
</tr>
<tr>
<td>( V_c )</td>
<td>( V_c = \frac{1}{3} \frac{(1 - \gamma^<em>)}{(1 + \gamma^</em>)} ) ( (18, \text{ solved from } 4.33) )</td>
</tr>
<tr>
<td>( V_o )</td>
<td>( V_o = \frac{V_p - A}{V_p} ) ( (20, \text{ solved from } 4.1) )</td>
</tr>
<tr>
<td>( L )</td>
<td>( L = V_p - A - R_m ) ( (21, \text{ solved from } 4.3) )</td>
</tr>
<tr>
<td>( \phi )</td>
<td>( \phi = \frac{1}{5} ) ( (23, 4.5) )</td>
</tr>
</tbody>
</table>
where \( b'_{3} \) is fractionation by Rubisco, 30\%{\text{o}} (Roeske and O’Leary, 1984), and \( b'_{4} \) represents the net effect of CO\(_{2}\) dissolution, hydration, and PEPC activity, which at 25 °C has a value of −5.7\%{\text{o}} (Farquhar, 1983; Henderson et al., 1992). There is some uncertainty about the fractionation values for respiration (\( e \)) and photorespiration (\( f \)) as discussed in subsequent sections.

Additionally, if carbonic anhydrase (CA) activity is limiting CO\(_{2}\) and HCO\(_{3}\) isotopic equilibrium, then the term \( b_{4} \) can be replaced by (Farquhar, 1983):

\[
b_{4c} = b_{4} \left( 1 - \frac{V_{p}}{V_{h}} \right) + (e_{s} + h) \left( \frac{V_{p}}{V_{h}} \right) - eR_{m} \left( \frac{V_{p}}{V_{h}} \right) \tag{27}
\]

where \( V_{h} \) is the rate of CO\(_{2}\) hydration and \( h \) (1.1\%{\text{o}}) is the catalysed fractionation during CO\(_{2}\) hydration (Cousins et al., 2006).

If \( C_{m} \) is expressed as a function of \( C_{i} \) and mesophyll conductance (\( C_{m} = C_{i} - A/g_{m} \)), Equation 24 transforms to:

\[
\Delta_{ms} = \frac{a(C_{a} - C_{i})}{C_{a}} + \frac{(e_{s} + a_{l})A}{g_{m}C_{a}} + \frac{b_{4}(C_{i} - C_{a} + \frac{A}{g_{m}}) + b_{4}(C_{i} - C_{a} + \frac{A}{g_{m}})}{C_{a} - C_{i} + \frac{A}{g_{m}}} + \phi \left( C_{i} - C_{a} + \frac{A}{g_{m}} \right) \times \frac{C_{i} - C_{a} + \frac{A}{g_{m}}}{C_{a}} \tag{28}
\]

Several simplifications are often applied to Equation 28. First, if \( C_{i} \) is much larger than \( C_{m} \), then \( C_{i}/(C_{i} - C_{m}) \) is ≈ 0. In this case, Equation 28 simplifies to:

\[
\Delta_{m} = \frac{a(C_{a} - C_{i})}{C_{a}} + \frac{(e_{s} + a_{l})A}{g_{m}C_{a}} + b_{4} + \phi \left( C_{i} - C_{a} + \frac{A}{g_{m}} \right) \times \frac{C_{i} - C_{a} + \frac{A}{g_{m}}}{C_{a}} \tag{29}
\]

Additionally, if \( g_{m} \) is considered infinite (\( C_{i} = C_{m} \)), Equation 28 simplifies to:

\[
\Delta_{is} = \frac{a(C_{a} - C_{i})}{C_{a}} + \left( b_{4} + \phi \left( C_{i} - C_{a} + \frac{A}{g_{m}} \right) \right) \times \frac{C_{m}}{C_{a}} \tag{30}
\]

Notice that in this equation, the notation \( C_{m} = C_{i} \) was kept even though \( C_{m} = C_{i} \) was assumed. Equation 30 can be further simplified by assuming that \( C_{a} \) is large, to get:

\[
\Delta_{i} = \frac{a(C_{a} - C_{i})}{C_{a}} + \left( b_{4} + \phi \left( C_{i} - C_{a} + \frac{A}{g_{m}} \right) \right) \times \frac{C_{m}}{C_{a}} \tag{31}
\]

When \( g_{m} \) is infinite, \( \phi \) can be derived from either Equation 30 (\( \phi_{is} \)) or Equation 31 (\( \phi_{i} \), assuming large \( C_{a} \)):

\[
\phi = \frac{(C_{a} - C_{m}) \times (b_{4}C_{m} - C_{a}A_{obs} + a(C_{a} - C_{i}) - A_{obs} - a(C_{a} - C_{i}) - b_{3}C_{s} + s(C_{a} - (C_{a} - C_{m}))}{C_{m}[C_{a}A_{obs} - a(C_{a} - C_{i}) - b_{3}C_{s} + s(C_{a} - C_{m})]} \tag{32}
\]

**Applying the models of C\(_{4}\) photosynthesis and discrimination**

To describe and discuss the effect of using different formulations for \( \Delta \) (Equations 28–31) in \( \phi \) calculations, a set of values was generated for \( A, C_{s}, \) and \( \phi \) for a range of incident light intensities (80–800 \( \mu \)mol quanta m\(^{-2}\) s\(^{-1}\)) using the \( C_{4} \) photosynthesis model for light-limited conditions (Equations 11, 15, 16, and 23). It was confirmed that light-limited equations were applicable in this range of irradiance by calculating \( A \) (data not shown) both with the light-and the enzyme-limited equations (von Caemmerer, 2000). \( C_{s} \) was obtained with an empirical fit to measured data in four \( F. \) bidentis plants (Fig. 5B, ML-21\% O\(_{2}\) data). The response of photosynthesis to light was modified to represent real measurements by adjusting the curvature parameter \( \theta \) (=0.1) and \( J_{\text{max}} \) (=280 \( \mu \)mol electrons m\(^{-2}\) s\(^{-1}\), Fig. 5A, ML-21\% O\(_{2}\) data). Calculations were performed assuming 21\% O\(_{2}\), \( \alpha \)=0.2 (von Caemmerer, 2000), \( \gamma^{*} \)=0.000193 (von Caemmerer et al., 1994), \( g_{s} \)=0.005 mol m\(^{-2}\) s\(^{-1}\) (middle range of the reported values for this variable, 0.001–0.01 mol m\(^{-2}\) s\(^{-1}\), von Caemmerer and Furbank, 2003), \( R_{d} \)=0.84 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) (value measured here in \( F. \) bidentis), and \( R_{m} \)=0.5\( R_{d} \) (von Caemmerer, 2000). Assuming \( g_{m} \)=\( \infty \), first the following were evaluated (i) the effect of including \( C_{s} \) in the calculations by comparing results from Equation 30 (\( \Delta_{u} \)) and Equation 31 (\( \Delta_{i} \)) for a range of \( g_{s} \) values; and (ii) the effect of respiratory and photorespiratory fractionations by calculating \( \Delta_{u} \) and \( \Delta_{i} \) for a range of \( e \) and \( f \) values. Secondly considering a finite \( g_{m} \), the effect of including \( g_{m} \) was evaluated by calculating \( \Delta_{ms} \) (Equation 28) and \( \Delta_{m} \) (Equation 29) for different values of \( g_{m} \) and results were compared with values obtained when \( g_{m} \) was infinite.

**Effect of CO\(_{2}\) concentration in the bundle-sheath cells**

The CO\(_{2}\) concentration in bundle-sheath cells, \( C_{s} \), can be calculated using either Equation 15 or Equation 16 (equivalent results, Table 2). Bundle-sheath conductance to CO\(_{2}\), or \( g_{s} \), is one of several variables used to determine \( C_{s} \). Reported values for \( g_{s} \) include 0.00065 mol m\(^{-2}\) s\(^{-1}\) for \textit{Digitaria sanguinalis} (Jenkins et al., 1989a), 0.00098 mol m\(^{-2}\) s\(^{-1}\) for \textit{Echinolochia crussgalli} (Jenkins et al., 1989a), 0.00113 mol m\(^{-2}\) s\(^{-1}\) for \textit{S. bicolor} (Brown and Byrd, 1993), and 0.00037–0.00235 mol m\(^{-2}\) s\(^{-1}\) for \textit{Z. mays} (Kromdijk et al., 2010). However, He and Edwards (1996) suggested that \( g_{s} \) might be higher, ranging between 0.005 mol m\(^{-2}\) s\(^{-1}\) and 0.02 mol m\(^{-2}\) s\(^{-1}\). For most \( C_{4} \) plants, \( g_{s} \) varies
between 0.001 mol m\(^{-2}\) s\(^{-1}\) and 0.01 mol m\(^{-2}\) s\(^{-1}\) (von Caemmerer and Furbank, 2003).

A 3-fold decrease of \(g_s\) (from 0.01 mol m\(^{-2}\) s\(^{-1}\) to 0.003 mol m\(^{-2}\) s\(^{-1}\)) increased \(C_s\) by 54% at PAR=100 (534 ppm versus 820 ppm) but caused an 82% increase in \(C_s\) at PAR=800 (776 ppm versus 1412 ppm) (Fig. 1A). Low \(C_s\) values resulted in large values of \(C_i/(C_s-C_m)\) (Fig. 1B), especially at low light, when \(C_m\) (assumed to equal \(C_i\)) was relatively large. It is often common to derive \(g_s\) from the photosynthetic discrimination equation assuming that \(C_i/(C_s-C_m)\) equals 1 (\(\Delta_s\), Equation 31). However, at PAR=100, \(C_i/(C_s-C_m)\) ranged from 1.8 to 2.9 as \(g_s\) increased from 0.003 mol m\(^{-2}\) s\(^{-1}\) to 0.01 mol m\(^{-2}\) s\(^{-1}\). Even at PAR=800, \(C_i/(C_s-C_m)=1.5\) when \(g_s\) was 0.01 mol m\(^{-2}\) s\(^{-1}\) (Fig. 1B).

Differences in \(C_s\) had little effect on modelled \(A\), especially at low light intensities (Fig. 1C). Despite minimal changes in \(A\), photosynthetic discrimination (\(\Delta_s\), Equation 30) was sensitive to variance in \(C_s\) by changing \(g_s\) (Fig. 1D). A 3-fold increase in \(g_s\) changed \(\Delta_s\) from 4.8\(^{\circ}\) to 11.3\(^{\circ}\) and from 0.3\(^{\circ}\) to 2.5\(^{\circ}\) for PAR=100 and 800, respectively. Only when \(C_s\) was large (\(g_s=0.003\) mol m\(^{-2}\) s\(^{-1}\)) and PAR >400 did \(\Delta_s\) (Fig. 1, stars) and \(\Delta_s\) produce similar results. However, when \(C_s\) was reduced as a result of low light intensities or larger \(g_s\) values, \(\Delta_s\) and \(\Delta_s\) diverged.

To illustrate further the effect of \(C_s\) on \(\Delta_s\) calculations, \(\Delta_s\) and \(\Delta_s\) were calculated for a range of \(C_i/C_a\) and \(\phi\) values (Fig. 2). It was assumed that \(\Delta_s\) decreased as light intensity increased so that \(\Delta_s\) was 351 \mu mol mol\(^{-1}\) and 104 \mu mol mol\(^{-1}\) at 100 and 750 PAR, respectively. The \(C_4\) light-limited photosynthesis equations were used to calculate \(C_s\) for each combination of \(C_i/C_a\) and \(\phi\) values under two scenarios: \(g_s=0.003\) mol m\(^{-2}\) s\(^{-1}\) (Fig. 2A) and \(g_s=0.01\) mol m\(^{-2}\) s\(^{-1}\) (Fig. 2B). Figure 2 shows that when \(C_s\) was large (low \(C_i/C_a\) values, which corresponds to high irradiances in this example), \(\Delta_s\) ~ \(\Delta_s\). Under these conditions the relationship between \(C_i/C_a\) and \(\Delta\) was linear. However, when \(C_s\) was small (high \(C_i/C_a\) in this example), there was a curvature in the relationship between \(C_i/C_a\) and \(\Delta_s\). Data were included from \textit{F. bidentis} plants (21% O\(_2\); NU, unpublished data) to demonstrate the \(C_s\) effect on \(\phi\) calculations. At PAR=125, values for observed photosynthetic discrimination (\(\Delta_m\)) and \(C_i/C_a\) were 10.8\(^{\circ}\) (SE \pm 0.2) and 0.9 (SE \pm 0.01), respectively. Using these values, \(\phi=0.6\) with the simplified equation (\(\Delta_s\)); however, including \(C_s\) in the \(\Delta\) equation (\(\Delta_m\)) predicted \(\phi\) of either 0.4 or 0.3 at \(g_s\) values of 0.003 mol m\(^{-2}\) s\(^{-1}\) or 0.01 mol m\(^{-2}\) s\(^{-1}\), respectively. Consequently, whenever \(C_s\) is low, using the simplified equation will result in an overestimation of \(\phi\).
**Effect of respiration and photorespiration**

To understand the role of respiratory and photorespiratory fractionations in $\Delta$ equations, it is first necessary to understand the series of events that contribute to determining the C-isotope values ($\delta^{13}C$) in C4 plants. The dissolution of CO2 into water concentrates $^{13}C$ in the gas phase by 1.1% ($e_b$) (Mook et al., 1974). Subsequently, there is a strong isotope effect associated with the conversion of CO2 into HCO$_3^-$ ($e_b = -9\%$), which concentrates the heavy isotope in HCO$_3^-$. Thus, the net equilibrium fractionation during dissolution and hydration of CO2 is $-7.9\%$ at 25°C (Mook et al., 1974). The magnitude of the enrichment is temperature dependent; HCO$_3^-$ is 8.5% more enriched than gaseous CO2 at 20°C and 7.4% at 30°C (Mook et al., 1974). This enriched HCO$_3^-$ pool is the substrate for PEPc, which discriminates against the heavy isotope by $b_{4*} = 2.2\%$ (O’Leary, 1981) much less than the C$_3$ photosynthetic enzyme Rubisco, 30% (Roeske and O’Leary, 1984). The effects of CO2 dissolution, hydration, and PEPc activity can be grouped into a single fractionation factor, $b_{4'} (= e_b + b_{4*})$, which at 25°C has a value of $-5.7\%$ (Farquhar, 1983). If Rubisco consumed all released CO2 produced from the C4 cycle then there would not be further fractionation. However, some of the CO2 and HCO$_3^-$ leaks out of the bundle-sheath cells determining the extent of Rubisco fractionation (Berry and Farquhar, 1978).

So far the events that alter the $\delta^{13}C$ values of atmospheric CO2 as it gets processed through photosynthesis have been described. However, the CO2 within a leaf is a mixture of CO2 from different sources. The majority of it comes from ambient air ($\delta^{13}C$ is $\sim -8\%$), but it also includes isotopically different CO2 released during mitochondrial respiration and photorespiration. To account for this, the terms $b_{4'}$ and $b_{3'}$ are modified by subtracting a fractionation factor $e$ (respiration) and $f$ (photorespiration). The isotopic effects of the added CO2 from these processes are weighed by the ratio of respiration to carboxylation rates (Equations 25 and 26).

There is some debate about the magnitude of the fractionation factors $e$ and $f$. For example, reported values for $f$ range from 0% to 10% (von Caemmerer and Evans, 1991; Scartazza et al., 1998) to 8% (Rooney, 1988; Gillon and Griffiths, 1997) and 11.6% (Lanigan et al., 2008). The values of $f$ are positive, meaning that photorespiration favours the lighter isotope. Alternatively, it is believed there is extensive enrichment in $\delta^{13}C$ of CO2 respired by C3 leaves ($e=0$ to $-10\%$) (Ghashghaei et al., 2001; Barbour et al., 2007; Battellier et al., 2008). To our knowledge there is only one study reporting $\delta^{13}C$ values for CO2 respired by C4 leaves. In this study, by Sun et al. (2010), leaf respirated CO2 was as much as 4% more enriched than leaf biomass in the C4 species *Sporobolus wrightii*. Additionally, the effect of respiration can be magnified during $\Delta$ measurements if the reference gas has $\delta^{13}C$ values different from air in growth conditions. Generally, CO2 tanks used for $\Delta$ measurements are derived from fossil fuels and are very depleted in $^{13}C$ (i.e. $\sim 30\%$). In this case, the value of $e$ is modified following Wingate et al. (2007):

$$e' = e + \delta^{13}C_{\text{measurements}} - \delta^{13}C_{\text{growth}}$$  \hspace{1cm} (34)

To illustrate the impact of $e'$ and $f$ fractionations on photosynthetic discrimination calculations, $\Delta_{is}$ (Equation 30) and $\Delta_i$ (Equation 31) were calculated for different values of $e'$ and $f$ (Fig. 3). Values of $V_p$, $V_c$, and $V_o$ were modelled for a range of light intensities (80-800 μmol quanta m$^{-2}$ s$^{-1}$) using the equations for C4 light-limited photosynthesis (Equations 17–20). Dark respiration ($R_d$) was 0.84 μmol m$^{-2}$ s$^{-1}$, measured in *F. bidentis* plants, and $R_m$=0.5R_d. We assumed $g_s = 0.005$ mol m$^{-2}$ s$^{-1}$ and $\phi$ was derived with the C4 light-limited photosynthesis model (Equation 23).
Respiration

To investigate the effect of respiration alone, \( f = 0 \) was assumed and \( e' \) was varied from 0\(^{\%}_\infty \) to –30\(^{\%}_\infty \) (Fig. 3A, B). This range includes \( e \) values reported in the literature (0\(^{\%}_\infty \) to –10\(^{\%}_\infty \)) and the artefact of using a depleted reference tank. The inclusion of the respiratory fractionation resulted in an increased value for \( b_3 \) (Fig. 3A), especially at low light intensities, when \( V_c \) values were small (\( V_c \) ranged from 5.7 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) to 26.6 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) between 100 and 800 PAR). The respiratory fractionation also increased (less negative) values for \( b_4 \) (Fig. 3A). Because \( e' \) is divided by a small \( V_p \) at low light intensities, \( b_4 \) was considerably larger than –5.7\(^{\%}_\infty \) under these conditions. For example, at \( \text{PAR} = 100 \), \( V_p \) would be 6.5 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) and if \( e' = –30^{\%}_\infty \) and \( R_m = 0.42 \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \), then \( e' R_m/V_p = –1.9^{\%}_\infty \). This would cause \( b_4 \) to be –3.8\(^{\%}_\infty \) [\( b_4 = –5.7–(–1.9) \)]. However, at \( \text{PAR} = 800 \), \( V_p \) would be 29.2 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \), \( e' R_m/V_p = –0.4^{\%}_\infty \), and \( b_4 = –5.7–(–0.4) = –5.3^{\%}_\infty \). Notice that the calculation of \( V_p \) is dependent on \( J_{\text{max}} \), the maximum electron transport, and the model assumed \( J_{\text{max}} = 280 \mu \text{mol electrons} \text{ m}^{-2} \text{s}^{-1} \). However, an increase in \( J_{\text{max}} \) results in a larger estimate of \( V_p \) and a smaller effect of \( e' \) on \( b_4 \) calculations.

The influence of \( e' \) on both \( b_3 \) and \( b_4 \) fractionations resulted in an increase in photosynthetic discrimination calculated with either the simplified (\( \Delta_i \)) or the more complete (\( \Delta_a \)) equation. In both cases, \( \Delta \) was most affected at low light when the change in \( b_3 \) and \( b_4 \) values was large. At \( \text{PAR} = 100 \) changing \( e' \) from 0\(^{\%}_\infty \) to –30\(^{\%}_\infty \) increased values for \( \Delta_i \) and \( \Delta_a \) by 3\(^{\%}_\infty \).

Photorespiration

To study the effect of photorespiration alone, \( e' \) was considered to equal 0 and \( f \) was varied from 0\(^{\%}_\infty \) to 11.6\(^{\%}_\infty \). Because \( f \) is positive, its effect is to reduce \( b_3 \) by the magnitude \( fV_p/V_c \). The ratio \( V_p/V_c \) was close to 0, ranging from 0.12 at \( \text{PAR} = 100 \) to 0.08 at \( \text{PAR} = 800 \). Therefore, even using the largest value for \( f (11.6^{\%}_\infty) \), the overall effect was small (i.e. reduction of \( b_3 \) by 1.4\(^{\%}_\infty \) at \( \text{PAR} = 100 \) and by 1\(^{\%}_\infty \) at \( \text{PAR} = 800 \), Fig. 3C). At \( \text{PAR} = 100 \), using a value for \( f \) of either 11.6\(^{\%}_\infty \) or 0\(^{\%}_\infty \) resulted in changes in \( \Delta \) (either \( \Delta_i \) or \( \Delta_a \)) <0.6\(^{\%}_\infty \). At \( \text{PAR} = 800 \), varying \( f \) in the same range altered \( \Delta \) by <0.1\(^{\%}_\infty \).

Finally, it is worth noting that because reported values for \( e' \) and \( f \) are of opposite signs (respiration favours \( ^{13}\text{C} \) while photorespiration favours \( ^{12}\text{C} \)), their effects will partially cancel out when considered together. For example, in the present modelling exercise, a light intensity of 100 PAR using \( e' = –30^{\%}_\infty \) and \( f = 0^{\%}_\infty \) resulted in \( \Delta_i = 10.3^{\%}_\infty \) (Fig. 3). Alternatively, if \( e' = 0 \) and \( f = 11.6 \), then \( \Delta_a = 6.6^{\%}_\infty \). However, considering both fractionations together, \( \Delta_a = \ldots \)
Similarly, 4B). For example, using the lowest for photosynthetic discrimination formulations for photosynthetic leakage calculated using different 

\[ \text{Discrimination} \]

\[ \text{ Shoot conductance (L) and } \text{ mesophyll conductance (M)} \]

\[ \text{Leakiness calculated using different formulations for photosynthetic discrimination} \]

\( \text{To illustrate the effect of choosing different formulations for } \Delta \text{ to estimate } \phi, \text{ three sets of data were used where } \Delta_{\text{obs}} \text{ was measured in } F. \text{ bidentis using a LI-6400 (Licor, Inc., Lincoln, NE, USA) coupled to a tunable diode laser (TDL: Campbell Scientific, Inc., Logan, UT, USA). Two of the data sets were compiled by Pengelly et al. (2010) using plants grown at irradiances of either 500 (ML) or 150 PAR (LL) and measured in a 2% O}_2 \text{ atmosphere. The third data set was from } F. \text{ bidentis plants grown at ML and measured in a 21% O}_2 \text{ atmosphere (NU, unpublished data). For all three data sets, } A \text{ and } C_i \text{ were modelled as a function of light (Fig. 5). Values for } g_m \text{ of 0.01 mol m}^{-2} \text{s}^{-1} \text{ and } 0.007 \text{ mol m}^{-2} \text{s}^{-1} \text{ for ML and LL plants were chosen to match predicted and observed photosynthetic discriminations (Kromdijk et al., 2010), and } J_{\text{max}} \text{ was 280 } \mu \text{mol m}^{-2} \text{s}^{-1}. \]
electrons m$^{-2}$ s$^{-1}$ and 180 μmol electrons m$^{-2}$ s$^{-1}$ for ML and LL plants, respectively.

The equations for light-limited C4 photosynthesis were used to derive $C_t$ (Equations 15 or 16, Fig. 6A), $V_o$, $V_c$, $V_p$ (Equations 17–20, Fig. 6B, C), and $\phi$ (Equation 23, Fig. 7B, C, lines), and values for $R_d$ and $f$ were 0.84 μmol m$^{-2}$ s$^{-1}$ and 11.6%, respectively, in all cases. The inclusion of a finite $g_m$ had a negligible impact on $\Delta$ calculations; therefore, for simplicity, $g_m=\infty$ was assumed for all calculations. For the Pengelly et al. (2010) data sets, $e'=−5.1\%$ (value reported by the authors) and for Ubierna’s data, $e' = −31\%$, calculated using Equation 34 where the $\delta^{13}$C of the reference gas was $−33\%$, and $e = −6\%$. Using these parameters there was good agreement between the model $\Delta_{\text{is}}$ (Equation 30, Fig. 7A, lines) and measured $\Delta_{\text{obs}}$ (Fig. 7A, circles) in the three data sets.
Subsequently, \( \phi_\text{is} \) (Equation 32, Fig. 7B, circles) and \( \phi_i \) (Equation 33, Fig. 7C, circles) were calculated for each data set using measured \( C_i \) and \( \Delta_{\text{obs}} \) with modelled \( C_s \) values. The results were compared with modelled \( \phi \) obtained with the light-limited C4 photosynthesis equations where \( \phi = L/V_p \).

Figure 7B and C reveals that while there was a good agreement between \( \phi_\text{is} \) and modelled \( \phi \) (Fig. 7B, compare lines and circles), \( \phi_i \) largely overestimated \( \phi \) at all light intensities (Fig. 7C, compare lines and circles).

At PAR = 125, \( \phi_i \) was 5-, 4-, and 2-fold larger than \( \phi_\text{is} \) in ML-2% O2, LL-2% O2, and ML-21% O2 plants, respectively. Because \( g_s \) was large in *F. bidentis*, the simplified equation also resulted in an overestimation of \( \phi \) at higher light intensities (\( \phi_i \) was about twice the value of \( \phi_\text{is} \)). Additionally, the simplified equation could not account for differences in \( C_s \) originating from different \( g_s \) values. Therefore, when the simplified equation was used, it resulted in lower \( \phi_\text{is} \) for LL plants than ML plants measured at 2% O2 (Fig. 7C, compare filled and open circles). However, when the complete equation was used and values for \( g_s \) were accounted for, this difference disappeared (Fig. 7B).

Lastly, under low O2 concentrations, the C4 photosynthesis model predicted very low \( C_s \) values at all light environments (Fig. 6A). Using the simplified equation resulted in similar \( \phi_\text{is} \) for ML plants measured at either 2% or 21% O2 (Fig. 7C, compare filled and grey circles). However, the complete equation showed larger \( \phi_\text{is} \) for the 21% O2 than for the 2% O2 measurements.

**Model analysis**

Leakiness (\( \phi \)), the proportion of carbon fixed by PEP carboxylation that subsequently leaks out of the bundle-sheath cells, is an important parameter describing the efficiency of the C4 photosynthetic pathway. The quantification of \( \phi \) is difficult because this variable cannot be directly measured. Instead, \( \phi \) is usually estimated by comparing observed and theoretical values for \( ^{13}\text{C} \) photosynthetic discrimination (\( \Delta \)). The calculation of \( \Delta \) includes factors of uncertain value (fractionations during respiration or photorespiration) or those that are difficult to quantify for C4 plants (mesophyll and bundle-sheath conductance). Additionally, the impact of these factors on \( \Delta \) calculations is not constant but rather depend on rates of photosynthesis. For example, fractionations associated with respiration are weighted by the ratio of \( \text{respiration} : \text{carboxylation} \), which is larger when photosynthesis is low (i.e. low light intensities). Conversely, the effect of mesophyll conductance is larger under high photosynthetic rates, since the drop in \([\text{CO}_2]\) between \( C_i \) and \( C_m \) is dependent on \( A/g_m \). Additionally, bundle-sheath conductance influences values of \( C_s \), the \( \text{CO}_2 \) concentration in the bundle-sheath cells. It is often assumed that \( C_s \) is much measured at 21% O2 (ML, 21% O2, grey circles and dotted lines).

Error bars represent \( \pm 1 \text{ SE} \).
larger than $C_m$, the CO2 concentration in the mesophyll cells, and therefore calculations for discrimination are simplified from Equation 30 ($\Delta_{m}$) to Equation 31 ($\Delta_i$). Using a modelling approach, we have described how values for these different parameters affect $\Delta$ calculations for a range of light intensities. In particular, it is shown that artefacts in $\Delta$ calculations could result in an overestimation of $\phi$ under low light environments. Understanding the response of $\phi$ to both diurnal light gradients and different light environments during growth is required to evaluate the efficiency of C$_4$ canopies, where considerable self-shading may occur.

As previously reported (Henderson et al., 1992), fractionation during photorespiration ($f$) had little impact on $\Delta$ calculations (Fig. 3). This term is included in $\Delta$ calculations by using the formulas for $b_3$ and $b_4$ presented in Equations 25 and 26. These equations show that $f$ is weighted by the oxygenation to carboxylation ratio ($V_r/V_c$), which was small under all light environments (Fig. 6B, C). Therefore, even assuming a large value for $f$ (11.6$\%$), the impact on $\Delta$ calculations was negligible.

Additionally, the respiratory fractionation ($e'$) is weighted by the ratio of respiration to carboxylation ($R_d/V_c$ and $A_{m}/V_{p}$), which increases as light intensity decreases. Under low light conditions and if $e'$ is very large (i.e. $-30\%$), significant variation can be introduced into $\Delta$ calculations ($3\%$). Notice that in the present modelling exercise a value for $R_d$ of 0.84 $\mu$mol m$^{-2}$ s$^{-1}$ was used, which corresponds to rates measured in F. bidentis. However, respiration rates can be significantly higher in other C$_4$ species, which will accentuate the impact of $e'$ in $\Delta$ calculations. Large $e'$ values are typically not the result of a biological process but an artefact of the measurement technique. This effect can be avoided by choosing a gas for measurements that has $\delta^{13}$C values similar to ambient air during plant growth or can be accounted for by using Equation 34 (Wingate et al., 2007).

The value for $b_3$ could be further altered if CA was limiting the isotopic equilibrium between CO$_2$ and HCO$_3^-$ (see Equation 27). In this study, an analysis of the influence of CA on $\Delta$ has not been included. In wild-type F. bidentis, CA activity was not limiting $^{13}$C isotopic equilibrium and had little effect on $\Delta$; however, this may not be true in other C$_4$ species, especially grasses which typically have lower CA activity (Cousins et al., 2006, 2008). Further research is needed to investigate the variation in CA activity among different C$_4$ species.

The present calculations also demonstrated that including a finite mesophyll conductance ($g_m$) did not affect $\Delta$ values, especially under low light conditions. At low light intensities, $A/g_m = -0$ and thus $C_m \sim C_i$, which is equivalent to assuming an infinite $g_m$. At higher light intensities, including a range of $g_m$ values from 0.4 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$ to 1.2 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$, altered $\Delta$ by $<0.6\%$, compared with values obtained assuming $g_m = \infty$. Even though actual $g_m$ values for C$_4$ plants have not been extensively quantified, the current understanding is that $g_m$ may be larger in C$_4$ than in C$_3$ plants (Evans and von Caemmerer, 1996). In C$_4$ species, the initial CO$_2$ fixation occurs in the mesophyll cytosol, while in C$_3$ plants, the CO$_2$ needs to diffuse deeper into the chloroplast stroma. While reported values for $g_m$ in C$_4$ plants range from 0.1 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$ to 0.4 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$ (Flexas et al., 2008), values in C$_4$ plants are from 0.6 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$ to 1.0 $mol$ m$^{-2}$ s$^{-1}$ bar$^{-1}$ (Kromdijk et al., 2010; Pengelly et al., 2010).

The largest variation in $\Delta$ calculations originated from the use of the simplified equation that ignores the term $C_i$. For similar values of $e'$, $f$, and $g_m$, the difference between $\Delta_i$ (ignores $C_i$) and $\Delta_0$ (includes $C_i$) was as much as 6$\%$ when $C_i$ was low. This can have a large impact on $\phi$ estimates, since $\phi$ is derived comparing measured values of $\Delta$ with a theoretical formulation for this variable. The present model simulations showed that $C_i$ was low when: (i) low light intensities reduced the flows through the C$_4$ and C$_3$ cycles; (ii) $g_s$ values were large; and (iii) under low O$_2$ concentrations.

At low light intensities and $g_s$ values ranging from 0.003 $mol$ m$^{-2}$ s$^{-1}$ to 0.01 $mol$ m$^{-2}$ s$^{-1}$, $C_i$ was $\sim 700$ ppm (Fig. 1A). Because $C_m$ values were also larger at low light, the ratio $C_i/(C_m-C_i)$ became substantially different from 1 (in the present simulation close to 2), which invalidated the simplification for the $\Delta$ equation. Values of $\Delta$ calculated using the simplification ($\Delta_0$) were lower than when $C_i$ was included in the calculations ($\Delta_i$; see Fig. 1D). Accordingly, large $\Delta_{obs}$ compared with low theoretical values resulted in large $\phi$ values. For example, when $\phi$ was calculated using the simplified equation ($\phi_s$, Equation 33) in F. bidentis measured at 21% O$_2$, it increased from 0.27 at PAR=750 to 0.51 at PAR=125 (88% increase). Conversely, when $\phi$ was calculated using the equation that includes $C_i$ ($\phi_m$, Equation 32), $\phi$ was 0.18 and 0.24 for 750 and 125 PAR, respectively (33% increase). Notice that using the complete equation still resulted in higher $\phi$ at low light, as suggested by previous studies (Henderson et al., 1992; Cousins et al., 2006; Tazoe et al., 2006, 2008; Pengelly et al., 2010), but the magnitude of change was different. This increase in $\phi$ (Fig. 7B) probably results from an incomplete inhibition of photorespiration because $C_i$ values under low light were approximately double those at ambient light, while a 7-fold increase of $C_i$ is needed to inhibit photorespiration effectively (Jenkins et al., 1989b). This is supported by the results from Kromdijk et al. (2010) in Z. mays, where the increase in $\phi$ at low light (calculated including $C_i$) was attributed to photorespiration. Furthermore, their results provided additional support for the photorespiratory role by demonstrating that measured $\Delta$ and calculated $\phi$ increased with increasing O$_2$ partial pressures.

Kromdijk et al. (2010), in addition to including $C_i$ in their calculations, also allowed for changes in the factor $x$, the partitioning of energy between the C$_4$ and C$_3$ cycles. In the present study, for simplicity and consistency with the original assumptions of the C$_4$ light-limited photosynthesis model (von Caemmerer, 2000), $x$ was considered to equal 0.4. If $x=0.4$, 40% of the total electron flux is used in the C$_4$ portion of the cycle to ensure regeneration of PEP and an efficient CO$_2$ pump. Raising $x$ above 0.4 reduces
photorespiration but also could result in an increase in $\phi$ as the capacity of the C₄ cycle increases beyond that of the C₃ cycle. In the case of the data of Kromdijk et al. (2010), this adjustment was required to explain high $\Delta$ values at low light intensities (i.e. 20%o). In the example with *F. bidentis*, where discrimination at low light was lower (maximum 10%o), the increase of $\phi$ was completely explained by the incomplete inhibition of photorespiration. Because there are feedbacks between the C₄ and C₃ cycles (Furbank et al., 2000) and key enzymes are regulated by light (Furbank et al., 1997), a long-term imbalance between the C₄ and C₃ activities would not be expected. However, it is possible that short-term imbalances in the C₄ and the C₃ cycles could occur due to slow changes in the light energy partitioning between the mesophyll and bundle-sheath cells in response to changes in light conditions. For example, it has been suggested that $x$ may vary with irradiance (Kromdijk et al., 2010), O₂ evolution in the bundle-sheath cells, and bundle-sheath conductance (von Caemmerer, 2000). It is beyond the scope of this study to discuss and analyse variations on this value further; for additional information see Peisker (1988) and von Caemmerer (2000).

Low $C_s$ values can also be observed when bundle-sheath conductance ($g_s$) is relatively large. This finding implies that comparisons among species, or species grown under different conditions, are compromised if different values for $g_s$ are expected. As illustrated with the *F. bidentis* data, the use of the simplified equation showed different $\phi$ for ML and LL plants measured at 2% O₂, while those differences disappear when the complete equation was used (Fig. 7). Undoubtedly, choosing a value for $g_s$ is complicated, since this variable cannot be quantified directly. In the present example, the approach by Kromdijk et al. (2010), of selecting $g_s$ to match observed and calculated $\Delta$, was followed. The resulting $g_s$ values (0.01 mol m⁻² s⁻¹ and 0.007 mol m⁻² s⁻¹ for ML and LL plants, respectively) were within the range of values predicted for this variable (0.01–0.001 mol m⁻² s⁻¹; von Caemmerer and Furbank, 2003) and agreed with observations of reduced $g_s$ in plants grown at low light (Kromdijk et al., 2010; Pengelly et al., 2010).

Finally, very low $C_s$ values (400–500 ppm) were predicted with the C₄ light-limited photosynthesis model under low O₂ concentrations. Low O₂ levels efficiently suppressed modelled photorespiration ($V_o/V_c$ was ~0; Fig. 6B). Consequently, the model predicted no need for a CO₂ pump and $V_o/V_c$ ~ 1 (under 21% O₂, $V_o/V_c$ was ~1.1–1.2; Fig. 6C). In other words, this scenario assumes that most of the CO₂ delivered to the bundle-sheath cells was fixed by Rubisco, which resulted in very little $\phi$. The rationale behind decreasing O₂ concentrations during measurements is to reduce fractionations associated with photorespiration. As previously noted, the impact of photorespiratory fractionations on $\Delta$ calculation was minimal. By artificially eliminating photorespiration with low [O₂] levels, the need for a strong C₄ pump is also eliminated. This results in a very low $C_s$ value predicted by the C₄ photosynthesis model at all light intensities. Currently it is not known whether these low $C_s$ values are real or an artefact of the model. This is problematic, as very different results are obtained whether $C_s$ values are included in the calculations or not. As illustrated by the *F. bidentis* example, while $\phi_1$ (simplified) was similar for 2% and 21% O₂ measurements, $\phi_1$ includes $C_s$ was larger for measurements at 21% O₂, as would be expected under higher photorespiration conditions. Future research is needed to measure $\Delta$ at different [O₂] to investigate whether down-regulation of the C₄ pump under low O₂ environments is real or an artefact of the model.

Conclusions

When leakiness is derived using the isotope method, care should be taken in choosing the equation to represent C₄ photosynthetic discrimination. At low light, the effects of photorespiratory fractionations and mesophyll conductance were negligible, accounting for a variation in $\Delta$ of 0.6%o and 0.3%o respectively. Respiratory fractionation has the potential to introduce larger changes (3%o) under low irradiances and when the value for $e'$ was very large (i.e. –30%o). The largest error in estimating $\phi$ was introduced by assuming that $C_s$ is much larger than $C_m$ under all circumstances. In fact, this assumption does not hold at (i) low light levels; (ii) large $g_s$ values; or (iii) low O₂ concentrations. In these cases, the use of the simplified equation (i) overestimated $\phi$; (2) invalidated comparisons among species with different $g_s$ values; and (iii) invalidated comparison across O₂ concentrations. It is not known whether $C_s$ values calculated using the C₄ light-limited photosynthesis equations are accurate under all circumstances, but currently there are limited alternative methods. Future research should attempt to confirm model outputs for $C_s$ or provide alternatives for estimating this variable.

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