# Chapter 7

# Respiratory Effects on the Carbon Isotope Discrimination Near the Compensation Point

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## Summary

The carbon isotope discrimination associated with net photosynthesis ($\Delta_{obs}$) when photosynthetic rates are low, such as approaching the light and $CO_2$ compensation points, has rarely been measured but may contain useful information on day respiration ($R_d$). In fact, at low assimilation rates, the relative importance of respiratory $CO_2$ release is larger and its isotopic signal can be captured. In this chapter, we describe the measurement of $\Delta_{obs}$ in cocklebur, spinach and magnolia leaves at very low irradiance and $CO_2$ concentration. The carbon isotope fractionation associated with day respiration appears to be similar when approaching the light and $CO_2$ compensation points, and not strongly affected by oxygen concentration. Under the experimental conditions imposed, the apparent fractionation associated with day respiration was found to be −100‰ for cocklebur and spinach, and −62‰ for magnolia. These strongly negative values were due to the use of $^{13}C$-depleted $CO_2$ during gas exchange measurements and the use of respiratory carbon fixed prior to gas exchange measurements. Theoretical considerations allowed estimation of the proportion of newly-fixed carbon as a respiratory substrate, which was found to be zero for all species when a single respiratory source is assumed. When two respiratory sources are assumed (with a respiratory pool in photosynthesizing cells and a photosynthetically disconnected pool in heterotrophic, non-photosynthesizing cells), the heterotrophic component dominated day respiration in cocklebur and magnolia leaves, with newly-fixed carbon contributing little to total efflux in magnolia, but representing about one half in cocklebur. In contrast, respiration from photosynthesizing cells dominated $R_d$ in spinach leaves, but newly-fixed carbon formed just 11% of the respiratory substrate. Therefore, day respiration appears to be mostly fed by "old" carbon sources, and this can lead to a considerable isotopic difference between net fixed $CO_2$ and $CO_2$ liberated by day respiration at the same moment.

## I. Introduction

Stable carbon isotopes have emerged over the last four decades as an important tool in understanding photosynthesis at scales from molecules to whole plants (Farquhar and Richards 1984; Cernusak et al. 2013; von Caemmerer et al. 2014). This is due to that fact that the rare $^{13}C$ atoms (1.11% of carbon) in $CO_2$ are discriminated against during carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, the main carboxylating enzyme in $C_3$ photosynthesis), resulting in measureable differences in the isotope composition of plant carbon pools and fluxes (O'Leary 1981). Although less widely studied than photosynthesis, stable carbon isotopes are also useful in understanding plant respiration, and again at a range of scales (Bowling et al. 2008; Cernusak et al. 2009). For example, natural abundance stable isotope compositions have been used to partition ecosystem respiration (e.g. Tu and Dawson 2005), disentangle leaf respiratory biochemistry (e.g. Ghashghaie et al. 2003; Barbour et al. 2007), trace carbon through ecosystems (e.g. Barbour et al. 2005; Bowling et al. 2008) and determine rates of leaf respiration in the light at the ecosystem scale (Wehr et al. 2016). A number of studies have also used $^{13}C$ labeling techniques at the leaf (e.g. Tcherkez et al. 2005) and mesocosm scales (e.g. Tcherkez et al. 2010; Barthel et al. 2011) to understand fluxes through biochemical pathways. Most of these studies focused on respiration in the dark or during the light-dark transition. In fact, $^{13}C$ studies were the first to demonstrate a direct biochemical link between use of malate as a respiratory substrate and the light-enhanced dark respiratory peak in respiration (LEDR) immediately following the darkening of illuminated leaves (Ghashghaie et al. 2003; Barbour et al. 2007; Gessler et al.

2009). This effect is also evident at the ecosystem scale (Barbour et al. 2011). See also Chap. 3 in this volume.

In contrast, isotope effects during respiration in the light are poorly studied and little understood due to technical difficulties in measuring a small flux within a large flux in the opposite direction. Hanson et al. (2016) recently reviewed approaches and challenges involved in the measurement of day respiration and photorespiration, demonstrating the importance of accurately quantifying these small fluxes as a component of the larger photosynthetic flux. In particular, the influence of respiration on observed photosynthetic carbon isotope discrimination ($\Delta_{obs}$) was assessed, leading to the conclusion that photorespiratory and day respiratory isotope fractionations (*f* and *e*, respectively) during photosynthesis can strongly effect $\Delta_{obs}$, particularly when photosynthetic rates are low. Wingate et al. (2007) recommended that disequilibria between purely photosynthetic discrimination ($\Delta^{13}C_A$) and the isotope composition of $CO_2$ respired in the light ($\delta_{resp}$) is taken into account when interpreting $\Delta_{obs}$, introducing the concept of apparent fractionation during day respiration (sometimes denoted as *e**). Assumptions regarding respiratory fractionations can also influence estimates of mesophyll conductance to $CO_2$ diffusion (Gu and Sun 2014).

Despite the technical difficulties of quantifying the very small respiratory flux within the larger photosynthetic flux, a limited number of studies have been conducted, demonstrating the respiratory flux in the light to be slightly depleted compared to organic molecules at both the mesocosm scale (Tcherkez et al. 2010) and at the leaf level (Tcherkez et al. 2011). This result is in contrast to leaf respiration in the dark, which is usually enriched compared to putative substrates (e.g. Duranceau et al. 1999; Ghashghaie et al. 2001). By measuring leaf fluxes in the light in a $CO_2$ environment with a depleted isotope composition compared to the growth environment, Tcherkez et al. (2011) were able to show that the respiratory substrate must have been carbon fixed prior to the start of the gas exchange measurements. Similarly, Hanson et al. (2016) report a strongly negative $\Delta_{obs}$ in leaves during short-term exposure to $CO_2$ strongly enriched in $^{13}C$ (+148 ‰), again suggesting an isotope disequilibrium between current photosynthesis and respiration, and use of older carbon.

The contribution of photorespiration to leaf $CO_2$ exchange may be assessed by exposing the leaf to a non-photorespiring environment, such as low oxygen concentration, but assessing the influence of day respiration is less straightforward. There are two widely-used gas exchange methods to estimate day respiration rate, $R_d$, namely the Kok method (Kok 1948) and the Laisk method (Laisk 1977). In the only study of its kind to date, Villar et al. (1994) found reasonable agreement between the two techniques for two woody species (*Heteromeles arbutifolia* and *Lepechinia fragans*), although the Laisk-derived estimates of $R_d$ were 55% higher than the Kok-derived estimates. Peisker and Apel (2001) developed a third method using leaves with a range of $CO_2$ compensation points which gave similar estimates to the Laisk method for tobacco leaves of differing ages. More recently still, a fourth method was developed by Yin et al. (2009) using combined gas exchange and fluorescence measurements. This method produced values that agreed with Laisk measurements but were consistently higher than Kok-derived estimates (Yin et al. 2011). Decisions by researchers on which technique to apply typically depend on ease of measurement (i.e. the Laisk, and Peisker and Apel methods are technically more challenging) and on the particular experimental design. For example, Ayub et al. (2011) used the Kok method because they required estimates of $R_d$ at the growth $CO_2$ concentration, which varied between 280 and 640 μmol $mol^{-1}$.

The Kok method estimates $R_d$ from an extrapolation to zero light of the linear relationship between net photosynthetic rate and light over a range of low light levels (Kok 1948). A small correction is commonly made to account for the influence of increasing internal $CO_2$ concentration ($c_i$) on photosynthetic rate as light level is reduced (following Kirschbaum and Farquhar 1987). The Laisk method of $R_d$ estimation measures net photosynthetic rate close to the $CO_2$ compensation point, typically over a range of low $CO_2$ concentrations at three different low light levels. $R_d$ is then estimated from the intersection of the three linear regressions for the relationships between leaf internal $CO_2$ concentration and net photosynthetic rate. Given that estimates of $R_d$ vary between measurement techniques, it is possible that these approaches actually measure different processes. Tcherkez et al. (2011) quantified $e$ at differing $CO_2$ concentrations (mimicking a Laisk approach) but it is not known whether isotope fractionations associated with day respiration vary with $CO_2$ or light during Laisk and Kok measurements. A direct comparison of $e$ during Laisk and Kok measurements may be enlightening with respect to underlying biochemistry and may help to determine appropriate values for $e$ and $f$. In fact, at low and very low values of $A$ (near the compensation point) the relative influence of respiratory efflux is larger and thus its impact on $\Delta_{obs}$ should also be larger.

As an aid in clarifying the impact of day respiratory isotopic exchange, we address four questions in the current chapter:

1. Is the $^{12}C/^{13}C$ fractionation associated with net photosynthesis ($\Delta_{obs}$) quantitatively similar when approaching the light compensation point and the $CO_2$ compensation point?
2. Does the $^{12}C/^{13}C$ fractionation associated with day respiration vary between species with differing degrees of light suppression of respiration?
3. Does photorespiration alter observed $^{12}C/^{13}C$ fractionation associated with day respiration?
4. Does the $^{12}C/^{13}C$ fractionation associated with day respiration influence estimates of mesophyll conductance ($g_m$) at low light and low $CO_2$ concentration?

## II. Coupled Gas Exchange and Carbon Isotope Measurements

Coupled on-line gas exchange and stable carbon isotope measurement techniques are now well-established (Evans et al. 1986), both with isotope ratio mass spectrometers (e.g. Tcherkez et al. 2011) and with optical spectrometers such as tunable diode lasers (TDL; e.g. Barbour et al. 2007; Tazoe et al. 2009). However, there are a number of issues that must be considered for accurate interpretation of the measurements when $CO_2$ fluxes are low, such as approaching the light and $CO_2$ compensation points. Firstly, the precision and accuracy requirements for carbon isotope measurements are high, and most isotope measurement systems struggle with precision at low $CO_2$ concentrations. A solution is to use a large leaf area chamber to maximize the difference between inlet and outlet chamber $CO_2$ concentrations and isotope compositions. One such chamber is described by Loucos et al. (2015), able to enclose 38 $cm^2$ of leaf area in a chamber of volume 57 $cm^3$. Such a large chamber requires a compromise between a large $CO_2$ concentration difference and regulating water vapor concentration below dew point temperature to avoid condensation (particularly for high flux leaves). The second issue relates to concentration dependence of the stable isotope measurements, a problem typ-

ical of optical spectrometers (Tazoe et al. 2011) and also common in mass spectrometers. In both cases, concentration dependence can be accounted for in the instrument calibration procedure. The third issue relates to accurate assignment of isotope fractionation factors (e.g. Barbour et al. 2010; Gu and Sun 2014) during interpretation of $\Delta_{obs}$ (particularly for $g_m$ estimation).

In the data reported here, a TDL (TGA100A; Campbell Scientific Inc) calibrated using four standard cylinders across a range of $CO_2$ concentrations from 100 to 1100 ppm was used (Barbour et al. 2007), with a photosynthesis system (Li6400xt; LiCor Inc) fitted with a red-green-blue light source (Li6400-18) set to produce white light and a custom built chamber (Loucos et al. 2015) which enclosed the entire leaf and was sealed around the petiole. These arrangements maximized the accuracy and precision of isotope measurements. A number of studies have explored the influence of values assumed for $^{12}C/^{13}C$ fractionations on $\Delta_{obs}$ (e.g. Barbour et al. 2010; Douthe et al. 2012), and concluded that if values for $e$, $f$, $R_d$ and the $CO_2$ compensation point in the absence of $R_d$ ($\Gamma^*$) are constrained within the range of likely values, then differences in estimates of $g_m$ between plants and with environmental conditions likely reflect real physiological differences. Here, measurements were made at differing $CO_2$ concentrations and light levels, with records taken after stabilization of gas exchange parameters (15–90 min depending on environmental conditions and species). The carbon isotope composition of growth $CO_2$ was −8.1‰ inside the growth cabinet and measurement $CO_2$ was −34.7‰, both measured on the TDL. We assume $\delta^{13}C$ of $CO_2$ to be −8‰ outdoors.

The data presented in this Chapter were obtained from spinach (*Spinacia oleraea*, cultivar Popeye, Erica Vale, Brisbane, Australia), cocklebur (*Xanthium strumarium*, seed collected from naturalized plants growing in Sydney, Australia) and magnolia (*Magnolia grandiflora* "Little gem", purchased from a local nursery). Spinach and cocklebur plants were grown from seed in a controlled environment growth cabinet in 1-L pots filled with commercial potting mix and amended with slow-release complete fertilizer (Osmocote Exact, Scotts, Sydney). The cabinet was controlled at 400 μmol $mol^{-1}$ $CO_2$, 23 °C/15 °C day/night, 75% RH throughout and 700 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) during the 16-h day. Magnolia plants were grown outdoors on the Camden campus of the University of Sydney in 20-L pots filled with potting mix and amended with slow-release complete fertilizer (Osmocote). All plants were well-watered throughout, and four replicate plants of each species used for measurements.

## III. Calculating Carbon Isotope Fractionation During Day Respiration and Mesophyll Conductance

The carbon isotope fractionation associated with net photosynthesis is given by Eq. (7.1) below (Farquhar et al. 1989). Here, we neglect ternary effects (Farquhar and Cernusak 2012) which are indeed very small for $^{13}C$.

$$\Delta_{obs} = a\frac{c_a - c_i}{c_a} + a_e\frac{c_i - c_c}{c_a} + b\frac{c_c}{c_a} - \frac{eR_d}{kc_a} - f\frac{\Gamma^*}{c_a} \tag{7.1}$$

where $c_a$, $c_i$ and $c_c$ are $CO_2$ mole fractions in atmosphere, intercellular spaces and at carboxylation sites, respectively. $a$, $a_e$ and $b$ are fractionations associated with diffusion in air (4.4‰), dissolution and diffusion in water (1.8‰) and during carboxylation (29‰), respectively. $k$ is carboxylation efficiency,

given by $k = v_c/c_c$ where $v_c$ is the carboxylation rate, and $\Gamma^*$ is the $CO_2$ compensation point in the absence of $R_d$. The following section describes how to provide an explicit way to extract the fractionation associated with day respiration $e$ (and also $R_d$) using the observed fractionation at low $A$. The assumption is that neither $e$ nor $R_d$ change with $A$ (even at low $A$). It should be recalled that similarly, common methods used to measure $R_d$ are all carried out at low $A$ (Laisk or Kok methods) and thus under comparable photosynthetic conditions. Here, we use the symbol $e$ to denote the fractionation associated with day respiration assuming there is a single respiratory source and expressed relative to current photosynthetic discrimination, as originally defined in Farquhar et al. (1989). It should be noted that this definition facilitates calculations (in practice, simplifies the expression of $\Delta_{obs}$ in Eq. 7.1), but has important numerical consequences, as explained below.

### A. *Standard Model with One Respiratory Source*

Tcherkez et al. (2011) suggested the use of the offset of $\Delta_{obs}$ with respect to $b$, multiplied by $c_a$. This technique can be improved slightly using an expression that comprises an intercept tending to $e$ when $A$ is vanishingly small ($A \to 0$). To do so, we use Eq. (7.1) and the common relationships: $A = g_s(c_a - c_i) = g_m(c_i - c_c) = g_t(c_a - c_c)$, where $g_s$, $g_m$ and $g_t$ are stomatal conductance, mesophyll conductance and total conductance, respectively. Thus, we have:

$$\Delta_{obs} = \frac{A}{c_a}\left(\frac{a}{g_s} + \frac{a_s}{g_m} - \frac{b}{g_t}\right) + b - \frac{eR_d}{kc_a} - f\frac{\Gamma^*}{c_a} \qquad (7.2)$$

Since $v_c$ can be written as $v_c = (A + R_d)/(1 - \Gamma^*/c_c)$, $c_c/v_c = (c_a - A/g_t - \Gamma^*)/(A + R_d)$. Therefore, Eq. (7.2) gives:

$$\Delta_{obs} = \frac{A}{c_a}\left(\frac{a}{g_s} + \frac{a_s}{g_m} - \frac{b}{g_t} + \frac{\frac{eR_d}{A+R_d}}{g_t}\right) + b - \frac{eR_d}{A+R_d}\cdot\frac{c_a - \Gamma^*}{c_a} - f\frac{\Gamma^*}{c_a} \qquad (7.3)$$

Equation (7.3) can be re-arranged easily to:

$$\left(\Delta_{obs} - b + f\frac{\Gamma^*}{c_a}\right)\frac{c_a}{c_a - \Gamma^*} = A\frac{P}{c_a - \Gamma^*} - \frac{eR_d}{A + R_d} \qquad (7.4)$$

where $P$ stands for the parenthesis in Eq. (7.3). The quantity in the left term is here defined as $\theta_a$ (subscript "$a$" refers to $c_a$, as explained below):

$$\theta_a = \left(\Delta_{obs} - b + f\frac{\Gamma^*}{c_a}\right)\frac{c_a}{c_a - \Gamma^*} \qquad (7.5)$$

In Eq. (7.4), $P$ is in ‰ $m^2$ s $mol^{-1}$. Ordinarily, conductance tends to increase with $A$, so that $P$ is expected to increase as $A \to 0$. That is, the slope that multiplies $A$ is not constant in this relationship. In the non-linear regressions applied here, $P/(c_a - \Gamma^*)$ is empirically modeled as $\alpha/(A + \beta)$ where $\alpha$ and $\beta$ are constants. Also, if $R_d$ is assumed constant, the right term of Eq. (7.4) tends to $e$ when $A \to 0$. In other words, a plot showing $\theta_a$ as a function of $A$ has $e$ as an intercept. Note that the transformation from Eq. (7.2) to (7.3) could also be made using $c_c = c_i - A/g_m$ to express $c_c/v_c$. This would thus lead to:

$$\theta_i = \left(\Delta_{obs} - b + f\frac{\Gamma^*}{c_a}\right)\frac{c_a}{c_i - \Gamma^*} = A\frac{P}{c_i - \Gamma^*} - \frac{eR_d}{A + R_d} \qquad (7.6)$$

Nevertheless, either $\theta_i$ or $\theta_a$ can be used simply because when $A$ tends to 0, both $c_i$ and $c_c$ tend to $c_a$ and the expression converges to the same quantity. That is why it will be simply referred to as $\theta$ thereafter.

In Eqs. (7.4) and (7.6), some parameters have to be fixed to compute $\theta$ : $b$, $f$ and $\Gamma^*$. The impact of $f$ (standard value of 11‰, Tcherkez 2006) is quite small because $\Gamma^*/c_a$ is about 0.1 under ordinary conditions (ambient $CO_2$). In what follows, the effect of changing $b$ and $\Gamma^*$ is examined. It is found that the effect is very small (i.e. in the order of 1‰ while the value of $e$ is about −62 or −100‰).

It should also be noted that a mathematically strictly equivalent way of obtaining $e$ is the direct utilization of Eq. (7.3): when $A \to 0$, the first term disappears while the right terms only remain. That is:

$$\Delta_{obs}^{A\to 0} = b - e \cdot \frac{c_a - \Gamma^*}{c_a} - f\frac{\Gamma^*}{c_a} \tag{7.7}$$

that can be re-arranged to:

$$e = \frac{\Delta_{obs}^{A\to 0} - b + f\frac{\Gamma^*}{c_a}}{\frac{\Gamma^*}{c_a} - 1} \tag{7.8}$$

In practice, the use of Eq. (7.8) is less convenient because getting a good estimate of $\Delta_{obs}$ at $A = 0$ is difficult and requires curve fitting. The plot of $\Delta_{obs}$ against $A$ forms a steep apex when $A \to 0$ (at least, steeper than the plot of $\theta$ against $A$), and so the estimate of $\Delta_{obs}$ at $A = 0$ is a little less reliable. Also, when different experiments (at different $c_a$) are plotted together, the value of $c_a$ we should use to apply Eq. (7.8) is quite arbitrary. The graphical method based on Eqs. (7.4) or (7.6) is thus preferable.

### B. *Two-Source Model*

In equations given above including Eq. (7.1), it is assumed that day respiration is fed by a carbon pool that reflects net fixed $CO_2$, yielding the apparent fractionation ($e$). In fact, it should be recalled that $e$ is defined by the isotope ratio of evolved $CO_2$ ($R_{resp}$) with respect to that of net fixed carbon ($R_{new}$) (Farquhar et al. 1989):

$$e = \frac{R_{new}}{R_{resp}} - 1 = \frac{\delta_{new} - \delta_{resp}}{\delta_{new} + 1}$$

Even at the leaf level, day respired $CO_2$ could originate from a pool that is disconnected from current photosynthesis (and thus, with an isotope ratio different from $R_{new}$): either a metabolically distinct pool in photosynthetic cells or by heterotrophic leaf cells. Mathematically, this extra source can be accounted for by adding a term of the form $e_h R_h/A$ in Eq. (7.1) (where the subscript "h" stands for this extra source) (for the mathematical evidence, see Tcherkez et al. 2010, 2011). The derivation of equations is rather similar, except that the expression of $v_c$ must account for this extra respiration, as follows:

$$v_c = \frac{A + R_d + R_h}{1 - \frac{\Gamma^*}{c_c}} \tag{7.9}$$

Therefore, this gives:

$$\theta = A\frac{P}{c_i - \Gamma^*} - \frac{eR_d}{A + R_d + R_h} - \frac{e_h R_h}{A} \cdot \frac{c_a}{c_i - \Gamma^*} \tag{7.10}$$

In Eq. (7.10), it should be noted that the denominator of the last term is $A$ instead of $A + R_d$, and thus the quotient diverges to infinity when $A \rightarrow 0$. This makes the non-linear regression more sensitive to experimental errors. The increased number of parameters to be determined ($e_h$ and $R_h$) also means that the estimation of $e$ is potentially more difficult (more demanding of experimental data).

### C. Fraction of "New" Carbon in Respired $CO_2$

The value of $e$ can be exploited to get the isotope composition of day respired $CO_2$ and thus its % of "new" carbon (that is, the % of carbon that comes from recent net photosynthesis), denoted as $x$. The isotope composition of "new" carbon when $A \rightarrow 0$ is given by:

$$\delta_{new} = \frac{\delta_{outlet} - \Delta_{obs}^{A\rightarrow 0}}{1 + \Delta_{obs}^{A\rightarrow 0}} \qquad (7.11)$$

The isotope composition of "old" carbon (net fixed before the experiment) is:

$$\delta_{old} = \frac{\delta_{atm} - \Delta_{obs}^{st}}{1 + \Delta_{obs}^{st}} \qquad (7.12)$$

where the superscript "st" means "under standard conditions before the experiment". The observed isotope composition of day respired $CO_2$ when $A \rightarrow 0$ is:

$$\delta_{resp} = \frac{\delta_{new} - e}{1 + e} \qquad (7.13)$$

From this point, we have to differentiate the apparent fractionation $e$ obtained experimentally (under a background of atmospheric $CO_2$ with a controlled isotope composition potentially causing a large difference between respired $CO_2$ and net fixed carbon), and the intrinsic enzymatic fractionation of the metabolic pathway. The latter is denoted as $e_{int}$. The mass balance between "old" and "new" carbon gives:

$$\delta_{resp} = x\frac{\delta_{new} - e_{int}}{1 + e_{int}} + (1 - x)\frac{\delta_{old} - e_{int}}{1 + e_{int}} \qquad (7.14)$$

Combining (11) and (14) gives:

$$x = \frac{(\delta_{new} - e)(1 + e_{int})}{1 + e} + e_{int} - \delta_{old} \approx \frac{\delta_{new} - e}{1 + e} - \delta_{old} \qquad (7.15)$$

The approximation shown on the right hand side of Eq. (7.15) is valid if $e_{int}$ is very small. This might be the case here since $e_{int}$ is probably a few per mil while $\delta_{new}$ is large (very negative) due to the use of highly $^{13}C$-depleted industrial $CO_2$ during experiments.

### D. Calculation of Mesophyll Conductance

Mesophyll conductance to $CO_2$ diffusion ($g_m$) can be calculated from combined measurements of carbon isotope discrimination and leaf gas exchange following equations outlined in Evans et al. (1986), and Barbour et al. (2010). We have chosen to leave the values uncorrected for ternary effects (Farquhar and Cernusak 2012) for consistency with equations described above. If ternary corrections were included for the measurements described here, estimates of $g_m$ would be between 3 and 20% lower and the responses to changes in $CO_2$ concentration and light would be slightly reduced, but the direction of responses would remain the same.

## IV. $\Delta_{obs}$ Approaching the Compensation Point

The three species studied here had different leaf day respiration rates (as estimated using the Kok method) and respiration rates in the dark, with lower values found for magnolia. The species also differed in the degree of light inhibition of $R_d$, from no inhibition for cocklebur at 21% $O_2$ and magnolia at 2% $O_2$ to 41% inhibition for spinach at 21% $O_2$. There was no relationship between the estimates of $R_d$ at differing oxygen concentrations. Laisk estimates of $R_d$ were higher than Kok estimates for cocklebur and magnolia at 21% $O_2$, but lower for spinach (Table 7.1).

Photosynthetic carbon isotope discrimination increased approaching both the light and the $CO_2$ compensation points, for all three species studied here (Fig. 7.1). $\Delta_{obs}$ was as high as 100‰ for cocklebur and spinach, and as high as 50‰ for magnolia. There was no significant difference in $\Delta_{obs}$ at a given photosynthetic rate ($A$) between variable light conditions and variable $CO_2$ concentration (for all three species), and no significant difference in $\Delta_{obs}$ between measurements made at 21% and 2% $O_2$ (for spinach and magnolia). There was also no clear difference between species with little (cocklebur) and moderate (spinach) light inhibition of respiration, although the species with strong light inhibition of respiration at 21% $O_2$ (magnolia) had lower $\Delta_{obs}$ at the same $A$ compared to the other species.

These results suggest that the $CO_2$ released by respiration in the light was $^{13}C$-enriched compared to chamber inlet $CO_2$, thereby increasing $\Delta_{obs}$ substantially and implying that at least some of the respired $CO_2$ was from carbon fixed prior to the leaf gas exchange measurements. Further, the data point to similar respiratory substrates being used during the approach to the light and $CO_2$ compensation points, and to a limited influence of photorespiration on the $^{12}C/^{13}C$ fractionation during day respiration.

## V. Carbon Isotope Fractionation Associated with Day Respiration

Using the curve fitting approach outlined above, and assuming that current photosynthesis forms the substrate for respiration, we estimate that apparent fractionation during day respiration ($e$) is −100‰ for both cocklebur and spinach, and −62‰ for magnolia (Fig. 7.2; Table 7.2). That is, day-respired $CO_2$ is $^{13}C$-enriched compared to current photosynthates. However, the strong $^{13}C$ depletion of the $CO_2$ used for gas exchange measurements ($\delta_{inlet}$ = −35‰) compared to growth $CO_2$ ($\delta_{atm}$ approx. –8‰) needs to be considered. Assuming that in the growth cabinet the photosynthetic carbon isotope discrimination was between 17 and 22‰, this would give $\delta^{13}C$ of carbohydrates formed under growth conditions between −25 and −30‰. In contrast, if the photosynthetic carbon isotope discrimination under measurement conditions were the same as under growth conditions, then the $\delta^{13}C$ of carbohydrates formed under measurement condi-

*Table 7.1.* Measured respiration rate in the dark ($R_{dark}$) and estimated respiration rate in the light ($R_d$) using the Kok and Laisk methods at 21% and 2% $O_2$ for cocklebur, spinach and magnolia (all in μmol $m^{-2}$ $s^{-1}$). Also shown is the percent light inhibition of respiration, which is calculated from the ratio of the Kok-estimated $R_d$ and $R_{dark}$ for the same leaf after at least 20 min in the dark. Values are averages, $n$ = 4

| | 21% $O_2$ | | | | 2% $O_2$ | |
|---|---|---|---|---|---|---|
| Species | $R_{dark}$ | Laisk $R_d$ | Kok $R_d$ | % inhibition | Kok $R_d$ | % inhibition |
| Cocklebur | 1.0 ± 0.1 | 1.3 ± 0.2 | 0.9 ± 0.1 | 5 ± 22 | nd | nd |
| Spinach | 2.6 ± 0.2 | 1.2 ± 0.2 | 2.2 ± 0.2 | 16 ± 6 | 1.4 ± 0.1 | 29 ± 5 |
| Magnolia | 0.6 ± 0.2 | 0.7 ± 0.2 | 0.4 ± 0.1 | 41 ± 12 | 0.7 ± 0.2 | 0 ± 20 |

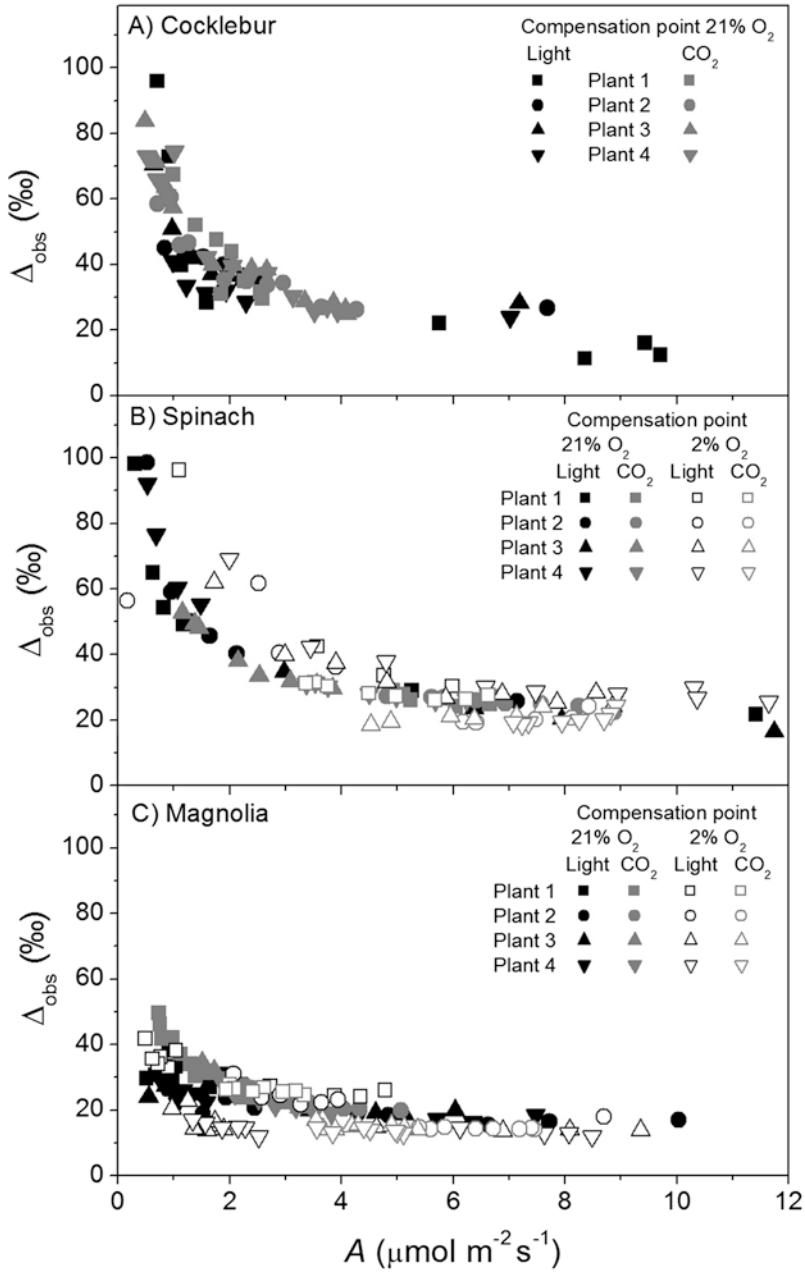


*Fig. 7.1.* Photosynthetic carbon isotope discrimination for (**a**) cocklebur, (**b**) spinach, and (**c**) magnolia, under conditions of varying light and $CO_2$ concentration, at 21% and 2% $O_2$. Measured values for the four replicate leaves are shown to demonstrate that all leaves responded similarly

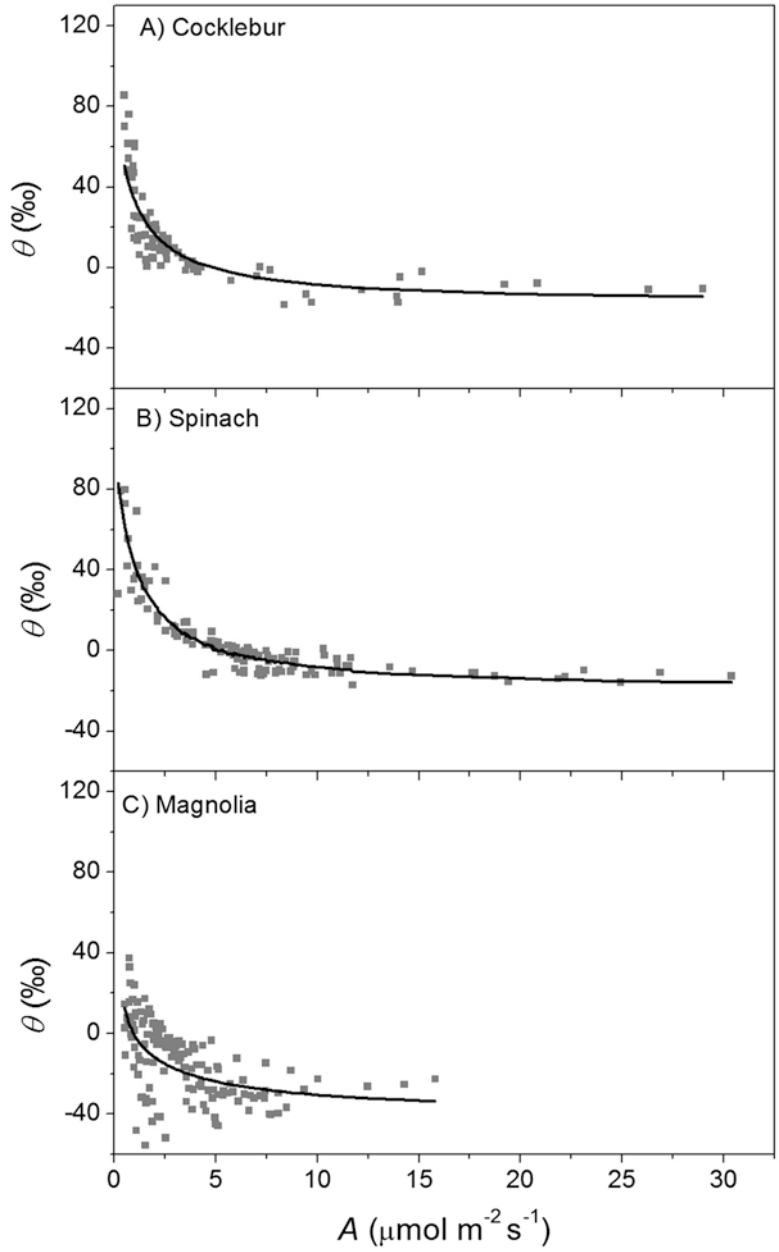


*Fig. 7.2.* Variation in $\theta$ calculated using Eq. (7.6) as photosynthetic rate varies with light, $CO_2$ concentration, and oxygen concentration for cocklebur (**a**), spinach (**b**) and magnolia (**c**). The bold lines are fitted relationships, assuming a single respiratory carbon source that is photosynthetically-linked, predicted by fitting $e$ to be −107, −100 and −62‰ for the three species, respectively

tions would be between −52 and −57‰. Here, in practice, the net photosynthetic fractionation is about 100‰ at low *A* in spinach, meaning that new photosynthates are at about −35–100 = −135‰. Day respired $CO_2$ is found to be enriched by 100‰ (that is, $e$ = −100‰) thus has a $\delta^{13}C$ value of about −135–(−100) = −35‰. A similar calculation can be done with the two other species. Hence, respired $CO_2$ is considerably $^{13}C$-enriched compared to current photosynthates but isotopically similar to old photosynthates.

Assuming current photosynthates as a respiratory source and standard values for $b$ and Γ* (29‰ and 40 μmol mol$^{-1}$, respectively), we fit $R_d$ of 1.05, 1.08 and 0.66 μmol m$^{-2}$ s$^{-1}$ for cocklebur, spinach and magnolia, respectively. These values are close to estimates using both the Kok and the Laisk gas exchange methods. Using either lower $b$ (i.e. 27‰) or lower Γ* (i.e. 35 μmol mol−1) does not significantly alter the fitted values for $e$ or $R_d$ (Table 7.2)

Treating the possible carbon sources for day respiration more rigorously, accounting for both photosynthetically-connected carbon and photosynthetically-disconnected (heterotrophic) substrates, yields interesting results. Cocklebur uses almost entirely new carbon (88%) for photosynthetically-connected respiration, and almost half new carbon (43%) for heterotrophic respiration, but heterotrophic respiration accounts for most of the respiratory flux in the light. Spinach uses very little new carbon (11%) for photosynthetically-connected respiration with a strongly negative fractionation of −100‰, and just over half (59%) new carbon for heterotrophic respiration, but heterotrophic respiration accounts for little of the respiratory flux. In contrast, magnolia uses entirely new carbon for photosynthetically-connected respiration but this forms an

*Table 7.2.* Calculation of respiratory parameters using the graphical method based on plotting $\theta$ against $A$

| | One respiratory source | | Two respiratory sources | | | |
|---|---|---|---|---|---|---|
| | $e$ (‰) | $R_d$ (μmol m$^{-2}$ s$^{-1}$) | $e$ (‰) | $R_d$ (μmol m$^{-2}$ s$^{-1}$) | $e_h$ (‰) | $R_h$ (μmol m$^{-2}$ s$^{-1}$) |
| **Cocklebur** | | | | | | |
| Standard parameters | −107 | 0.84 | −12 | 0.00 | −59 | 0.39 |
| $b$ = 27‰ | −126 | 0.84 | −66 | 0.05 | −60 | 0.38 |
| Γ* = 35 μmol mol$^{-1}$ | −108 | 0.34 | −14 | 0.00 | −125 | 0.33 |
| **% new C** | **<0** | | **88** | | **43** | |
| **Spinach** | | | | | | |
| standard parameters | −100 | 1.08 | −99 | 1.11 | −45 | 0.00 |
| $b$ = 27‰ | −102 | 1.11 | −104 | 1.07 | −46 | 0.02 |
| Γ* = 35 μmol mol$^{-1}$ | −99 | 1.07 | −97 | 1.14 | −45 | 0.00 |
| **% new C** | **9** | | **11** | | **59** | |
| **Magnolia** | | | | | | |
| standard parameters | −62 | 0.66 | −1.0 | 0.00 | −67 | 0.13 |
| $b$ = 27‰ | −64 | 0.70 | −0.7 | 0.00 | −62 | 0.16 |
| Γ* = 35 μmol mol$^{-1}$ | −62 | 0.66 | −0.9 | 0.00 | −68 | 0.12 |
| **% new C** | **<0** | | **98** | | **<0** | |

The apparent fractionation associated with day respiration $e$ (with respect to net fixed carbon) and the day respiration rate are calculated following two hypotheses: *(i)* day respired $CO_2$ comes from photosynthetic cells only (one respiratory source) or *(ii)* there is an additional source disconnected from photosynthesis, e.g. from leaf heterotrophic tissues (two respiratory sources). "Standard parameters" means that the following parameterization was used: $b$ = 29‰, Γ* = 40 μmol mol$^{-1}$ and $f$ = 11‰. The percentage of "new" net fixed carbon in respired $CO_2$ was calculated using mass balance between "old" carbon ($\delta_{air}$ corrected for net photosynthetic fractionation under ordinary gaseous conditions) and "new" carbon ($\delta_{outlet}$ corrected for net photosynthetic fractionation when $A \rightarrow 0$) under standard parameterization

undetectably small part of the total flux, while heterotrophic respiration dominates the flux again with a strong negative fractionation of −67‰ (Table 7.2). However, it should be stressed that these conclusions are limited by instrument precision, both due to low $CO_2$ concentrations and small concentration differences between chamber inlet and outlet air streams. Further measurements would be required, particularly with $\delta_{outlet}$ closer to $\delta_{atm}$, and with $\delta_{outlet}$ more enriched than $\delta_{atm}$ (as described by Hanson et al. 2016)

A strong fractionation effect during $R_d$, as suggested here, does not imply that there are pools of metabolites in the leaves with strongly negative carbon isotope compositions, and there is little experimental evidence of large changes in $\delta^{13}C$ of leaf carbon pools. However, it should be kept in mind that $R_d$ is a small flux compared to $A$, the size of leaf carbon pools, and even the respiratory flux in the dark. It is not surprising under these experimental conditions of extremely low photosynthetic rates that the $\delta^{13}C$ value of evolved $CO_2$ was relatively close to that of carbon fixed under growth conditions because the low rate of carbon fixation would have been insufficient to support the turn-over of respiratory pools. In other words, the influx of new carbon in metabolism was tiny, simply because net photosynthesis was close to zero (compensation point). Thus, catabolism used carbon reserves, and probably to a greater extent in magnolia than in spinach, perhaps due to differences in leaf structure and leaf mass per unit area. Under normal conditions, far from the compensation point, the influx of new carbon participates in sustaining day respiration to a larger (but still appreciably small) extent (Tcherkez et al. 2011)

Wingate et al. (2007) suggested a simple approach to allow for isotopic disequilibria between growth and measurement $CO_2$ by calculating the respiratory fractionation, denoted here as $e^*$, as:

$$e^* = e - \delta_{outlet} + \delta_{atm} \quad (7.16)$$

which yields $e^* = -73‰$ for cocklebur and spinach and −35‰ for magnolia. For comparison, Tcherkez et al. (2011) report $e$ between −14 and −32‰ in *Pelargonium* leaves under industrial $CO_2$ at −45‰, giving $e^*$ between +5 and +23‰. Treating fractionation during day respiration simply using $e^*$ is mathematically convenient, but obscures the complexity of photosynthetically-linked respiration and heterotrophic respiration which drives values of $e$ to seemingly (metabolically) unrealistic values at low photosynthetic rates (i.e. to values that cannot reflect a real enzymatic fractionation). However, this approach is relevant when $A$ is large relative to $R_d$.

## VI. Influence of Day Respiration Fractionation on Mesophyll Conductance

Mesophyll conductance to $CO_2$ diffusion ($g_m$) has been the focus of increasing interest in the last decade, due to recognition of the significant and variable limitation it places on photosynthetic rate (Warren 2008; Flexas et al. 2008). The development of an online, real-time stable isotope method to estimate $g_m$ (Tazoe et al. 2009; Barbour et al. 2010), building on off-line techniques (Evans et al. 1986), has contributed to a rapid expansion of published values for $g_m$. However, the technique requires assumptions for the values of the major $^{12}C/^{13}C$ fractionations ($b$, $e$ and $f$), none of which are well constrained. The value for $b$ is most important when $A/R_d$ is high, but estimates of $g_m$ are extremely sensitive to values for $e$ and $f$ when $A/R_d$ is low, such as approaching the light or $CO_2$ compensation points. Given that we have fitted values for $e$ of −100‰ and −62‰, we explore the influence of these values on $g_m$ estimates.

Using gas exchange and $\Delta_{obs}$ measurements presented above, we calculated $g_m$ using $e = -30‰$ (i.e. using the Wingate et al.

simplification (Eq. 7.16) assuming $e^* = -3‰$; Bickford et al. 2009), and found $g_m$ values were negative when $A$ was less than about 5 µmol $m^{-2}$ $s^{-1}$. A negative value for $g_m$ is physically impossible, so it is obvious that $e = -30‰$ is inappropriate here. Using actual values of $e$ of −100‰, we find that $g_m$ is positive for all measurements in cocklebur and spinach, albeit comparatively low. For cocklebur, $g_m$ declined with decreasing light below 100 µmol $m^{-2}$ $s^{-1}$ PAR, when measured at $c_a$ around 380 µmol $mol^{-1}$; from 0.018 to 0.005 mol $m^{-2}$ $s^{-1}$ $bar^{-1}$ (Fig. 7.3a). Also in cocklebur, $g_m$ increased with decreasing $c_i$, and was lower at lower light levels (Fig. 7.3b); $g_m$ declined from 0.14 to 0.01 mol $m^{-2}$ $s^{-1}$ between $c_i$ of 100 and 200 µmol $mol^{-1}$. Estimated $g_m$ also declined with increasing $c_i$ in spinach, with $g_m$ being less sensitive to $c_i$ for the same leaf when measured under 2% compared to 21% $O_2$ (Fig. 7.4). The very low fluxes measured in magnolia meant that $g_m$ estimates were highly variable, but the general trends in $g_m$ were also observed (data not shown).

The observation of increasing internal conductance $g_m$ with increasing light and decreasing $CO_2$ has been widely observed (Flexas et al. 2007, 2008; Hassiotou et al. 2009; Vrabl et al. 2009; Douthe et al. 2011; Tazoe et al. 2011; Xiong et al. 2015), and may relate to the activity of carbonic anhydrase (Makino et al. 1992), or to variable activity or expression of $CO_2$-permeable aquaporins in the plasma membranes or chloroplast envelopes (Terashima and Ono

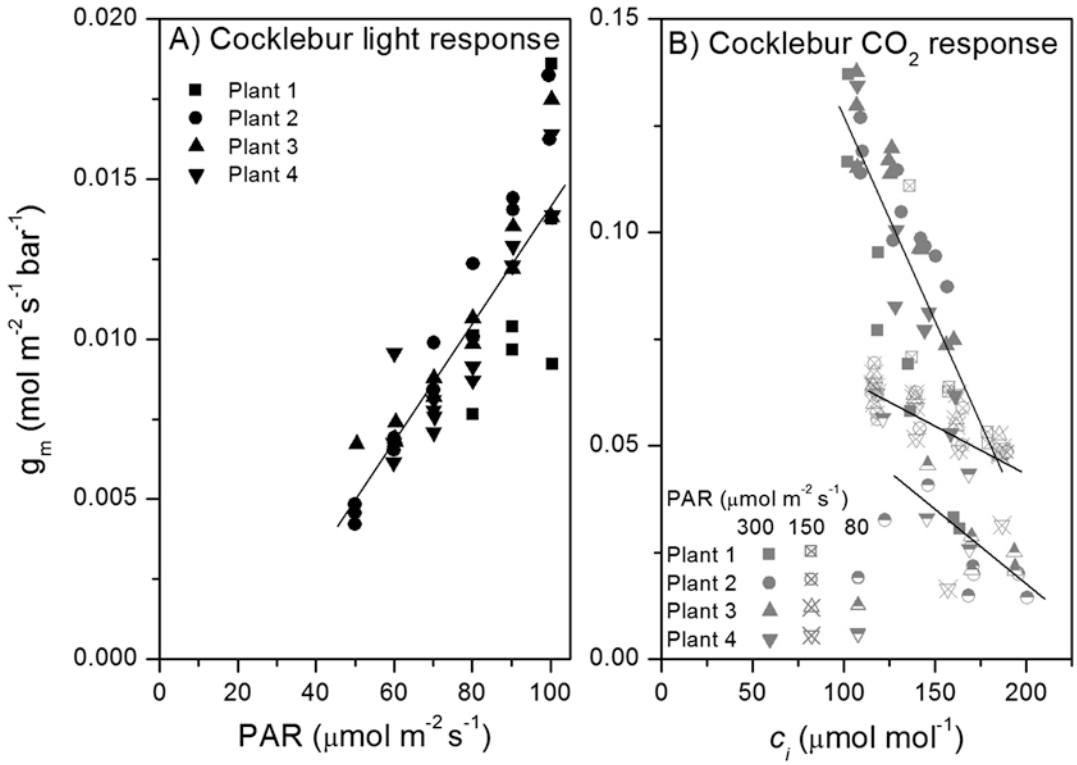


*Fig. 7.3.* The response of mesophyll conductance ($g_m$) to irradiance (**a**) and leaf internal $CO_2$ partial pressure at differing low irradiances (**b**) for cocklebur. The lines represent linear regressions: in (**a**) $g_m = 0 + 2.00 \times 10^{-4}$ PAR, $R^2 = 0.77$, $P < 0.0001$; in (**b**) $g_m = 0.25–11.5 \times 10^{-4}$ $c_i$, $R^2 = 0.67$, $P < 0.0001$ for 300 µmol $m^{-2}$ $s^{-1}$ PAR, $g_m = 0.106–3.1 \times 10^{-4}$ $c_i$, $R^2 = 0.15$, $P = 0.019$ for 150 µmol $m^{-2}$ $s^{-1}$ PAR, $g_m = 0.088–3.6 \times 10^{-4}$ $c_i$, $R^2 = 0.57$, $P = 0.0001$ for 80 µmol $m^{-2}$ $s^{-1}$ PAR. Measured values for the four replicate leaves are shown to demonstrate that all leaves responded similarly

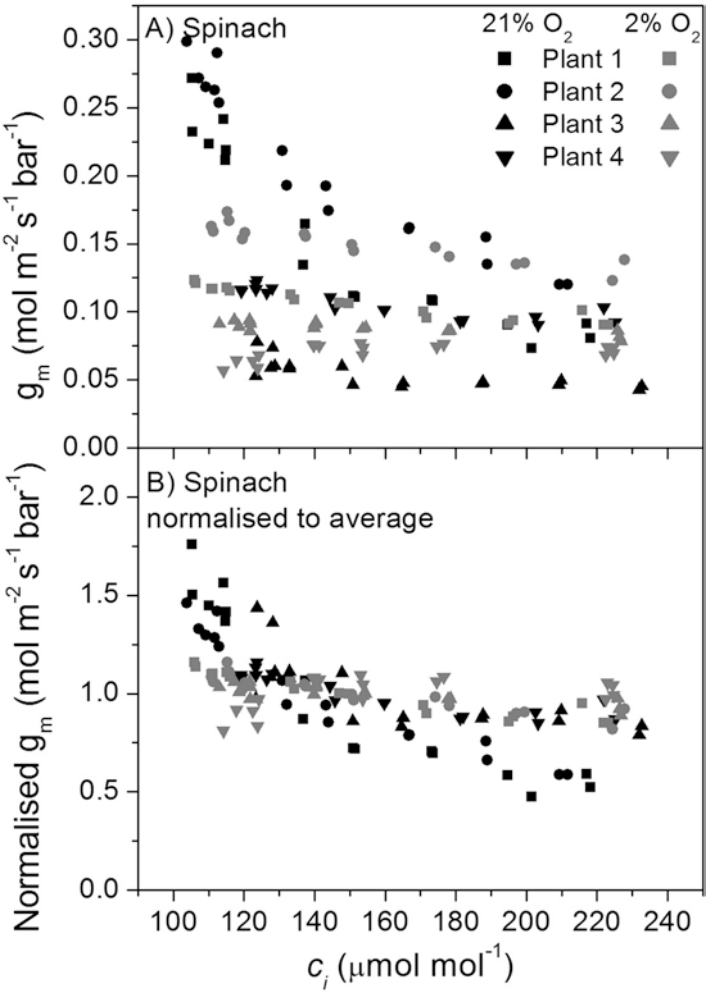


*Fig. 7.4.* The response of mesophyll conductance ($g_m$) to leaf internal $CO_2$ partial pressure for spinach when measured at 21 and 2% $O_2$. In (**b**) $g_m$ values are normalized to the average $g_m$ for that leaf at the given $O_2$ concentration, to facilitate comparison between leaves and $O_2$ concentrations. Measured values for the four replicate leaves are shown to demonstrate that all leaves responded similarly

2002; Flexas et al. 2006; Uehlein et al. 2003, 2008). Indeed, aquaporins have been shown to influence $CO_2$ membrane permeability in plasma membrane vesicles isolated from *Arabidopsis* and pea leaves, despite the absence of a correlation between water and $CO_2$ permeability of the membranes (Zhao et al. 2016). The data presented here confirm $g_m$ responsiveness to light and $CO_2$ concentration approaching the compensation points. Finally, it should be noted that assuming $e = -100‰$ for spinach and cocklebur did not significantly alter the estimates of $g_m$ at photosynthetic rates further from the light and $CO_2$ compensation points, even though such a negative value of $e$ is inappropriate when the respiratory flux forms a very small component of net $CO_2$ exchange.

## VII. Conclusions

The data and calculations presented in this Chapter suggest that isotope effects during leaf day respiration are quantitatively similar when approaching the light and $CO_2$ compen-

sation points, and are not strongly influenced by photorespiration. We found apparent fractionation during $R_d$ to be −100‰ for cocklebur and spinach, and −62‰ for magnolia. These values, strongly negative, simply stem from the definition of $e$ in equations describing $\Delta_{obs}$, whereby it is expressed relative to current net photosynthetically fixed carbon. In other words, the apparent very negative values are mostly a consequence of *(i)* the use of $^{13}C$-depleted $CO_2$ during gas exchange measurements, and *(ii)* the prevalence of $CO_2$ respiratory efflux from an "old" carbon source at low $A$, causing very large $\Delta_{obs}$ values. The $\delta^{13}C$ of $CO_2$ released by $R_d$ was close to the estimated $\delta^{13}C$ of photosynthates formed under growth conditions prior to conducting measurements by gas exchange. The approach described here provides estimates of $R_d$ assuming either a single substrate of current photosynthates or two substrate pools, and values of $R_d$ were similar to measured values using either the Kok or the Laisk method.

Again, the approach of linking $\delta^{13}C$ of day respired $CO_2$ to current photosynthetic discrimination (e.g. Farquhar et al. 1989) is mathematically convenient but causes seemingly strange effects approaching zero net carbon exchange (positive, as here, or negative as in Hanson et al. 2016). The isotopic disequilibrium approach suggested by Wingate et al. (2007) partly addresses this issue but obscures the complexity of photosynthetically-linked respiration and heterotrophic respiration, both of which may use either newly-fixed carbon or that fixed under previous conditions, and this complexity can affect $\Delta_{obs}$ and $e$ when $A$ is low. Finally, $g_m$ was found to increase approaching the $CO_2$ compensation point, but decrease approaching the light compensation point in cocklebur and spinach, provided the actual value of $e$ was used (e.g. –100‰ in spinach). Strongly negative values of $e$ did not affect estimates of $g_m$ at higher photosynthetic rates. However, strongly negative $e$ values are unlikely to be relevant at higher photosynthetic rates when $R_d$ is sustained by current photosynthates to some extent, and $\Delta_{obs}$ is much lower because $R_d$ is proportionally much smaller than $A$.

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