Photorespiration Methods and Protocols Book Chapter

Title: *Using gas exchange to study CO_2 release during photosynthesis with steady- and non-steady-state approaches*

Authors: Stephanie C. Schmiege[1,2], Berkley Walker[3,5], Thomas D. Sharkey[1,3,4]

[1]Plant Resilience Institute, Michigan State University, East Lansing, MI 48824 USA

[2]Department of Biology, Western University, London, Ontario, Canada N6A 5B7

[3]MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824 USA

[4]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824 USA

[5]Department of Plant Biology, Michigan State University, East Lansing, MI 48824 USA

Corresponding author: Stephanie C. Schmiege, schmie18@msu.edu

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ABSTRACT

Measures of respiration in the light and C_i* are crucial to the modeling of photorespiration and photosynthesis. This chapter provides background on the equations used to model C_3 photosynthesis and the history of the incorporation of the effects of rubisco oxygenation into these models. It then describes three methods used to determine two key parameters necessary to incorporate photorespiratory effects into C_3 photosynthesis models: respiration in the light (R_L) and C_i*. These methods include the Laisk, Yin and isotopic methods. For the Laisk method we also introduce a new rapid measurement technique.

KEY WORDS

Respiration in the light, Laisk method, Kok method, Yin method, isotopes, Γ_*, C_i*

INTRODUCTION

Net gas exchange measurements have been essential for linking photorespiration to carbon assimilation. Evidence for photorespiration and other respiration in the light has been examined for the last 100 years. For example, Warburg [1] used gas exchange methods to discover that oxygen inhibited photosynthesis [2]. Decker [3] showed that CO_2 evolution immediately after imposing darkness was significantly greater than a somewhat stable rate of CO_2 evolution reached several minutes after imposing darkness. (This Post-Illumination Burst is explored further in this book.) The interpretation was that there is one or more processes that release (or "respire") CO_2 and that are stimulated by light. This was variously called photorespiration e.g. Rabinowitch [4] and or light respiration (R_L). The possibility of light stimulated CO_2 release was

47 called “a nightmare oppressing all who are concerned with the exact measurement of
48 photosynthesis” by Rabinowitch [4]. Given the importance of gas exchange to the discovery of
49 photorespiration and the importance of photorespiration to predicting gas exchange of
50 photosynthesis, we present here a summary of the discoveries of photorespiration by gas
51 exchange and how these discoveries informed the subsequent equations used to model it. This
52 historical perspective is helpful to illustrate the various assumptions integrated into commonly
53 used forms of these models. We then present methods for exploring photorespiration using these
54 models and gas exchange methods based on steady- and non-steady-state assumptions. In this
55 chapter we will focus specifically on C_3 photosynthesis.

56 A significant part of CO_2 release during photosynthesis was found to be associated with,
57 but not necessarily coming directly from, glycolate metabolism in peroxisomes [5]. The source
58 of the glycolate was found to be dependent on rubisco [6]. The metabolism that involves CO_2
59 released during metabolism of glycolate produced by oxygenation of ribulose 1,5-bisphosphate
60 (RuBP) is now the definition of photorespiration, while other possible CO_2-releasing processes
61 occurring in the light were collectively called day respiration [7]. This nomenclature was the
62 result of the initial belief that this CO_2 release comes from mitochondrial reactions and is the
63 same as respiration in the dark [8] and so called R_d. To allow that there may be other sources of
64 CO_2 release during photosynthesis that are important to understanding gas exchange behavior,
65 this daytime “dark respiration” was rebranded as “day respiration” so that the abbreviation could
66 be retained. Here we will define respiration in the light (R_L) as respiration (i.e., CO_2-release) in
67 the light that is not photorespiration. While this could involve mitochondrial metabolism, isotope
68 studies have consistently shown that there is very little activity of the tricarboxylic acid cycle in
69 the light [9-11].

The discovery of rubisco-catalyzed RuBP oxygenation as the initial event in photorespiration opened the door to a quantitative description of the effect of oxygenation on net photosynthesis [12]. Thus,

$$A = v_c - t \cdot v_o \qquad \text{Equation 1}$$

where A is net CO_2 assimilation (what we measure in a gas exchange system), v_o and v_c are the velocities of the oxygenase and carboxylase respectively, and t is the proportion of carbon released as CO_2 during metabolism resulting from one oxygenation event. The actual velocities are denoted by lower case v's as is the norm in enzymology, to distinguish from theoretical maximum velocities, V_{max}. The value of t has been taken to be 0.5 [13] based on the glycolate metabolism pathway proposed by Tolbert [14]. The relationship between v_o and v_c, that is, the ratio of oxygenation to carboxylation, is labeled Φ and given by

$$\frac{v_o}{v_c} = \frac{V_o K_c}{V_c K_o} \cdot \frac{O}{C} = \Phi \qquad \text{Equation 2}$$

where V_o and V_c are V_{max} for the oxygenase and carboxylase activities, K_c and K_o are the Michaelis constants for carboxylation and oxygenation, and O, and C are the partial pressures or concentrations of oxygen and CO_2. From Equation 2 it is clear that the ratio of oxygenation to carboxylation is linearly dependent on oxygen and inversely dependent on CO_2. Keck, Ogren [13] used equations 1 and 2 to derive the CO_2 partial pressure (or concentration as long as the

92 K_m's are in the same units) at which CO_2 assimilation by carboxylation is equal to CO_2 release
93 by oxygenation, the compensation point, Γ.
94
95 $\Gamma = \frac{tV_oK_cO}{V_cK_o}$. Equation 3
96
97 Farquhar et al. [8] pointed out that to apply the above equations to gas exchange measurements
98 of photosynthesis and related photorespiration, it was necessary to account for R_L. Thus,
99 Equation 1 becomes
100
101 $A = v_c - t \cdot v_o - R_L$. Equation 4
102
103 The rubisco compensation point as defined in Equation 3 will occur when gas exchange is
104 showing a CO_2 release equal to R_L. Farquhar et al. [8] renamed Γ as defined initially by Equation
105 3 as $\Gamma*$ since it is the rubisco compensation point, not the point where net leaf CO_2 exchange is
106 zero [see 15 for full derivations]. Thus, the CO_2 partial pressure at which CO_2 exchange is
107 independent of light intensity is $\Gamma*$ (but see below regarding C_i*). And, at $\Gamma*$, the measured CO_2
108 exchange is a measure of R_L.
109
110 Using Φ as defined in Equation 2, Equation 4 is
111
112 $A = v_c(1 - t\Phi) - R_L$. Equation 5
113
114 Equations 2 and 3 can then be used to derive that

$\frac{v_o}{v_c} = \frac{1}{t}\frac{\Gamma_*}{C}$ typically shown to be $\frac{2 \cdot \Gamma_*}{C}$ $if\ t = 0.5$. Equation 6

Laisk [16] pointed out that at $\Gamma*$, A will be independent of light and so a series of CO_2 response curves at limiting light levels will cross over at $\Gamma*$. This makes it possible to use gas exchange measurements to determine a parameter that combines many rubisco characteristics, or in other words, provides a powerful validation of the above theory when they do cross over *in vivo* at a value predicted by rubisco kinetics *in vitro*. However, $\Gamma*$ depends on t, and so if t is variable, $\Gamma*$ will be variable.

However, the point at which various CO_2 response curves cross over will not be $\Gamma*$ if there are other sources of CO_2 release or if there is diffusion resistance between the intercellular air spaces and rubisco. This is because the diffusion resistance encountered by the net flux of CO_2 across the mesophyll will cause the apparent $\Gamma*$ to be at a lower CO_2 partial pressure than the true $\Gamma*$. This apparent $\Gamma*$ is called C_i* [17]. The relationship between $\Gamma*$ and C_i* is

$$\Gamma_* = C_i^* + \frac{R_L}{g_m} \quad \text{Equation 7}$$

where g_m is the diffusion conductance between the intercellular air spaces and rubisco. Notably, if there are large diffusive barriers between the mitochondrial release of CO_2 and rubisco, equation 7 is not as valid and an additional theoretical framework involving multiple resistances are needed [18].

Gas exchange measurements can be used to estimate both C_i* and R_L, which, as demonstrated through the theoretical equations above, are important parameters to estimates of rubisco oxygenation and thus photorespiration. Here we outline several gas exchange methods used to estimate C_i* and R_L, any recent advances in these methods, and any important considerations when using these methods. Both C_i* and R_L can be measured using the Laisk method [Laisk [19] as described in English in Laisk [16]], whereas R_L can also be measured by several other techniques including the Kok [20] and Yin [21,22] methods, and by using isotopes [23,24]. Materials for these methods are listed below followed by the protocols themselves. Finally, we present additional insight on isotopic methods gained from metabolic flux analysis studies.

MATERIALS

Laisk, Kok, and Yin materials

1. Infra-Red Gas analyzer (IRGA)-based gas exchange system. The system should have the capability to measure both gas exchange and chlorophyll fluorescence simultaneously for the Yin method.

Isotopic materials

1. IRGA
2. Gas tanks: 99% $^{13}CO_2$, 99% $^{12}CO_2$, N_2 and O_2
3. 5 mass flow controllers

4. Swagelok T-joint attached to the back of the IRGA measurement head to facilitate easy switching from $^{12}CO_2$ to $^{13}CO_2$
5. Bev-A-line IV tubing (more gas tight than Teflon tubing)
6. Bubbler to humidify airstream
7. Tunable diode laser (TDL) or equivalent method capable of identifying $^{12}CO_2$ emission from a leaf in a $^{13}CO_2$ background

METHODS

This chapter does not provide detailed instructions on best practices when using gas exchange systems as many such guides already exist in the literature (see for example [25]); nonetheless, we do feel it is important to have a basic understanding of the measures and units used to describe the proportions of a gas in air. As such, we have included a basic primer on this topic in Appendix 1.

Laisk method for estimating C_i and R_L*

The Laisk method [16,19] estimates C_i* and R_L by collecting at least two but in practice typically three to five photosynthetic CO_2 response curves at different light intensities such that the curves intersect at a single point where the x and y coordinates are equal to C_i* and R_L, respectively (Figure 4). While the expectation is that all the CO_2 response curves should cross over at the same point, it has frequently been documented that the cross-over points can differ among pairs of curves [18]. For this reason, several methods have been developed to identify a common crossover point from the curves including averaging the values obtained from the intersection of

each pair of curves and the slope-intercept regression method [26,27]. The CO_2 response curves used to identify this cross over point are collected either by steady-state gas exchange techniques, or by employing the new dynamic assimilation technique (DAT; Figure 1) [28]. The DAT technique significantly reduces the time required to collect the CO_2 response curves allowing for higher throughput and leaving less time for the physiology of the leaf to change in response to holding them at or below the compensation point. Both steady-state and DAT techniques provide comparable estimates of R_L, although estimates of C_i* may vary slightly between the two techniques [29]. At this stage it is not fully clear why there are slight differences between techniques in determining C_i* but it is possible that these small shifts may be due to slight changes in g_m or in glycine export from the photorespiratory pathway [29].

Steady-state Laisk protocol

1. Identify at least three light intensities[1,2] that provide evenly spaced differences in the initial slopes of the CO_2 response curves.
2. Identify CO_2 concentrations that span the linear portion of the CO_2 response curve but minimize the amount of time spent at very low CO_2 concentrations[3]. Stay at each CO_2 concentration for at least 30 s but no more than 120 s. Point matching should be employed before each measurement in IRGA's with two separate detectors[4].
3. Run CO_2 response curves at each light intensity, returning to 420 ppm between each curve until all light levels have been completed.

DAT Laisk protocol (using the LI-6800)

The LI-6800 is currently the only IRGA capable of running DAT curves, so specific instructions are provided for this particular instrument.

1. Set up DAT on the LI-6800[5]. We have found that this technique works best with LI-6800s that have the most recent processor installed. This processor decreases lagging in the measurements during the CO_2 ramps.
    a. Enable dynamic equations.
    b. Test dynamic tuning using an empty chamber and your chosen flow rate (usually between 300 – 600 μmol s^{-1})
    c. Set up range matching
2. Light intensities for each of the curves should be identified as above.
3. At the first light intensity, ramp the reference CO_2 concentration from high to low such that you collect the approximately linear portion of a traditional A/C_i curve[6].
4. Return to 420 ppm (ambient atmospheric CO_2 concentration) before running the curve at the next light level to minimize time spent at low CO_2 concentrations.
5. Point match before running next curve, especially if the LI-COR range match was set up before the IRGA was fully warmed up (within the first hour of starting up the LI-COR).
6. Repeat steps two through four until curves at all light levels have been completed.

Laisk curve analysis protocol

This method extracting C_i* and R_L from the Laisk curves uses the slope-intercept regression method [26,27].

1. If using DAT-collected Laisk curves, remove the first five or so points from each curve as they show the initial adjustment to the CO_2 ramp and are not linear.
2. Visually assess the linearity of the data you have collected. Subset to datapoints in the linear portion. We have found these to be the points below 85 – 100 ppm.
3. Fit linear regressions to the CO_2 response curves at each of the light intensities.
4. Extract the slope and intercept of the linear regressions[7].
5. Fit a linear regression to the slopes and intercepts from step 4 with the slopes on the *x*-axis and the intercepts on the *y*-axis. The slope and intercept of this line provide estimates of C_i* and R_L, respectively.

Kok and Yin methods for estimating R_L

In addition to the Laisk method, R_L can also be estimated via the Kok or Yin method. In these methods, R_L is estimated by collecting a photosynthetic light response curve with particular attention to low light intensities around the light compensation point. The Kok method derives its name from Bessel Kok, who discovered a subtle shift in the response of photosynthesis to light intensity around the light compensation point, now called the "Kok effect" [20]. The point where this shift occurs has been called the breakpoint. Biologically, it has been interpreted as the point where leaf mitochondrial respiration is suppressed by light. Consequently, if a linear regression is fit to the points above the breakpoint, the *y*-intercept will provide an estimate of R_L. In contrast, if a linear regression is fit to the points below the breakpoint, an estimate of respiration in the dark (R_D) is gained instead. An important update for this method requires accounting for the fact that internal CO_2 concentrations (C_i) increases as light intensity decreases [30]. The higher C_i at low light levels suppresses photorespiration relative to carboxylation

resulting in higher measured photosynthetic rates in the linear portion of the curve [31]. The result is a lower slope in the linear relationship through the observed data than would be observed if the C_i were constant, resulting in an underestimation of R_L. Correcting for changes in C_i should be undertaken and methods for this are fully described in Kirschbaum, Farquhar [32] and Ayub et al. [30].

More recently, an additional modification to the Kok method has been proposed. The Kok method assumes that photosystem II electron transport efficiency (Φ_{II}) is constant across all light levels used in the analysis; however, Φ_{II} declines at high light levels (Figure 2). This has led to the advent of the Yin method, developed by Xinyou Yin [21,22], which incorporates this decline in Φ_{II}. Φ_{II} is estimated by taking simultaneous measurements of chlorophyll fluorescence during gas exchange. At each light intensity of the photosynthetic light response curve, Φ_{II} is calculated as $1 - F_s/F_m'$. Then, Φ_{II} is incorporated into the traditional light response curve by plotting photosynthesis as a function of $I_{inc}\,\Phi_2/4$ where I_{inc} is the incident irradiance. R_L is estimated from this modified light response curve by extracting the intercept from the linear regression of photosynthesis to $I_{inc}\,\Phi_{II}/4$ in the lower portion of the response curve. These estimates of R_L often slightly larger than estimates from the Kok method, but comparable to estimates acquired via the Laisk method [22].

Kok and Yin protocol

1. Data for Kok and Yin methods are acquired using steady-state gas exchange techniques. Data for the Kok method can be collected using an IRGA with precise light intensity

control. Data for the Yin method must be collected with an IRGA capable of acquiring simultaneous chlorophyll fluorescence and gas exchange measurements.

2. For the Kok and Yin methods, a larger number of datapoints should be acquired at lower light intensities[8].

Isotopic methods for estimating R_L

In addition to gas exchange methods, there are also isotopic methods that have been used to estimate R_L. Although these methods are not easily employed in field settings, limiting their application, they have been lauded for their ability to measure R_L under high light and photosynthetic conditions. The fact that Laisk, Kok and Yin methods require altering CO_2 concentrations and/or light conditions at conditions near the CO_2 or light compensation point limits our ability to assess the magnitude of this flux under ambient photosynthetic conditions. One of the first isotopic methods used to assess R_L under high light and high photosynthetic conditions was the method developed by Francesco Loreto [23,24]. In this technique the leaf is rapidly transitioned to 99.9% $^{13}CO_2$ environment and the $^{12}CO_2$ emission from the leaf (measured using an IRGA with reduced sensitivity to $^{13}CO_2$) is measured as an estimate of R_L. Theoretically this method provides accurate estimates of R_L because CO_2 released by R_L comes from older stored pools of carbon that will not be labeled by exposure to $^{13}CO_2$. The advantage of this method is that R_L can be assessed under a variety of different light conditions and CO_2 concentrations. Nevertheless, there are several caveats that are important to be aware of when using this method.

The first of these is that the intermediates of the Calvin Benson cycle label quickly during the first 5-10 minutes, but are not fully labeled for many hours [33]. We know that the Calvin

293 Benson cycle intermediates are 80 to 90% labeled after 20 min and can take this degree of label
294 into account when estimating R_L. However, because the Calvin Benson cycle is incompletely
295 labeled, we can infer that photorespiration also remains incompletely labeled to some degree.
296 Up until now, there have been few estimates of the degree of label in photorespiration, making it
297 challenging to ascertain whether the total measured $^{12}CO_2$ efflux from the leaf is due to R_L or the
298 incomplete labeling of photorespiratory CO_2 release. However, a recent metabolic flux analysis
299 by Xu et al. [10] may provide the information necessary to estimate this (see below).
300 An additional factor that must be accounted for is the fact that CO_2 released in the cell
301 can be refixed in the Calvin Benson cycle or released into the atmosphere. Any estimates of R_L
302 via this isotopic labeling method must take refixation into account. In fact, a simple
303 mathematical method was used by Loreto et al. [23] in which reassimilated $^{12}CO_2$ was calculated
304 from the ratio of $^{12}C_i$ to $^{13}C_i$ multiplied by the photosynthetic rate. In the second appendix to this
305 chapter, we provide a more complex accounting for refixation that considers the updated
306 understanding of $^{13}CO_2$ labeling time courses, the potential for competitive interactions between
307 the isotopes, and photorespiration effects on the relationship between photosynthesis and the
308 velocity of carboxylation (see Appendix 2).
309 In the method below we present a modified version of the setup used by Loreto, where
310 instead of a $^{13}CO_2$-insensitive IRGA, we use a TDL tuned to wavelengths that can sensitively
311 detect $^{12}CO_2$ in an enriched $^{13}CO_2$ background with great sensitivity and precision.
312
313 *$^{13}CO_2$ labeling protocol*
314 System setup to prepare the air mixture that will be fed into the gas exchange system
315 1. Connect the O_2, 99% $^{12}CO_2$ and 99% $^{13}CO_2$ to flow controllers

2. For the N_2 divide the airstream into two separate tubing paths with separate flow controllers. One path will pass through a bubbler, the other through desiccant. This will allow some control of the humidity in the airstream.
3. The humid and dry N_2 airstreams should then be joined to the O_2 airstream.
4. Install the Swagelok T-joint on the air inlet of the IRGA head.
5. Connect the N_2, O_2 tube, and the $^{13}CO_2$ and $^{12}CO_2$ lines such that turning the four way switching valve will rapidly add either $^{13}CO_2$ or $^{12}CO_2$ to the N_2, O_2 airstream entering the leaf chamber (see Figure 3 for a flow path diagram of the system setup).
6. Flow controllers should be set to provide 80% N_2, 20% O_2 and 420 ppm of either $^{13}CO_2$ or $^{12}CO_2$.
7. Connect the chamber (sample) and reference air outlets from the IRGA to a TDL or equivalent system to measure the $^{12}CO_2$ in the exhaust chamber air.

Measurements

1. Let the leaf acclimate in $^{12}CO_2$ until photosynthesis and stomatal conductance are stable (often approximately 20 min). Record photosynthesis, transpiration, and flow rate on the gas exchange system. These will be used later to calculate $^{12}CO_2$ concentrations.
2. After acclimation, switch from $^{12}CO_2$ to $^{13}CO_2$ (Figure 4). Record the $O_{16}C_{12}O_{16}$ peak of the TDL absorbance spectra for 20 min.

Calculating $^{12}CO_2$ efflux

1. To calculate the total $^{12}CO_2$ efflux from the $O_{16}C_{12}O_{16}$ peak of the TDL absorbance spectra, you will need to take into account your leaf area and correct your flow rate to account for transpiration as water vapor efflux from the leaf increases total the flow rate [see appendix 2 in 34].

INSIGHT FROM METABOLIC FLUX ANALYSIS

One of the concerns with isotopic methods for estimating R_L is the fact that we do not know how much of the $^{12}CO_2$ efflux from the leaf is due to R_L as opposed to incomplete labeling of photorespiration with $^{13}CO_2$. Metabolic flux analysis provides a unique opportunity to examine the contributions of different CO_2 releasing processes to total $^{12}CO_2$ emission. Xu et al. [10] provide a unique dataset that allows a first approximation of the contributions from the various CO_2 releasing processes in the leaf occurring during photosynthesis. Their dataset includes the degree of label in a variety of different metabolites along with the velocity of CO_2 release from the enzymes catalyzing the processes. From these data, we can calculate the total ^{12}C emission from a leaf as the sum of the CO_2 releasing fluxes multiplied by the degree of ^{12}C label remaining in the metabolites after 30 minutes in a 99% pure $^{13}CO_2$ environment if we assume that the enrichment of these metabolites is the same during a $^{13}CO_2$ experiment measured with the TDL. Thus, contributing pathways could include the glucose 6-phosphate (G6P) shunt, fatty acid synthesis, photorespiration and the tricarboxylic acid (TCA) cycle such that:

$$^{12}C = (1 * R_{UDPG} * v_{6PGD}) + (1 * R_{PEP} * v_{PDH.c}) + (0.5 * R_{RUBP} * v_o) + (1 * R_{PEP} * v_{PDH.m}) + (1 * R_{ICI} * v_{IDH}) + (1 * R_{ICI} * v_{KGDH}) \quad \text{Equation 12}$$

where ^{12}C is the total ^{12}C emission from the leaf, v is the velocity or rate of flux contributing to the total ^{12}C emission, and R is the % ^{12}C label remaining in the metabolites. UDPG, UDP glucose; 6PGD, 6 phosphogluconate dehydrogenase; PEP, phosphoenolpyruvate; PDH.c,

chloroplastic pyruvate dehydrogenase; RUBP, ribulose-1,5-bisphosphate, v_o, velocity of oxygenation; PYR.m, mitochondrial pyruvate; PDH.m, mitochondrial pyruvate dehydrogenase; ICI, isocitrate; IDH, isocitrate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase.

Note that in some cases the nearest upstream metabolite was not available and so nearby representative metabolites were chosen instead. Thus, in the G6P shunt, we have used % ^{12}C release from UDPG instead of 6-phosphogluconate (6PG) as 6PG is very hard to estimate. In fatty acid synthesis, we have used PEP instead of chloroplastic pyruvate (PYR.c) as evidence from work on isoprene by Sharkey et al. [33] indicates that sources of carbon for the methyl erithritol pathway, glyceraldehyde 3-phosphate and pyruvate, are labeled to a similar degree as CBC intermediates. In photorespiration, we have used RUBP instead of glycine because glycine can also be stored in the vacuole making it challenging to differentiate between slow and fast pools of this metabolite. The % label in glycine would need to be the total of both slow and fast pools. Thus, the % ^{12}C label in glycine would be most accurately characterized according to the following equation:

$$R_{GLY} = (v_o * R_{RUBP} + v_{GLY.v} * R_{GLY.v})/(v_o + v_{GLY.v}) \quad \text{Equation 13}$$

Where the total label in glycine includes the rate of exchange with, and ^{12}C label in, the slow, vacuolar pool of glycine (GLY.v). As we do not have estimates of GLY.v, we have used RUBP to set the degree of label in glycine. Finally, in the TCA cycle CO_2 releasing reactions, we have used the label in PEP instead of in mitochondrial pyruvate because there is large variability in the pool of pyruvate and we have used the label in ICI instead of α-ketoglutarate as we have no estimate of the % ^{12}C label in this α-ketoglutarate.

385 It is worth noting here that there is a possible additional CO_2 releasing pathway in which
386 malate is decarboxylated by malic enzyme to form pyruvate. This reaction has not previously
387 been considered in flux analysis studies. Consequently, we do not know the velocity of this
388 reaction compared to other CO_2 releasing reactions, and have therefore excluded it from this
389 current assessment. Future studies could examine this further.
390 By using the % ^{12}C label from the identified metabolites from Xu et al. [10], and
391 converting the velocities from μmol metabolite $g^{-1}FW\ hr^{-1}$ to μmol $m^{-2}\ s^{-1}$ using the ratio of fresh
392 weight to area of 550 g m^{-2}, we have calculated the contribution to $^{12}CO_2$ release from each of
393 the processes (Table 1). From these calculations we estimate that the processes usually
394 considered to contribute to R_L (the G6P shunt, fatty acid synthesis and the TCA cycle) release a
395 total of 0.374 μmol $m^{-2}\ s^{-1}$ CO_2 while photorespiration releases 0.245 μmol $m^{-2}\ s^{-1}$ CO_2. Thus,
396 we can see that photorespiration comprises a large fraction of the total $^{12}CO_2$ release as measured
397 using isotopic methods, and accurately accounting for this photorespiratory contribution is
398 critical to accurate estimations of R_L via this technique. Not only is this new accounting
399 important for isotopic methods, it highlights that photorespiration contributes a large proportion
400 to the total CO_2 release during photosynthetic daylight hours.
401
402
403 NOTES
404 [1]When setting the light intensity on a LI-COR gas exchange instrument such as the LI-6800, we
405 set the proportion of red light and blue light reaching the leaf is 50:50, which might be better for
406 keeping the stomata open during multiple rounds of variation in light and CO_2 [35].

[2]An initial light response curve can be used to select light intensities that will give an even spread of slopes during the Laisk measurement.

[3]Generally, it is not recommended to go below 25 ppm when taking steady-state gas exchange measurements. We have used the following CO_2 concentrations with good results: 150, 100, 75, 50, 25 ppm.

[4]Some gas exchange systems are not capable of measuring at CO_2 levels below the cross over point. It is possible to use projections from the higher CO_2 concentrations, but this is not optimal.

[5]For all Laisk measurements, if using a gas exchange system with fluorescence capabilities, make sure that fluorescence is turned off.

[6]We have found that starting at 150 ppm and ramping to 0 ppm at a rate of 50 ppm min^{-1} works well.

[7]There is a spreadsheet available in the supplemental information of [27] to perform the analysis according to the slope-intercept regression method.

[8]We recommend collecting the photosynthetic and fluorescence measurements at the following light intensities: 100, 75, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5 $\mu mol_{photons}\, m^{-2}\, s^{-1}$. These measurements can be combined with a full light response curve if desired.

APPENDICES

Appendix 1 – A primer on gas exchange – measures of the proportion of a gas in air

There are several aspects of using gas exchange to study photorespiration that can be confusing initially. To start, how should one describe the amount of gas being used? Gases dissolve into liquids in proportion to their partial pressure (Henry's law). In ideal gases, the total pressure is

the sum of the pressures that would be exerted by each component (Dalton's law). A common unit of pressure is the standard atmosphere (at sea level). The SI unit for pressure is the Pascal (Pa), one standard atmosphere is 101.3 kPa. People use bar, which is convenient because 1 bar is 1.013 atmospheres. The atmosphere with a sea-level pressure of 101.3 kPa total pressure would have about 78 kPa nitrogen, 21 kPa oxygen, 1 kPa argon, 42 Pa CO_2, and zero to ~4 kPa water vapor.

For photorespiration studies we want to know the availability of CO_2 and oxygen. According to Henry's law, CO_2 dissolves into the water-saturated cell walls inside a leaf according to its partial pressure. To illustrate, at the top of a mountain the ratio of oxygen partial pressure to total pressure is the same as at sea level, but the total pressure is less and so the partial pressure of oxygen is less, making it hard to breath.

Most often people express CO_2 in parts per million (and oxygen in %). These are unitless ratios (% and PPM are not units, they are used when units cancel). A very useful fact is that the partial pressure of a gas divided by the total pressure is the same as the partial volume of a gas divided by the total volume or the number of moles of the gas divided by the total number of moles of all gases present. This is mole fraction and denoted χ. Since there are only 0.00042 moles of CO_2 in a mole of air, we express this as moles of CO_2 per million moles of air, ppm. This is different from the ppm used in fertilizer studies. In that usage, 1 ppm is 1 mg per liter. Since milligrams and liters are not the same, use of ppm in this context is often frowned upon, but in gases, expressing mole fraction is defensible. However, the criticism of mg per liter as ppm spilled into gas studies so now to avoid saying ppm we use $\mu l\ l^{-1}$ or, because lower case l is confused with

the numeral one, μL L^{-1}. Others use μmol mol^{-1} and also μbar bar^{-1} or μPa Pa^{-1}. They are all mole fraction and identical.

$$\text{mole fraction}, \chi = \frac{\text{mol}}{\text{mol}} = \frac{\text{Pa}}{\text{Pa}} = \frac{\text{L}}{\text{L}} \qquad \text{Equation A1.1}$$

So, when should mole fraction be used and when should partial pressure be used? When communicating about how CO_2 affects photosynthesis, it is best to use partial pressure. That way, the effective CO_2 availability is the same regardless of total pressure. A CO_2 response curve reported in partial pressure will be the same at sea level (101.3 kPa atmospheric pressure) and in Denver Colorado, USA (84 kPa). If you report in mole fraction, then the effective CO_2 availability for photosynthesis in Denver will be only 83% of what was available at sea level. On the other hand, mole fraction is often the more convenient measure in the lab. Most mass flow meters report the molar flow of a gas. If you mix two gas streams, you will know the ratio of the molar flux of each. If you mix them at high pressure and reduce the pressure, the mole fraction will stay the same while the partial pressure will change. This is especially applicable to isotope studies. We routinely start with a pressure vessel with a known amount of $^{13}CO_2$ or $^{14}CO_2$ and then pressurize the tanks. In this case, partial pressure can be ignored, just the molar ratios need to be considered.

Mole fraction and partial pressure issues also apply to water vapor but there is another consideration for water vapor, the dew point. This is the temperature at which humid air has as much water vapor as possible. Any colder and condensation will occur. Condensation is an all-too-common disaster in gas exchange systems. So, in addition to mole fraction and partial pressure, there are two additional ways to describe how much water vapor is in the air. The first

475 is the dew point of the air, that is the temperature at which dew (condensation) would occur if the
476 air comes to that temperature, regardless of the current air temperature. The partial pressure of
477 water vapor above liquid water, often denoted e_0, is a function of absolute temperature and
478 appears exponential. Thus, an empirical equation to determine the partial pressure above liquid
479 water (e_0) in kPa, where T is temperature in degrees Celsius [36] is
480
481 $$e_0 = 0.61121^{\left(\left(18.678-\frac{T}{234.5}\right)\cdot\left(\frac{T}{257.14+T}\right)\right)}$$ Equation A1.2
482
483 In gas exchange we estimate the partial pressure of water vapor in the airspaces inside the leaf
484 by knowing the leaf temperature and looking up in a table (or using an empirical equation) to
485 determine the partial pressure of water vapor for pure water at that temperature. On the other
486 hand, relative humidity is very often used to describe the amount of water vapor in air. This is
487 the partial pressure of water vapor divided by the partial pressure that the air at that temperature
488 could hold before condensation would occur. Table 1 shows how these measures of water vapor
489 are related at three temperatures.
490
491
492 Table A1.1. Expressing the amount of water vapor in air at 25, 30, and 35°C. The water vapor
493 pressure above liquid water at the indicated temperature is in the second column. The remainder
494 of the columns are for a relative humidity of 60%, a common target humidity used in gas
495 exchange studies. The mole fraction assumes an atmospheric pressure of 101.3 kPa. From this
496 table it is clear that in order to make gas exchange measurements at 35°C to examine the effect
497 of temperature on photorespiration, it would be necessary to do the experiment in a warm
498 greenhouse or growth chamber or accept less than 60% relative humidity (the alternative of
499 risking condensation in the gas exchange system is not advised). Table 10 shows that it is
500 difficult to set humidity to be constant at higher temperatures. If you use relative humidity, then
501 the absolute humidity (partial pressure) will vary. If you set the vapor pressure difference
502 between the leaf and air constant, then relative humidity will be different.

Temperature, °C	Vapor pressure, kPa	Relative humidity, %	Partial pressure, kPa	Mole fraction, %	Dew point °C
Constant relative humidity of 60%					
25	3.17	60	1.90	1.88	16.7
30	4.24	60	2.54	2.51	21.4
35	5.63	60	3.38	3.33	27.8
Constant Vapor pressure difference of 1.5 kPa					
25	3.17	53	1.67	1.64	14.7
30	4.24	65	2.74	2.37	20.2
35	5.63	73	4.13	4.08	29.3

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511 ***Appendix 2 – Accounting for refixation in isotopic methods***

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513 Loreto et al. (2001) originally accounting for refixation according to the following equation:

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515 $R_{LR} = {}^{12}C_i/{}^{13}C_i \cdot AE$ Equation A2.1

516

517 where R_{LR} is released $^{12}CO_2$ that is reassimilated, $^{12}C_i$ is calculated below and $^{13}C_i$ is calculated

518 from gas exchange.

519

$$^{12}C_i = R_L/g_s$$ Equation A2.2

Where R_L is the rate of respiration in the light and g is stomatal conductance to CO_2.

Given our updated understanding of $^{13}CO_2$ labeling it may be necessary to elaborate on the original equations. Thus, the ratio of $^{12}CO_2$ carbon fixation to $^{13}CO_2$ carbon fixation can be described as:

$$\frac{^{12}v_c=\cancel{V_{cmax}}\cdot{}^{12}C/(^{12}C+(K_C\cdot(1+{}^{O}/_{K_O}+{}^{13}C/K_C))}{^{13}v_c=0.97\cdot\cancel{V_{cmax}}\cdot{}^{13}C/(^{13}C+(K_C\cdot(1+{}^{O}/_{K_O}+{}^{12}C/K_C))}$$ Equation A2.3

(V_{cmax} for $^{13}CO_2$ is 0.97 times that for $^{12}CO_2$). Let us call the ratio of these two equations $^{12/13}R$. In these equations we use the CO_2 concentrations inside the chloroplast by:

$$^{13}C = {}^{13}C_a - \frac{1.6\cdot A\cdot 0.97}{g_s} - \frac{A\cdot 0.97}{g_m}$$ Equation A2.4

and

$$^{12}C = {}^{12}C_a + \frac{1.6\cdot R_L}{g_s} + \frac{R_L}{g_m}$$ Equation A2.5

Assuming similar diffusion paths for $^{12}CO_2$ and $^{13}CO_2$ but opposite directions of flux.

If we know total A we can estimate $^{13}v_C$.

$$A = {}^{T}v_C(1 - 0.5\phi) - R_L \qquad \text{Equation A2.6}$$

where ${}^{T}v_C$ is the total velocity of carboxylation. Then assume ${}^{12}v_C$ is negligible relative to ${}^{13}v_C$ (we estimate 1%), then ${}^{T}v_C \approx {}^{13}v_C$ and so

$${}^{13}v_C = \frac{(A+R_L)}{(1-0.5\phi)} \ . \qquad \text{Equation A2.7}$$

Then the rate of carboxylation of ${}^{12}CO_2$, i.e. refixation, is

$${}^{12}v_C = {}^{12/13}R \cdot {}^{13}v_C \ . \qquad \text{Equation A2.8}$$

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678 **Table 1.** Remaining % ^{12}C label in metabolites at 30 minutes (means, n = 3), and velocities of
679 ^{12}C emission [10] used to calculate $^{12}CO_2$ release from the leaf.

	% ^{12}C label		Velocity (μmol m^{-2} s^{-1})		$^{12}CO_2$ release (μmol m^{-2} s^{-1})
G6P Shunt	UDPG	0.214	v_{6PGD}	1.069	0.229
Fatty Acid Synthesis	PEP	0.11	$v_{PDH.c}$	0.061	0.007
Photorespiration	RUBP	0.063	v_o	7.792	0.245
TCA Cycle	PEP	0.11	$v_{PDH.m}$	0.141	0.015
	ICI	0.873	v_{IDH}	0.141	0.123
	ICI	0.873	v_{KGDH}	0.000	0.000
TOTAL $^{12}CO_2$ release					0.619

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