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ORIGINAL ARTICLE

Uncertainty in measurements of the photorespiratory CO_2 compensation point and its impact on models of leaf photosynthesis

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Abstract Rates of carbon dioxide assimilation through photosynthesis are readily modeled using the Farquhar, von Caemmerer, and Berry (FvCB) model based on the biochemistry of the initial Rubisco-catalyzed reaction of net C_3 photosynthesis. As models of CO_2 assimilation rate are used more broadly for simulating photosynthesis among species and across scales, it is increasingly important that their temperature dependencies are accurately parameterized. A vital component of the FvCB model, the photorespiratory CO_2 compensation point (Γ_*), combines the biochemistry of Rubisco with the stoichiometry of photorespiratory release of CO_2. This report details a comparison of the temperature response of Γ_* measured using different techniques in three important model and crop species (*Nicotiana tabacum, Triticum aestivum*, and *Glycine max*). We determined that the different Γ_* determination methods produce different temperature responses in the same species that are large enough to impact higher-scale leaf models of CO_2 assimilation rate. These differences are largest in *N. tabacum* and could be the result of temperature-dependent increases in the amount of CO_2 lost from photorespiration per Rubisco oxygenation reaction.

Keywords Rubisco · Photorespiration · Temperature response · Modeling photosynthesis

Introduction

Biochemical models of leaf photosynthesis are increasingly important as we develop more sophisticated simulations of plant carbon budgets and search for new strategies to improve crop productivity (Zhu et al. 2008; Dufresne et al. 2013; Long et al. 2015; Kromdijk and Long 2016). The widely adopted biochemical model of leaf photosynthesis of Farquhar, von Caemmerer, and Berry (FvCB) has proven invaluable since its development over 35 years ago and continues to be employed to represent photosynthesis from the cell to global scale (Farquhar et al. 1980; von Caemmerer and Farquhar 1981; von Caemmerer 2000). This model is characterized by its elegant combination of Rubisco kinetics with the physiology of photosynthesis and photorespiration to simulate net CO_2 assimilation rate in response to CO_2 partial pressures, making it useful both for predicting rates of carbon uptake as well as probing plant physiology and metabolism.

The photorespiratory CO_2 compensation point (Γ_*) is a critical parameter of the FvCB model. Γ_* integrates Rubisco specificity for reaction with CO_2 relative to O_2

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($S_{C/O}$) with the stoichiometry of CO_2 release per Rubisco oxygenation (α) to quantify photorespiratory CO_2 loss to net CO_2 assimilation rate. Γ_* is measured in three main ways, which can be understood in light of the following equation set:

$$\Gamma_* = \frac{\alpha O}{S_{C/O}} = \frac{\alpha C_c v_o}{v_c}, \quad (1)$$

where O, C_c, v_o, and v_c represent the oxygen partial pressure, chloroplastic CO_2 partial pressure, rate of Rubisco oxygenation, and the rate of Rubisco carboxylation, respectively (Ruuska et al. 2000; von Caemmerer 2000; Walker and Cousins 2013). Γ_* has been measured in vivo using the common intersection method by measuring CO_2 exchange under various CO_2 partial pressures and irradiances and requires no assumed α value (Laisk 1977; Brooks and Farquhar 1985). Γ_* can also be calculated from in vitro determinations of $S_{C/O}$ values as a function of O_2 partial pressure, assuming that α equals 0.5 as predicted from the commonly accepted biochemistry of photorespiration (von Caemmerer 2000; Hermida-Carrera et al. 2016). Γ_* can also be determined from net oxygen fluxes in and out of the leaf using online mass spectroscopy (Badger ; Ruuska et al. 2000; Walker and Cousins 2013). The oxygen exchange method de1985termines the $C_c v_o/v_c$ ratio and α is assumed to equal 0.5.

Recently, there has been a growing interest in parameterizing the FvCB model with species-specific temperature responses of Rubisco to better represent photosynthesis and identify optimal Rubisco kinetics for given environments (e.g., Zhu et al. 2004; Walker et al. 2013; Hermida-Carrera et al. 2016; Orr et al. 2016). These efforts predominantly employ calculations based on in vitro $S_{C/O}$ values due to the higher throughput of the technique, but it is not known how well in vitro $S_{C/O}$ values compare to in vivo approaches like CO_2 exchange and O_2 exchange.

A recent compilation of Rubisco kinetics explored the differences among various in vitro and in vivo values and their impact on leaf-level modeling of net CO_2 assimilation rate (Galmés et al. 2016). This meta-analysis supported the past work exploring the variability of Rubisco temperature responses from species adapted to different environments and contained an in-depth re-calculation of the past in vitro values with standard assumptions of ionic strength and gas solubilities. It also explored the differences between in vitro and in vivo methods and their sensitivity to modeled temperature responses, suggesting that both methods are useful for understanding and modeling the impact of Rubisco catalytic properties on photosynthesis based on their independent assumptions. The in vivo datasets analyzed in this paper were limited to those examining CO_2 flux (Harley et al. 1985; Bernacchi et al. 2001; Walker et al. 2013), leading us to wonder what additional insights could be determined from methods based on O_2 exchange, specifically in regards to measurements of Γ_* (Bernacchi et al. 2002). Additionally, Galmés et al. (2016) focused on methodological explanations of differences between the measured Rubisco kinetics, leading us to further question if there were any additional physiological explanations for the differences.

Differences among methods could indicate errors of some underlying physiological assumptions of the techniques. For example, it has been shown that Γ_* determined by CO_2 exchange increases more with temperature than Γ_* determined by O_2 exchange in *Arabidopsis thaliana* (Walker and Cousins 2013). Similar differences were observed between Γ_* determined in more extensive temperature response measurements in *Nicotiana tabacum* (Bernacchi et al. 2001, 2002, Fig. 1). Walker and Cousins (2013) suggested that the increased temperature response of Γ_* determined by CO_2 exchange could be the result of an increase in α with temperature, but this hypotheses could not be confirmed due to other possible explanations inherent to determining O_2 exchange using online mass spectroscopy (Walker and Cousins 2013). Like the O_2 exchange method, Γ_* determined from in vitro $S_{C/O}$ is also sensitive to the assumptions of α, so the secondary goal of this report was to observe if there were differences between Γ_* determined by CO_2 exchange and in vitro $S_{C/O}$ consistent with an increase in α. Measuring Γ_* from CO_2 exchange involves the additional complication of converting the CO_2 photocompensation point as measured from the intercellular CO_2 partial pressure (C_{i*}) to chloroplastic partial pressures using values of day respiration (R_d) and mesophyll conductance (g_m, see

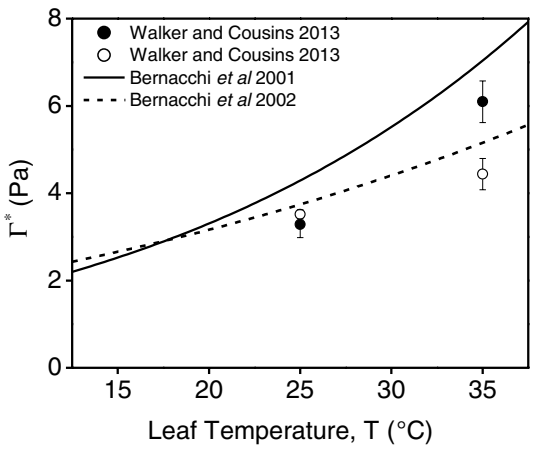

Fig. 1 Comparison of the temperature response of the photorespiratory CO_2 compensation point (Γ_*) measured from CO_2 gas exchange using the common intersection method (*closed circles* and Bernacchi et al. 2001) and from O_2 gas exchange (*open circles* and Bernacchi et al. 2002)

"Materials and methods" section). This conversion relies on the assumed values of g_m and could also play a role in explaining the differences in Γ_* as determined using various techniques.

In this report, the temperature response of Γ_* was measured using CO_2 exchange and in vitro $S_{C/O}$ in a C_3 model species (*N. tabacum*) and two major C_3 crop species (*Triticum aestivum* and *Glycine max*) to understand how comparable these methods are for use in simulating carbon assimilation at the leaf and canopy scale. This report demonstrates that there are differences between Γ_* determined by CO_2 exchange and in vitro $S_{C/O}$ that increase with temperature. These differences are most evident in *N. tabacum* and clearly present to a lesser extent in *T. aestivum* and *G. max*. The differences in the Γ_* temperature response, particularly for *N. tabacum*, are large enough to impact the output of leaf and canopy models of carbon assimilation parameterized with field data. Furthermore, differences in the Γ_* temperature response determined using CO_2 exchange, in vitro $S_{C/O}$, and O_2 exchange in *N. tabacum* are consistent with an increase in α with temperature. These findings have important implications to how the FvCB model is parameterized and raise questions concerning one of its underlying assumptions that the stoichiometry of CO_2 release per Rubisco oxygenation (α) is always 0.5.

Results

The common intersection measurements used to derive the slope–intercept regression values of C_{i*} and R_d produced consistent intersection points for a given temperature and species and were highly reproducible (Supplemental 1a–c). The different light intensities where the CO_2 response of assimilation (A–C_i) was measured produced an even distribution of slopes and intercepts for each temperature and species, with the exception of 15 and 20 °C in *G. Max* (Supplemental 1b). Additionally, due to the low values of CO_2 partial pressures used during the measurement, the linear regressions of each A–C_i curve used A–C_i data taken exclusively from the most linear region of the A–C_i curve. The common intersection point of these linear regressions showed typical variations for each temperature and species, but there was no consistent trend in how well the lines intersected as a function of temperature. When the slopes and the y-intercepts of these individual lines were used to determine C_{i*} using slope–intercept regression (Walker and Ort 2015; Walker et al. 2016a), there was no clear pattern in the residuals of the slope values between the linear regression and the measured values (Supplemental 2). This lack of pattern in the residual plots indicates that the slope–intercept regression was not measurably non-linear, indicating that a single g_m term is adequate to describe CO_2 transfer to and from the chloroplast (Tholen and Zhu 2011; Tholen et al. 2012; Walker and Ort 2015; Walker et al. 2016a).

The temperature response of Γ_* was steeper when measured using CO_2 exchange as compared to that calculated using Rubisco specificity in *N. tabacum, T. aestivum*, and *G. max* (Fig. 2). The differences were most pronounced in *N. tabacum* as compared to *T. aestivum*

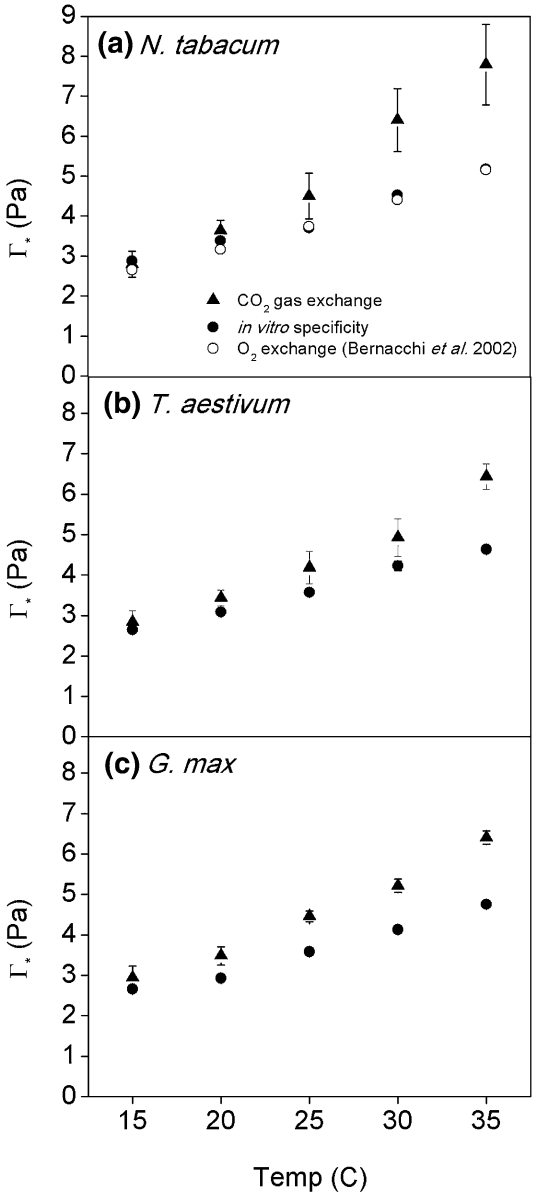

Fig. 2 Temperature response of the photorespiratory CO_2 compensation point (Γ_*) measured from CO_2 gas exchange using the common intersection method (*solid triangle*), calculated from Rubisco specificity values measured using the O_2 oxygen electrode method (*solid circles*) and from O_2 exchange (*open circles*) assuming CO_2 release per oxygenation = 0.5. Shown are the results from *N. tabacum* (**a**), *T. aestivum* (**b**), and *G. max* (**c**). *Bars* represent the means of $n = 5$–7 for the CO_2 gas exchange data and $n = 5$–16 for the in vitro assays ± *SE*

and *G. max* with the greatest differences being observed at 35 °C, the highest temperature measured. There was a close agreement between the temperature response of Γ_* calculated from Rubisco specificity and measurements from O_2 exchange in *N. tabacum* (Bernacchi et al. 2002, Fig. 2a).

A sensitivity analysis was performed to determine if the differences in Γ_* measured using CO_2 exchange as compared to Γ_* measured from Rubisco specificity could be explained by errors in the values of R_d and g_m used to convert C_{i*} to Γ_* (see "Materials and methods" section). This sensitivity analysis revealed that in *N. tabacum, G. max*, and *T. aestivum*, the values of g_m or R_d would have to be negative to explain the differences between Γ_* measured using CO_2 exchange and Γ_* measured from Rubisco specificity at all temperatures, i.e., 25 °C and above (Table 2). Furthermore, g_m or R_d would have to be negative or reduced by an order of magnitude to explain the differences in Γ_* measured using the two techniques at temperatures below 25 °C. Since the negative values of g_m and R_d are not possible, it follows that the temperature-dependent differences in Γ_* measured using the two techniques cannot be explained by incorrect assumptions of g_m or measurements of R_d. Thus, the differences in the values of Γ_* measured from CO_2 exchange vs. in vitro Rubisco specificity are both much too large and in the wrong direction to be explained by errors in g_m or R_d.

Alternatively, increases in α with increasing temperature could explain the differences between Γ_* observed when measured using CO_2 exchange, Rubisco specificity, or O_2 exchange (Fig. 3). The required increase in α necessary to explain the difference was largest in *N. tabacum* and consistent when calculated using the values from Rubisco specificity or O_2 exchange. An increase in α of 54% between 15 and 35 °C would be required to explain the difference in Γ_* derived by the different determination techniques. Putative increases in α were less pronounced, but still large, in *T. aestivum* using the values from Rubisco specificity amounting to a 30% increase in α between 15 and 35 °C; however, some of that increase was observed only at 35 °C. When the 35 °C value was removed, the differences in *T. aestivum* and *G. max* were explained by a 22 and 30% increase between 15 and 30 °C, respectively. We next explored how these different Γ_* values from the different determination techniques impact higher-scale models of leaf photosynthesis using the values from *N. tabacum*, since these *N. tabacum* parameters are most commonly used to parameterize the FvCB model.

Differences in modeled CO_2 response curves at the leaf level reflected the difference in Γ_* values (Fig. 4). The modeled gas exchange using Γ_* values measured from CO_2 exchange were lower than those measured from Rubisco specificity or using O_2 exchange at 25 and 35 °C by 5 to >40%. The difference increased substantially at 35 °C. The modeled differences were largest at lower CO_2 partial pressures, where Rubisco kinetics most limit photosynthesis and the model is most sensitive to differences in Rubisco kinetics. The rapid increase in the percent differences at 25 and 35 °C occur during the transition between Rubisco and RuBP regeneration-limited photosynthesis.

To understand how using these different temperature responses of Γ_* would impact the larger-scale models

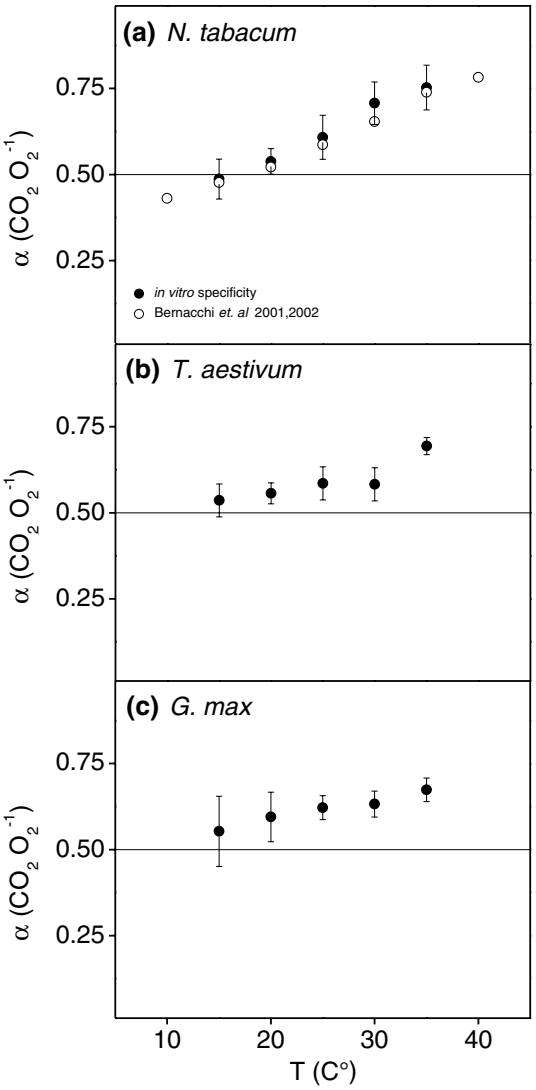

Fig. 3 Temperature response of the ratio of CO_2 release per Rubisco oxygenation (α) calculated from photorespiratory CO_2 compensation points (Γ_*) measured using the common intersection method and Rubisco specificity values determined using the O_2 oxygen electrode method (*solid circles*). Also shown are the hypothetical changes in α determined from the differences between Γ_* measured using CO_2 and O_2 exchange in Bernacchi et al. (2001, 2002, *open circles*). Shown are the results from *N. tabacum* (**a**), *T. aestivum* (**b**), and *G. max* (**c**). *Bars* represent the means of $n = 5$–7 for the CO_2 gas exchange data and $n = 5$–16 for the in vitro assays ± *SE*

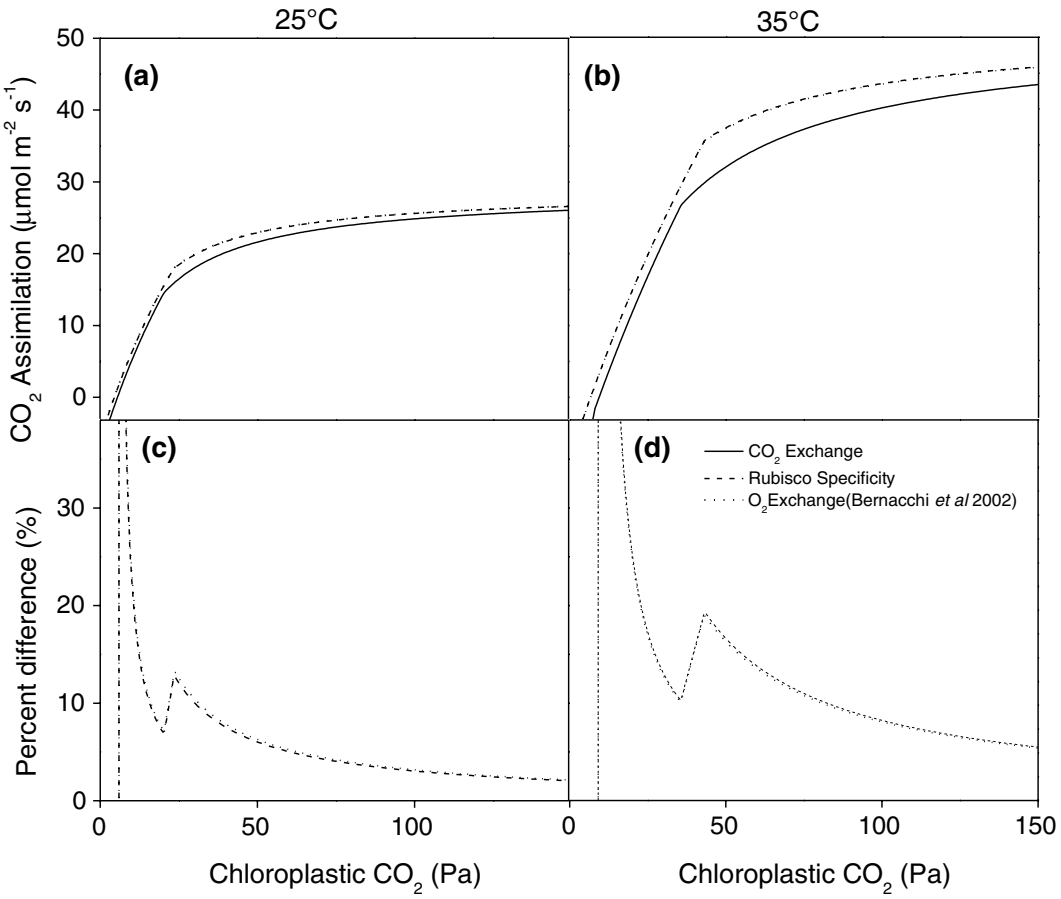

Fig. 4 Simulated impact of different assumptions of the photorespiratory CO_2 compensation point (Γ_*) on the net CO_2 assimilation rate at 25 °C (**a, c**) and 35 °C (**b, d**). *Lines* were modeled using the standard biochemical FvCB model of leaf photosynthesis, the temperature response of Rubisco kinetics, the maximum rate of electron transport determined in Bernacchi et al. (2001, 2002), and Γ_* assuming the temperature response measured in this study from CO_2 exchange using the common intersection method (*solid lines*) and from in vitro Rubisco specificity measured using the O_2 electrode method (*dashed lines*). Shown are the percent differences between net CO_2 assimilation rate simulated using Γ_* measured from CO_2 exchange and in vitro Rubisco specificity measured using the O_2 electrode method (*dashed lines*, **c, d**)

parameterized with field conditions, we next incorporated each method's temperature response into a well-validated multilayer canopy model of soybean (MLCan, Drewry et al. 2010a, b, Fig. 5). Since the impact of different Γ_* functions are influenced by temperature and CO_2 concentrations, we ran the model using field data modified according to the current and future climate predictions from the IPCC (Table 1) to produce a realistic range of the present and future conditions. Consistent with the CO_2 response curve modeling, simulations using Γ_* from Rubisco specificity and O_2 exchange simulate higher net assimilation rates under all conditions. Under the current and RCP 2.6 conditions, simulations using Γ_* from Rubisco specificity and O_2 exchange were 9% greater. Under RCP 8.5 the differences were 7% greater.

Discussion

The differences among Γ_* values measured using the different methods revealed an apparent inconsistent temperature response in a critical parameter of photosynthesis that impacts leaf- and canopy-scale simulations of carbon assimilation. Measurements of Γ_* derived from CO_2 gas exchange were the most sensitive to physiological temperature ranges (Fig. 2), and these differences were large enough to result in lower simulated photosynthetic rates as compared to the Γ_* values determined from Rubisco specificity or O_2 exchange. Simulated photosynthesis was especially lower in leaf-level simulations using CO_2 exchange-based Γ_* values under decreased CO_2 partial pressures at 35 °C (Fig. 4). The differences among methods resulted in more modest differences in photosynthesis when simulated

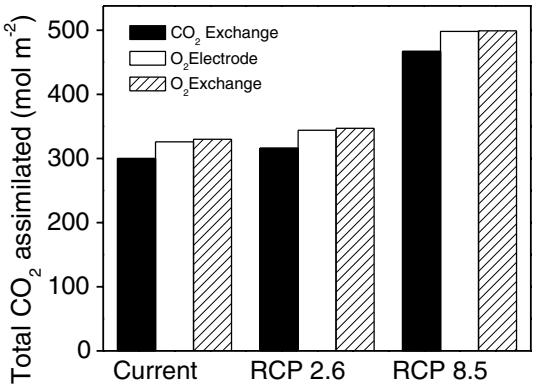

Fig. 5 Simulated impact of using different photorespiratory CO_2 compensation point (Γ_*) temperature response functions on canopy-level photosynthesis. A multilayer root–canopy model was parameterized with field data from 2002–2005 Bondville, Illinois AmeriFlux eddy covariance experiment assuming the current atmospheric CO_2 and temperature (400 PPM, no change to air temperature as measured in Bondville), IPCC scenario RCP 2.6 (450 PPM, +1 °C), and IPCC scenario RCP 8.5 (1000 PPM, +3.7 °C). Shown are the total simulated net moles of CO_2 fixed during the three modeled growing seasons

Table 1 Current and future representative concentration pathways (RCP) of mean global CO_2 and temperatures according to the 2014 IPCC report

Scenario	Ambient CO_2 (ppm)	Temp. increase (°C)
Current	400	0.0
100 years RCP 2.6	450	1.0
100 years RCP 8.5	1000	3.7

under field conditions at the current and future predictions of climate (Fig. 5). Since intercellular CO_2 partial pressure is reduced following stomatal closure, the differences in simulated photosynthesis would be greater under stress conditions including drought (Farquhar and Sharkey 1982). These simulations illustrate the sensitivity of the model to parameter values and the importance of understanding why different measurement techniques produce such different Γ_* values as the temperature increases.

Γ_* measured using CO_2 exchange does not require the assumption of $\alpha = 0.5$ that is made in both in vitro and oxygen exchange measurements. This difference can explain the increases in α with temperature relative to the other two methods. In *N. tabacum*, the increases in α required to harmonize the three methods were similar, indicating that photorespiration may release more carbon than the theoretical minimum as the temperature increases (Fig. 3a). If so, this increased CO_2 release would decrease the efficiency of photorespiratory recycling of glycolate under elevated temperatures even as the relative rates of Rubisco oxygenation increased due to decreased Rubisco specificity (Badger and Collatz 1978; Jordan and Ogren 1984; Walker et al. 2016b). An increase in α could arise through non-enzymatic decarboxylation reactions in the peroxisome of photorespiratory intermediates such as glyoxylate and/or hydroxypyruvate previously suggested to explain all of the photorespiratory CO_2 loss (Zelitch 1972; Halliwell and Butt 1974; Grodzinski 1978, 1979). This theory was later discounted by numerous lines of genetic and physiological evidence, but only at optimal temperatures (Ogren 1984). Alternatively, excess CO_2 could be released enzymatically, for example during the generation of carbon skeletons through starch degradation in a proposed glucose 6-phosphate shunt around the Calvin–Benson cycle or an as yet undescribed reaction(s) (Sharkey and Weise 2016). A recent series of isotopic labeling and fluxomic experiments on detached leaves support a stoichiometry of 0.5 in *Helianthus* annuus L. at 21 °C (Abadie et al. 2016), but this value has not yet been confirmed under elevated temperatures or in additional species. Interestingly, the trend in the calculated increases in α were not as pronounced in *T. aestivum* or *G. max* (Fig. 3b, c), indicating a potential improvement in photorespiratory efficiency with temperature through selective breeding for yield in these species compared to *N. tabacum*.

Measurements of Γ_* from CO_2 gas exchange require the assumptions of the transfer conductance between the intercellular airspace and the chloroplast (g_m) to accurately calculate chloroplastic CO_2 concentrations, unlike the measurements of Rubisco specificity or O_2 exchange (Badger 1985; von Caemmerer 2000; Furbank et al. 2009, Eq. 2). There are several methods for measuring g_m such as through curve-fitting of CO_2 response curves, combined gas exchange and chlorophyll fluorescence, and carbon isotope discrimination (Evans et al. 1986; Loreto et al. 1992; Tazoe et al. 2011). While many of these methods have been used to measure the temperature response of *N. tabacum* with similar results, they do show variation, especially at temperatures above 35 °C (Bernacchi et al. 2002; Evans and von Caemmerer 2012; Walker et al. 2013). Given this uncertainty, is it possible that the differences among Γ_* measuring techniques result from erroneous assumptions of g_m?

It does not seem probable that errors in the assumptions of g_m, or R_d for that matter, can explain the differences in Γ_* for several reasons. First, Γ_* values from CO_2 exchange were *higher* than those calculated from Rubisco specificity as the temperature increased (Fig. 2), even though Γ_* *decreases* with the inclusion of g_m in the calculation (Eq. 2). This means that g_m or R_d would need to *decrease* with temperature to explain the direction of the differences among Γ_* measurements, which has not been observed in

any reported temperature responses (Bernacchi et al. 2002; Warren and Dreyer 2006; Walker et al. 2013; von Caemmerer and Evans 2014). Furthermore, even if g_m were assumed to be a negligible value, Γ_* in *N. tabacum* at 35 °C would only be reduced from 7.8 to 7.6 Pa, which is insufficient to approach the value of 5.2 Pa determined from Rubisco specificity. This point is further illustrated in the sensitivity analysis of Γ_* calculations, where the impossibility of negative values of g_m or R_d are required to explain the differences between Γ_* measured from CO_2 exchange and in vitro Rubisco specificity (Table 2). Together, these observations and calculations indicate that the differences between methods of measuring Γ_* are not the result of incorrect assumptions or measurements of R_d and/or g_m.

Recent work concerning the validity of assumptions necessary for Γ_* measurements using the common intersection approach raise important considerations to ensure accurate determinations of Γ_*. One such concern is the appropriateness of using linear fits to determine the intersection point of non-linear A–C_i curves, which, according to simulations, results in underestimates of C_{i*} and, by extension, Γ_* (Gu and Sun 2014). To prevent assumptins of linearity from baising our common intercept determinations, we used measurement CO_2 partial pressures below 10 Pa CO_2 that further simulations demonstrate result in <1% underestimation of C_{i*} (Walker and Ort 2015). Additionally, the common intersection measurements did not show the "staggered" interceptions expected if their determination was biased by improper assumptions of linearity (Supplemental 1a–c). Finally, even if improper assumptions of linearity resulted in underestimates of C_{i*} measured in the temperature response of *N. tabacum, G. max*, and *T. aestivum*, this would only serve to *increase* the differences between the Γ_* values measured using the common intersection method and those from in vitro Rubisco specificity.

It has also been suggested that the current understanding of g_m needs to be revised, since it is commonly assumed that all CO_2 released from the mitochondria passes through the chloroplast, and multiple conductances of CO_2 between organelles and cytosol need to be considered (Tholen and Zhu 2011; Tholen et al. 2012). A new method of interpreting Γ_* measurements from CO_2 exchange indicates that the relationship between the slope and intercepts of a common intersection measurement would be non-linear in the presence of multiple inter-organellar fluxes from photorespired CO_2 (Walker and Ort 2015; Walker et al. 2016a). Non-linearity in the slope and intercept relationships was not observed under our growth and measurement conditions, suggesting that an assumption of a simple linear g_m was justified in this case (Table 2).

Table 2 Intercellular CO_2 partial pressure of the common intersection measurements (C_{i*}; Pa CO_2), the corresponding rates of day respiration (R_d; μmol CO_2 m^{-2} s^{-1}), the assumed mesophyll conductance (g_m; mol m^{-2} s^{-1} MPa^{-1}), and the final CO_2 photocompensation point (Γ_*; Pa CO_2) calculated from C_{i*}, R_d, and g_m

T_l	C_{i*}	R_d	$R_{d,S_{C/O}}$	g_m	$g_{m,S_{C/O}}$	Γ_*	$\Gamma_{*_{S_{C/O}}}$
N. tabacum							
15	2.58 ± 0.14	0.55 ± 0.14	0.09	3.32	0.54	2.75 ± 0.32	2.87 ± 0.02
20	3.33 ± 0.12	1.19 ± 0.07	0.01	4.41	0.05	3.60 ± 0.26	3.38 ± 0.07
25	4.27 ± 0.25	1.34 ± 0.10	−0.10	5.69	−0.42	4.51 ± 0.57	3.70 ± 0.06
30	6.15 ± 0.35	2.08 ± 0.22	−0.23	7.11	−0.78	6.44 ± 0.79	4.53 ± 0.08
35	7.59 ± 0.45	2.32 ± 0.24	−0.27	9.01	−1.04	7.85 ± 1.01	5.18 ± 0.03
G. max							
15	2.92 ± 0.27	0.05 ± 0.07	−0.10	2.63	−5.17	2.94 ± 0.60	2.66 ± 0.02
20	3.30 ± 0.23	0.59 ± 0.07	−0.09	4.09	−0.63	3.44 ± 0.50	2.93 ± 0.04
25	4.27 ± 0.14	0.89 ± 0.15	−0.14	4.85	−0.77	4.45 ± 0.31	3.58 ± 0.02
30	5.04 ± 0.17	1.17 ± 0.24	−0.17	5.40	−0.78	5.26 ± 0.39	4.13 ± 0.04
35	6.21 ± 0.20	1.70 ± 0.12	−0.23	6.35	−0.86	6.48 ± 0.44	4.75 ± 0.02
T. aestivum							
15	2.59 ± 0.12	0.63 ± 0.16	0.02	3.21	0.09	2.79 ± 0.27	2.65 ± 0.02
20	3.21 ± 0.09	0.70 ± 0.13	−0.04	3.32	−0.18	3.42 ± 0.20	3.09 ± 0.07
25	3.95 ± 0.18	0.88 ± 0.20	−0.10	3.94	−0.43	4.17 ± 0.40	3.57 ± 0.04
30	4.67 ± 0.20	1.17 ± 0.17	−0.11	4.01	−0.37	4.96 ± 0.46	4.23 ± 0.11
35	6.08 ± 0.14	1.88 ± 0.15	−0.38	3.76	−0.77	6.58 ± 0.32	4.64 ± 0.01

Also shown are the Γ_* value calculated from in vitro Rubisco specificity ($\Gamma_{*_{S_{C/O}}}$; Pa CO_2), the R_d value necessary to explain the differences between C_{i*} and $\Gamma_{*_{S_{C/O}}}$ ($R_{d,S_{C/O}}$), and the g_m value necessary to explain the differences between C_{i*} and $\Gamma_{*_{S_{C/O}}}$ ($g_{m,S_{C/O}}$), all according to Eqs. 1 and 2. All data are shown for leaf temperatures (T_l; °C) between 15 and 35 °C. The g_m values were determined according to the temperature responses measured previously for these species (von Caemmerer and Evans 2014). Shown are the means of $n = 5$–7 for the CO_2 gas exchange data and $n = 5$–16 for the in vitro assays ± *SE*

An alternative intriguing possibility is that the assumptions used to derive C_i are not always appropriate and result in a systematic error in the estimation of Γ_* in common intersection measurements. This argument rests on the assumption of the FvCB model that the vast majority of water loss occurs through the stomata and through the same path as CO_2 diffusion (Moss and Rawlins 1963). This assumption has recently been challenged after an analysis of its impact on gas exchange measurements, especially the ones sensitive to small fluxes as in Γ_* determination (Hanson et al. 2016). This re-evaluation of a common assumption is supported by the work demonstrating that water diffuses 20–40 times faster across the cuticle than CO_2 (Boyer et al. 1997; Boyer 2015b) and that many leaves transmit significant amounts of water through the cuticle, resulting in an over-estimation of stomatal conductance and consequently C_i, especially at lower rates of leaf water loss (Boyer 2015a). The impact of cuticular water loss on C_i estimation would be complex and require additional specialized measurements to determine if these effects could explain the differences observed using CO_2 gas exchange to measure Γ_*. Despite the added complexity, the possibility remains that cuticular water loss could explain the differences observed between the Γ_* values determined using CO_2 exchange and those determined based on in vitro Rubisco specificity.

There are two primary methods used to determine the in vitro Rubisco specificity. These alternatively monitor O_2 consumption via oxygenation of RuBP in an O_2 electrode system (Parry et al. 1989), or determine the ratio of ^{3}H-glycerate/^{3}H-glycolate produced from the consumption of ^{3}H-RuBP (Kane et al. 1994). While the absolute values produced do differ, there is consistency across methods as to the comparisons across species (e.g., both methods maintain that wheat Rubisco has a higher specificity than *N. tabacum* at 25 °C). Both methods have been employed in model species and a number of crop species under standard temperatures, and datasets incorporating temperature response are available for both methods (e.g., Galmés et al. 2005; Perdomo et al. 2015; Hermida-Carrera et al. 2016; Orr et al. 2016; Prins et al. 2016; Sharwood et al. 2016). However, the difference between methods has not been directly compared with temperature responses, due to a slight overlap of species with temperature response data using both methods. Recent efforts to compile and normalize in vitro Rubisco catalysis data (including $S_{C/O}$) from the available literature suggest that the methods available largely agree on the extent of temperature response once in vitro data were calculated accounting for the variation in equilibrium CO_2 concentration and the ionic strength of buffers (Galmés et al. 2016). This observation suggests that our findings should be relatively consistent with those from the other in vitro methods. The close agreement between Γ_* values determined from O_2 exchange and using Rubisco specificity determined using the O_2 electrode is remarkable. Clearly, if in vitro specificities are to be used in the modeling efforts of CO_2 exchange, the method used to collect them should be reported and carefully considered.

In this report, we demonstrate that there are significant differences in the temperature response of Γ_* dependent on the measurement method used and that these differences are large enough to impact leaf and canopy models of photosynthesis. While we have limited our discussion to the impact of these different Γ_* values to net CO_2 uptake, similar analysis could be performed to determine the impact to the measurements of g_m or carbon isotope exchange (Farquhar et al. 1989; Harley et al. 1992; Tholen et al. 2012; Gu and Sun 2014). Given the growing use of biochemical models of leaf photosynthesis to calculate carbon balance and productivity at all scales, it is critical to next reveal the mechanism for these differences in order to determine which methods should be used to accurately parameterize future work or explore novel physiology. The intent of this work is thus not to invalidate the measurements of Γ_* using the common intersection method, but rather to determine if more complete physiology can be learned by carefully comparing the Γ_* values measured using different techniques. Additionally, the source of these differences could provide insight into the efficiency of photorespiration in response to temperature or the biochemistry of Rubisco.

Materials and methods

Plant growth conditions

Plant material used for in vitro measurements was grown in a glasshouse at Rothamsted Research with a 16/8 h day/night cycle and accompanying diurnal temperatures of 26/19 °C. Plants were kept well watered. Young healthy leaves were collected, snap frozen immediately in liquid nitrogen, and then stored at −80 °C until analysis. For CO_2 gas exchange determination of Γ_* at the University of Illinois, *N. tabacum, T. aestivum*, and *G. max* seeds were grown in 2-L pots for 3–5 weeks until large enough for gas exchange. Plants were grown in a climate-controlled cabinet (Conviron, Winnipeg, Manitoba, Canada) set to mimic conditions in the Rothamsted glasshouse with day/night cycles of 16/8 h at 26/19 °C under an irradiance of 800 µmol m^{-2} s^{-1}.

In vitro Rubisco specificity measurements

Rubisco was purified from each species using the material grown in glasshouse conditions at Rothamsted Research, using the method described by Prins et al. (2016), and with

alterations as in Orr et al. (2016). The oxygen electrode method of Parry et al. (1989) was used to make a minimum of 12 replicate measurements of $S_{C/O}$ for each species, at 15 and 35 °C, and normalized to a known value for *T. aestivum* at each temperature, as described previously (Parry et al. 1989). For 20, 25, and 30 °C, the values from Orr et al. (2016) were used.

Γ_* and R_d measurements using the common intersection method

The youngest fully expanded leaves of 3- to 5-week-old plants were used for gas exchange. Gas exchange was performed using a LI-COR 6400 XT modified to reach low CO_2 partial pressures (LI-COR Biosciences 2010) using a 6 cm^2 chamber with a red/blue light source (LI-COR Biosciences, Lincoln, NE, USA). Assimilation measurements were corrected for CO_2 leakage according to the manufacturer's instruction. Γ_* was measured using the common intersection method by measuring the CO_2 response of photosynthesis under various sub-saturating irradiances (Laisk 1977; Brooks and Farquhar 1985). The common intersection was determined using slope–intercept regression to produce more accurate and consistent values of C_{i*} and R_d (Walker and Ort 2015; Walker et al. 2016a). To determine irradiances that would result in an even distribution of photosynthetic rates for Γ_* determinations, the photosynthetic light response of each species was first measured at 20 Pa CO_2. Prior to Γ_* determinations using the common intersection method, plants were acclimated under 250 µmol m^{-2} s^{-1} at 39 Pa CO_2 until photosynthesis reached steady state to activate Rubisco. Following initial acclimation, plants were measured at 15, 12, 9, 7, 5, and 3 Pa CO_2 under irradiances of 250, 165, 120, 80, and 50 µmol m^{-2} s^{-1} for *N. tabacum*, 250, 160, 100, 60, and 30 µmol m^{-2} s^{-1} for *T. aestivum*, and 250, 165, 120, 80, and 50 µmol m^{-2} s^{-1} for *G. max*. The x-intersection point represents C_{i*} which can be converted to Γ_* according to

$$\Gamma_* = C_{i*} + R_d/g_m, \quad (2)$$

where R_d is the y-intersection point (von Caemmerer 2000; Furbank et al. 2009). Species-specific temperature responses were used at each temperature for g_m (von Caemmerer and Evans 2014).

Leaf- and canopy-scale modeling of photosynthesis

Leaf-level modeling of the CO_2 response of net photosynthesis was modeled at 25 and 35 °C using the standard FvCB model of leaf photosynthesis. For 25 °C, the model was parameterized with $V_{cmax} = 80$ µmol m^{-2} s^{-1}, $K_c = 26.7$ Pa, $K_o = 16.3$ kPa, $R_d = 1$ µmol m^{-2} s^{-1}, and $J_{max} = 120$ µmol m^{-2} s^{-1}. Γ_* was assumed to be 4.74, 3.78, and 3.7 Pa for the common intersection method, O_2 exchange, and in vitro determinations, respectively. For 35 °C, the model was parameterized with $V_{cmax} = 187$ µmol m^{-2} s^{-1}, $K_c = 77.1$ Pa, $K_o = 22.2$ kPa, $R_d = 2$ µmol m^{-2} s^{-1}, and $J_{max} = 211$ µmol m^{-2} s^{-1}, respectively. Γ_* was assumed to be 7.88, 5.15, and 5.2 Pa for the common intersection method, O_2 exchange, and in vitro determinations, respectively.

For canopy-level implementation, we used a well-validated multilayer canopy–root–soil model (MLCan, Drewry et al. 2010a, b) with minor additions to include g_m (Walker et al. 2016b). The model was parameterized with field data from the Bondville, Illinois, AmeriFlux eddy covariance site measured during the 2002, 2004, and 2006 growing seasons (available from the AmeriFlux Database; http://ameriflux.lbl.gov/data/download-data). Full-field data can also be obtained from B. J. W. upon request.

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