Title: Racing against stomatal attenuation: rapid CO₂ response curves more reliably estimate photosynthetic capacity than steady state curves in a low conductance species

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Abstract

A/C curves are an important gas-exchange-based approach to understanding the regulation of photosynthesis, describing the response of net CO₂ assimilation (*A*) to leaf internal concentration of CO₂ (*C_i*). Low stomatal conductance species pose a challenge to the measurement of *A/C_i* curves by reducing the signal-to-noise ratio of gas exchange measures. Additionally, the stomatal attenuation effect of elevated ambient CO₂ leads to further reduction of conductance and may lead to erroneous interpretation of high *C_i* responses of *A*. Rapid *A/C_i* response (RACiR) curves offer a potential practice to develop *A/C_i* curves faster than the stomatal closure response to elevated CO₂. We used the moderately low conductance *Citrus* to compare traditional steady state (SS) *A/C_i* curves with RACiR curves. SS curves failed more often than RACiR curves. Overall parameter estimates were the same between SS and RACiR curves. When low stomatal conductance values were removed, triose-phosphate utilization (TPU) limitation estimates increased. Overall RACiR stomatal conductance values began and remained higher than SS values. Based on the comparable resulting parameter estimates, higher likelihood of success and reduced measurement time, we propose RACiR as a valuable tool to measure *A/C_i* responses in low conductance species.

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Introduction

Gas exchange is a powerful tool in plant physiology, allowing us to understand CO₂ and water relations in plants to compare plant performance in a range of environments (e.g. Stinziano & Way, 2017; Vincent et al., 2017; Smith et al., 2020), to understand why and how plants cope with stressful environments (e.g. Kumarathunge et al., 2019; Zhu et al., 2020), and for modeling plant-atmosphere interactions up to the global scale (e.g. Oleson et al., 2013; Rogers et al. 2017; Lombardozzi et al., 2018). However, gas exchange measurements require a sufficient

signal-to-noise ratio to obtain quality data for inferences. To obtain an adequate signal-to-noise ratio in a leaf, stomata need to be sufficiently open for a quality signal.

Challengingly, plant species present a wide variation in stomatal conductance depending on ecotype and environment, with many exhibiting low conductance, with maxima below $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Körner et al. 1986; Tezara et al. 1998), and rapidly stomatal closure whenever conditions are not ideal (Radin et al., 1994) or at a particular time of day (Steppe et al., 2006). For example, in a study of *Citrus x sinensis* (L.) and *Citrus reticulata* (Blanco) in which treatment did not affect stomatal conductance, 35% of gas exchange measurements exhibited stomatal conductance to water (g_{sw}) below $0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Vincent et al., *unpublished data*). When stomatal conductance is low, not only is the signal-to-noise ratio low in the gas exchange measurements, it becomes difficult to obtain a sufficient range in intercellular CO_2 concentration (C_i) to characterize the response of net CO_2 assimilation (A) to C_i (A/C_i curves) (Stinziano et al., 2020). Further compounding the signal-to-noise challenge in the context of A/C_i curves, plants respond to elevated CO_2 by stomatal closure on an order of minutes.

A/C_i curves are a powerful tool to which we can apply the Farquhar-von Caemmerer-Berry model of photosynthesis (Farquhar et al. 1980) to estimate photosynthetic biochemistry for comparing plant performance (e.g. Way & Sage, 2008), understanding photosynthetic acclimation (e.g. Kumarathunge et al. 2019), and modeling vegetative carbon uptake (e.g. Oleson et al., 2013). In species with low stomatal conductance or stomata that close rapidly at high CO_2 , these responses are very difficult to characterize. Stomatal limitations may further lead to incorrect conclusions on what is limiting photosynthesis. For example, a stomatal limitation may be incorrectly interpreted as a limitation due to rubisco carboxylation. The recent development of rapid A/C_i curves (RACiR, Stinziano et al. 2017, 2019ab), may be sufficiently fast to circumvent the difficulties of stomatal closure in such species.

Here we demonstrate the benefits of RACiR, relative to steady state A/C_i curves, in so-called “difficult” species using *Citrus x sinensis*, a species that is difficult to work with due to rapid stomatal fluctuations and moderately low stomatal conductance (Steppe et al. 2006; Syvertsen 1982).

Materials & Methods

Plant material

Citrus sinensis (L.), cultivar ‘Valencia,’ plants were grown in a greenhouse in Lake Alfred Florida (28.1021 °N, 81.7121 °W) in a natural diurnal cycle and daily maximum and minimum of ~25-34 °C. Plants were irrigated daily and fertilized according to standard practices. Steady-state and RACiR curves were performed on leaves of the same plants in a paired fashion on 19 plants for 19 RACiR curves and 19 steady-state (SS) curves.

Gas exchange

Response curves were measured using an infrared gas analyzer (LI-6800, LiCor Inc., Lincoln, NE). Leaf chamber settings were fixed at 60% relative humidity, $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, and set to maintain the ambient temperature (31-34 °C). For all measurements we used a threshold of g_{sw} of $0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$. If g_{sw} was below this threshold before beginning, the response curve was not initiated. RACiR data were gathered at 0.5 Hz, and began by stabilizing measurements at a reference CO_2 concentration of $100 \mu\text{mol mol}^{-1}$ at a ramping rate of $100 \mu\text{mol mol}^{-1} \text{min}^{-1}$ to $900 \mu\text{mol mol}^{-1}$. SS curves were collected starting at a reference CO_2 concentration of $380 \mu\text{mol mol}^{-1}$, then proceeding through the following

progression conditions 285, 190, 145, 100, 50, 380, 475, 570, 665, 760, 960, 1160, 1460, 1760, 1960 $\mu\text{mol mol}^{-1} \text{CO}_2$.

Analysis

RACiR data were calibrated using an empty leaf chamber run taken within 1 hr using the R package {racir} (Stinziano et al. 2019a; Stinziano, 2020). A/C_i curves were fit using the fitaci() command from the {plantecophys} package (Duursma, 2015) in R statistical software (R Core Team, 2019, version 3.6.2), with options selected to fit triose phosphate utilization (TPU) limitation rate and with no temperature correction. We extracted maximum rubisco carboxylation capacity (V_{cmax}), maximum electron transport rate under saturating light (J_{1200}), TPU , fitted respiration (R_d), the CO_2 to RuBP transition C_i ($C_{i\text{trans1}}$), and the RuBP to TPU transition C_i ($C_{i\text{trans2}}$) for analysis. To assess a success rate, curves where more than half the data points had $g_{\text{sw}} < 0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$ were considered to have failed.

Statistics

The resulting parameters from the fit curves were subjected to paired Student's t -tests to test for differences between steady state and RACiR curves after all points below the threshold had been removed.

Data and Code

Data and code are available in supplementary files S1, S2, and S3.

Results

Success rate of A/C_i curves

No RACiR curves failed when beginning at $g_{\text{sw}} > 0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$, however three SS curves out of 19 failed. Two of these curves lacked RuBP limitations (i.e. fitted $J_{1200} = 1,000,000$ - see source code of Duursma, 2015 for an explanation) or generated unreasonable estimates of $C_{i\text{trans1}}$ (2,000 $\mu\text{mol mol}^{-1}$) (Figure 1).

RACiR versus steady-state A/C_i curves

Overall, results from SS and RACiR curves were similar (Fig. 1). Notable differences are the lower R_d estimates by SS than by RACiR, and the small number of TPU estimates produced by RACiR, not surprising considering the preset range of leaf surface CO_2 concentration (C_a) values for RACiR.

Stomatal limitations on gas exchange

Considering A/C_i curves (Fig. 2), three curves reduced A noticeably as C_i exceeded 700 $\mu\text{mol mol}^{-1}$. However, a larger number of curves show g_{sw} dropping below our threshold of 0.08 $\mu\text{mol m}^{-2} \text{s}^{-1}$ as C_i increases or decreases relative to the “ambient” range of 380 $\mu\text{mol mol}^{-1}$. This stomatal sensitivity to changes in C_a has implications for the validity of the resulting estimates based on curve fitting. Most notably, when points with $g_{\text{sw}} < 0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$ were removed, estimates of TPU and the C_i of the RuBP- TPU limitation transition shifted upward for the SS curves. Additionally, variation between individual leaves in the responses to C_a , resulted in a wide range of maximum C_i values in SS curves, and the minimum g_{sw} values of most curves were below 0.08 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 2).

Figure 1. Box plots of V_{cmax} , J_{1200} , fitted R_d , TPU , and transitions points of limitation states of steady state (SS) and RACiR A/C_i curves of *Citrus x sinensis* leaves with all data included or points with $g_{sw} < 0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$ removed. C_i is the intercellular concentration of CO_2 . $C_{itrans1}$ represents the transition between CO_2 and RuBP limitations (i.e. V_{cmax} and J_{1200}). $C_{itrans2}$ is the transition from RuBP to TPU limitations (i.e. J_{1200} and TPU). To enable visualization one point measuring $2000 \mu\text{mol mol}^{-1}$ was removed from the SS All measurements group. Similarly, one J_{1200} estimate of $1,000,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was removed from the SS All measurements group (this value acts as a placeholder for the analysis and indicates no RuBP limitation, see Duursma, 2015). Open circles indicate individual data points. Closed circles represent distributions of outliers as determined by 95% confidence intervals. P-values indicate the probability that the SS and RACiR are not the same using a paired t-test assessing only the groups with g_{sw} above the threshold. All t-tests had 18 degrees of freedom.

Figure 2. Plots of net CO₂ assimilation (A) and stomatal conductance (g_{sw}) by intercellular CO₂ concentration (C_i), and distributions of maximum and minimum g_{sw} and C_i of leaves of *Citrus x sinensis* using steady state (SS) or RACiR methods. Black horizontal line represents the g_{sw} threshold of $0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$. P -values indicate results of a t-test with 18 degrees of freedom.

Discussion

Overall, results of A/C_i curve fits measured by steady-state or RACiR methods are similar with more precise estimates of $C_{i,trans1}$ for RACiR (Fig. 1), confirming previous studies that the fitted parameters V_{max} and J_{max} are statistically equivalent (Stinziano et al., 2017; Coursolle et al., 2019; Lawrence et al., 2019; Stinziano et al., 2019b). The single difference was found in the estimation of R_d , where the mean was approximately $1.75 \pm 0.52 \mu\text{mol m}^{-2} \text{s}^{-1}$ for RACiR and $0.22 \pm 0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the steady-state curves. On the same variety in a different study we found direct measurement of R_d to be $2.35 \pm 0.11 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Vincent et al., *unpublished data*).

Despite the similar resulting parameters, steady state curves are measured slowly relative to the time scale of leaf CO₂ attenuation of g_{sw} (Merilo et al., 2015). This leads to the reduction of g_{sw} in some curves below the threshold required for reliable gas exchange measurements. This can particularly affect the estimation of TPU , as stomatal attenuation may induce changes in A that are limited by stomatal conductance, rather than by enzymatic limitations despite the similarity in shape of the curve to the expected shape with TPU limitations. We observed g_{sw} falling below a critical threshold in 3 of 19 cases for the steady-state curves compared to 0 cases for the RACiR curves. The likely explanation for these differences is due to the higher minimum and maximum g_{sw} in the RACiR curves versus the steady-state curves, creating a larger buffer and less time for g_{sw} to fall before reaching the critical threshold. We do note, however, that the

traditional approach to A/C_i curves (starting near atmospheric CO_2 , going down, then up; e.g. Busch, 2018) may not be the best option for maximizing g_{sw} during the steady state, as starting at low CO_2 and monotonically increasing CO_2 may result in better A/C_i estimates (Sharkey, 2019), potentially avoiding the g_{sw} crash that can occur in *Citrus*.

To assess the A/C_i relationship, it is ideal to achieve a wide range of C_i values. The RACiR methods presented here did not achieve as wide a range of C_i as the SS methods. However, the very broad range of maximum C_i values demonstrates that the control of SS methods over this range is limited in such low conductance situations (Fig. 2). Additionally, depending on the species-specific responses, RACiR methods could continue to higher C_i values than those presented here, as the majority of curves ended with minimum g_{sw} well above the minimum threshold. In this case, the advantage of RACiR lies in that its short time “beats” the speed of stomatal attenuation, affording the ability to avoid stomatal impacts on the shape of the A/C_i curve. Additionally, we speculate that the speed of RACiR curves may allow for better estimation of TPU at high CO_2 , as such limitations are known to be transient and cryptic (Sharkey, 2019).

Therefore, based on our data, we recommend the use of RACiR in situations and species where rapid stomatal closure can limit the application of steady-state A/C_i curves.

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