# Phenotyping Photosynthesis on the Limit – A critical examination of RACiR.

We are now in the phenomic era. New, rapid screening approaches that provide quality data on plant physiological performance are a priority. A recently published advance, enabled by a next generation gas exchange system (LI-6800, LI-COR, Lincoln NE, USA), is the Rapid A-Ci Response (RACiR: Stinziano et al., 2017). This technique aims to allow rapid (~12 min) determination of the $A/c_i$ response (net $CO_2$ assimilation ($A$) response to intercellular $CO_2$ concentration ($c_i$)), which previously required ~40 minutes (standard $A/c_i$ approach). Evaluating RACiR as a means for higher throughput phenotyping reveals offsets to commonly estimated parameters from $A/c_i$ analyses; however, best practices for RACiR application and reporting should enable reliable comparisons with standard $A/c_i$ measurements.

The $A/c_i$ response is a powerful tool used by plant physiologists to establish factors limiting photosynthesis *in vivo* and *in situ*. In combination with biophysical models, $A/c_i$ measurements can establish apparent rates for Rubisco carboxylation ($V_{cmax}$), electron transport capacity ($J$), and, triose-phosphate utilisation (TPU), as limits affecting photosynthesis (Long & Bernacchi, 2003). The term apparent is used here, since the true values of the parameters requires the response of $A/c_c$, where $c_c$ is [$CO_2$] within the chloroplast, which can be determined by simultaneous measurement of mesophyll conductance ($g_m$) (Bernacchi *et al.* 2002). These parameters are used in applications from crop improvement (Kromdijk & Long, 2016) to global change modelling (Rogers *et al.*, 2014). An important application of $A/c_i$ response measurements is in understanding regulation of plant carbon and water balance by partitioning limits to photosynthesis into diffusive and biochemical components (Farquhar & Sharkey, 1982; Jones, 1985; Buckley & Diaz-Espejo, 2015). In addition, by establishing the $c_{i,trans}$ at which electron transport or TPU limitation replace carboxylation as limiting for photosynthesis, trends in $c_i$ under operating conditions can be used to identify limiting states under dynamic conditions (e.g., Taylor & Long, 2017).

Measurements of $A/c_i$ responses using the RACiR rely on comparisons of sample ($CO_{2,S}$) and reference ($CO_{2,R}$) [$CO_2$] under a continuous, linear ramp in $CO_{2,R}$; possible because of the LI-6800 photosynthesis system. Following Stinziano et al., (2017) the linear relationship between sample and reference IRGA [$CO_2$] from a measurement with an empty chamber is used to correct $A$.

$$A_{EMPTY} = m \cdot CO_{2,R} + c$$

$$A_{CORR} = A_{MEAS}|CO_{2,R} - A_{EMPTY}|CO_{2,R}$$

Where $A_{EMPTY}$ is the net $CO_2$ assimilation rate measured with the empty chamber; $m$ and $c$ are a slope and intercept respectively; $A_{CORR}$ is the corrected, $A_{MEAS}$ the measured, and $A_{EMPTY}$ the apparent empty chamber net $CO_2$ assimilation rate. In addition, $c_i$ must be corrected to match $A_{CORR}$. Substituting $A_{CORR}$ for $A$ in the manufacturer's equation:

$$c_{i,CORR} = \frac{\left(g_{tc} - \frac{E}{2}\right) CO_{2,R} - A_{CORR}}{\left(g_{tc} + \frac{E}{2}\right)}$$

Where $g_{tc}$ is the total leaf conductance to $CO_2$, and $E$ is the rate of transpiration. These corrections to $A$ and $c_i$ attempt to integrate correction of errors resulting from sources with different time constants: volume differences of the sample and reference flow paths, impacts of the particular flow rate used, physical distance between $CO_2$ injector and reference chamber, and cumulative calibration offsets (IRGA matching; Stinziano et al., 2017).

Experimental results suggest that RACiR measurements demonstrate a consistent offset towards lower $CO_2$ compensation points ($\Gamma$) (Fig. 1). This was tested using both a $C_3$ species (cowpea, *Vigna unguiculata*; Fig. 1a) and a species with $C_4$ photosynthesis, teff (*Eragrostis teff*), which we expected to show a compensation point close to zero. We found that the RACiR suggested an impossible, negative compensation point for teff (Fig. 1b). Returning to the data published by Stinziano et al., (2017) we also found unrealistic offsets in $\Gamma$ (Fig. 1c).

To establish the importance of these offsets, the Farquhar et al., (1980) model (FvCB model) was fit to RACiR data of Stinziano et al., (2017) using 500-0 µmol mol$^{-1}$ and 300-800 µmol mol$^{-1}$ ramps in $CO_{2,R}$, compared with overlapping data for standard $A/c_i$ measurements.

$$A = min(A_C, A_J)\left(1 - \frac{\Gamma^*}{c_i}\right) - R_d$$

$$A_C = V_{c,max} \frac{c_i}{c_i + K_{CO}}$$

$$A_J = J \frac{c_i}{4.5c_i + 10.5\Gamma^*}$$

$A_C$ is Rubisco-limited, $A_J$ electron transport-limited $CO_2$ assimilation, and $R_d$ dark respiration. The parameters Γ* (photorespiratory compensation point) and $K_{CO}$ ($K_{CO}=K_C(1+O/K_O)$: where $K_C$ and $K_O$ are temperature dependent, Rubisco Michaelis-Menten constants for $CO_2$ and $O_2$; and $O$ is atmospheric oxygen concentration) were obtained from Bernacchi et al., (2001). Estimating $V_{cmax}$, $J$ and $R_d$ from the data, led to similar $V_{cmax}$ and $J$ for the two methods (Table 1). However, there were significant offsets in $R_d$ (−95%), Γ (−11%), and $c_{i,trans}$ (−13%) for RACiR measurements (Table 1), consistent with the initial observation of an offset towards unrealistic Γ for $C_4$ and more negative Γ for $C_3$ leaves measured using RACiR (Fig. 1). Notably, the estimate of $R_d$ from the RACiR data was 0.04±0.036 $\mu$mol $m^{-2}$ $s^{-1}$ (mean±s.e.m.), compared with 0.81±0.215 for traditional $A/c_i$ measurements (paired t-test: $t = 3.98$, d.f. = 5, P = 0.01).

Further model fits were undertaken to determine if estimates of $V_{c,max}$ and $J$ obtained using RACiR were affected by assuming fixed values for all, or only some of the parameters Γ*, $K_{CO}$ and $R_d$. If none of the model's parameters were fixed, $R_d$ tended to zero for both RACiR and standard $A/c_i$ measurements (Supplementary Table 1). When we constrained Γ*, $K_{CO}$, and $R_d$ (based on the intial standard $A/c_i$ fit that fixed Γ* and $K_{CO}$), $V_{cmax}$ increased for the RACiR, resulting in a significant difference compared with the standard $A/c_i$ data (+6%; Table 1). Differences in $c_{i,trans}$ and Γ also remained significant (Table 1). The difference in $c_{i,trans}$ increased from 13 to 16%, while the difference in Γ decreased dramatically from 11% to almost zero, probably because of the proximity of Γ to Γ* and $R_d$.

To establish the potential impact of RACiR offsets on Γ*, we fit an FvCB model in which only $K_{CO}$ and $R_d$ (as above) were held constant. As anticipated, Γ* from the standard $A/c_i$ responses were close to (<0.01% greater than) values from Bernacchi et al., (2001; Table 1). By contrast, Γ* from RACiR was 20% lower and paralleled the lower Γ (Table 1). Compared with a model that fixed Γ*, fitting Γ* while holding $R_d$ and $K_{CO}$ constant resulted in smaller differences in $V_{cmax}$ (2.7% difference) and $c_{i,trans}$ (9% difference), while not inconsiderable in the case of $c_{i,trans}$ (39 $\mu$mol $mol^{-1}$), differences between RACiR and standard $A/c_i$ for these two parameters were not statistically significant. However, fitting Γ* to the data resulted in significantly lower $J$ (6.5%) for the RACiR than the standard $A/c_i$.

To determine whether estimating both Γ* and $R_d$ affected the match between $V_{cmax}$ and $J$ for the two techniques, a final fit was implemented fixing only the value of $K_{CO}$. This decreased $R_d$ for both measurement techniques and $J$ estimated from RACiR data, resulting in a significant (6%) difference between the standard and RACiR estimates of $J$. As expected, $\Gamma^*$, $\Gamma$ and $c_{i,trans}$ were also underestimated in RACiR data (Table 1).

These analyses show that for the dataset of Stinziano et al., (2017) fixing $\Gamma^*$ and $K_{CO}$ for $A/c_i$ fits results in reasonably close agreement of $V_{cmax}$ and $J$ for paired RACiR and standard $A/c_i$ measurements. Caution is still necessary, however, because the offsets in RACiR measurements shown in Fig. 1 mean that RACiR tends to estimate $V_{cmax}$ as higher and $J$ as lower than standard $A/c_i$ measurements. Since rates of flow, $CO_2$ ramping during RACiR measurements, and instrument-specific match parameters could be factors contributing to these offsets, we suggest that work using the RACiR technique should incorporate ground-truthing controls that compare paired RACiR standard $A/c_i$ measurements. This will allow empirical corrections to be applied to RACiR offsets.

The standard $A/c_i$ technique does take longer than RACiR. However, it allows modulated fluorescence measurements and estimation of mesophyll conductance and $c_c$ by the variable $J$ method (Harley et al., 1992) that can separate variation in $g_m$ from variation in $V_{cmax}$. For investigators focussed on understanding the biochemical factors limiting crop photosynthesis and the impacts of rising [$CO_2$], it is particularly important to realise that RACiR measurements can result in large underestimates of $R_d$, $\Gamma$, and $c_{i,trans}$. These offsets will impact identification of biochemical factors limiting photosynthesis and affect analyses that aim to partition the impacts of biochemical and stomatal limitations on photosynthesis.

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## Figure Legends

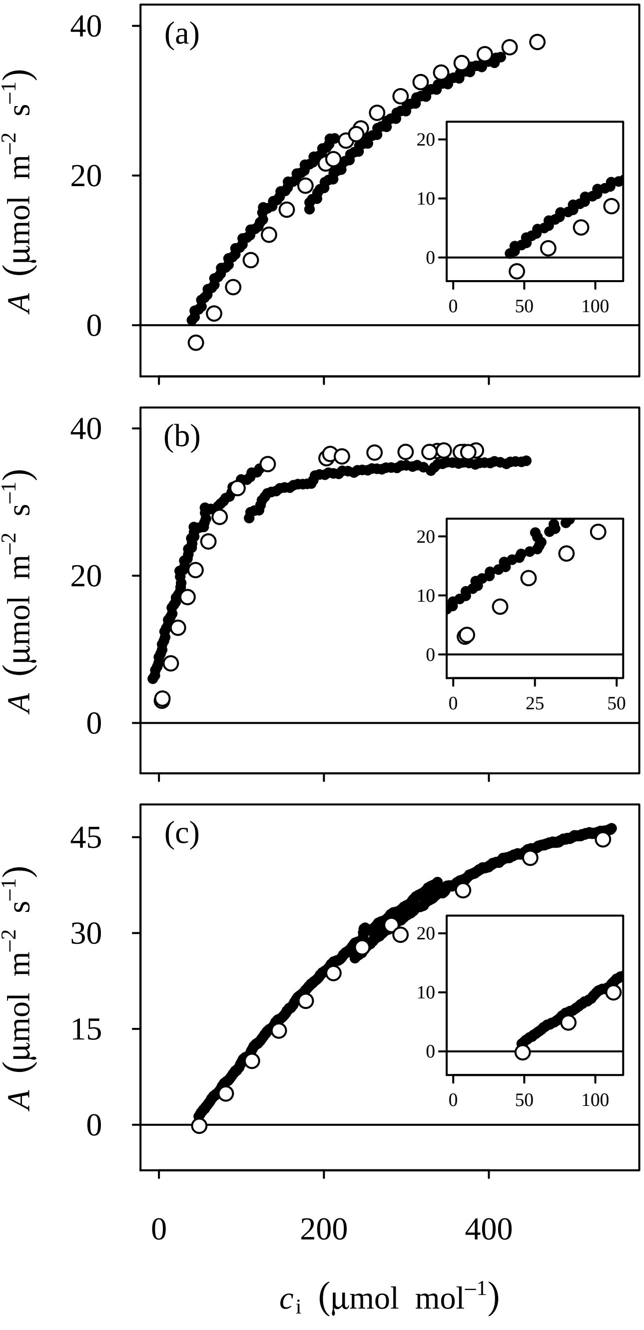
### Figure 1

Standard $A/c_i$ (open) and RACiR (closed) measurements made for (a) cowpea (*Vigna unguiculata*); (b) the $C_4$ grass teff (*Eragrostis teff*); and (c) selected data for *Populus deltoides* from Stinziano et al., (2017). Insets show the region around $\Gamma$ for each set of measurements. Measurements of *V. unguiculata* and *E. teff* used respective flow rates of 500 and 600 µmol $s^{-1}$; incident photosynthetic photon flux density (PPFD), 1500 µmol $m^{-2}$ $s^{-1}$; air temperature, 30 °C; leaf vapour pressure deficit ($VPD_{leaf}$), 1.7 kPa; and chamber over-pressure, 0.1 kPa. For *P. deltoides*: flow rate, 600 µmol $s^{-1}$; leaf temperature, 25 °C; $VPD_{leaf}$, 1.2 kPa; overpressure, 0.2 kPa (Stinziano et al., 2017)

**Table 1** Parameter choice affects statistical outcomes when comparing standard $A/c_i$ and RACiR measurements made by Stinziano et al., (2017).

| Fixed parameters | | $V_{cmax}$ | $J$ | $R_d$ | $\Gamma^*$ | $\Gamma$ | $c_{i,trans}$ | $K_{CO}$ |
|---|---|---|---|---|---|---|---|---|
| $\Gamma^*$, $K_{CO}$ | standard $A/c_i$ | 110±5.2 | 230±11 | 0.81±0.215 | 42.9 | 48.6±1.53 | 432±16.4 | 714 |
| | RACiR | 114±5.1 | 223±11 | 0.04±0.036 | 42.9 | 43.1±0.265 | 374±22.6 | 713 |
| | Δ% | 3.6 | −3.0 | −95.1 | - | −11.3 | −13.4 | - |
| | P (paired t-test, d.f.=5) | 0.223 | 0.273 | **0.011** | - | **0.011** | **0.016** | - |
| | | | | | | | | |
| $\Gamma^*$, $K_{CO}$, $R_d$ | standard $A/c_i$ | 110±5.3 | 230±11.3 | 0.81 | 42.9 | 48.58±0.284 | 434±19.5 | 714 |
| | RACiR | 117±5.3 | 228±11 | 0.81 | 42.9 | 48.16±0.226 | 364±22.6 | 713 |
| | Δ% | 6.4 | −0.01 | - | - | −0.01 | −16.1 | - |
| | P (paired t-test, d.f.=5) | **0.014** | 0.585 | - | - | **0.029** | **0.006** | - |
| | | | | | | | | |
| $K_{CO}$, $R_d$ | standard $A/c_i$ | 110±5.1 | 231±11 | 0.81 | 43.2±1.44 | 48.9±1.49 | 431±15.6 | 714 |
| | RACiR | 113±4.6 | 216±10 | 0.81 | 34.2±1.09 | 39.7±1.25 | 392±21.1 | 713 |
| | Δ% | 2.7 | −6.5 | - | −20.8 | −18.8 | −9.0 | - |
| | P (paired t-test, d.f.=5) | 0.341 | **0.033** | - | **$4\times10^{-5}$** | **$2\times10^{-5}$** | 0.056 | - |
| | | | | | | | | |
| $K_{CO}$ | standard $A/c_i$ | 110±5 | 232±12 | 0.34±0.262 | 46.2±1.68 | 48.7±1.47 | 428±16.7 | 714 |
| | RACiR | 112±4.5 | 218±11 | 0.3±0.31 | 37.5±2.75 | 39.8±1.22 | 389±20.2 | 713 |
| | Δ% | 1.8 | −6 | −11.8 | −18.8 | −18.3 | −9.1 | - |
| | P (paired t-test, d.f.=5) | 0.377 | **0.026** | 0.934 | **0.021** | **$3\times10^{-5}$** | **0.049** | - |

Using the R Language and Environment (R Core Team, 2017) function *optim*, the residual sum of squares (RSS) for $A_C$ and $A_J$ was used to establish best fitting parameter combinations with $c_{i,trans}$ values from 50 to 499 μmol mol$^{-1}$, then RSS was used to further select the $c_{i,trans}$ at which the optimisation had produced the best fit.


(a)
(b)
(c)
40
20
0
45
30
15
A (μmol m$^{-2}$ s$^{-1}$)
20
10
0
0
50
100
0
25
50
0
200
400
$c_i$ (μmol mol$^{-1}$)

## Supplementary Material

# Phenotyping Photosynthesis on the Limit – A critical examination of RACiR

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**Supplementary Table 1** FvCB model fits made allowing all parameters to be estimated from the data, for six paired measurements from the Supplementary data of Stinziano et al., (2017).

| ID | Data collection method | $V_{cmax}$ | $J$ | $R_d$ | $\Gamma^*$ | $K_{CO}$ | $\Gamma$ | $c_{i.trans}$ |
|---|---|---|---|---|---|---|---|---|
| LI1A | Standard | 102 | 262 | 0 | 49.1 | 553 | 49.1 | 472 |
|  | RACiR | 98 | 240 | 0 | 40.6 | 500 | 40.6 | 389 |
| LI1B | Standard | 75 | 180 | 0.9 | 41.5 | 547 | 48.4 | 411 |
|  | RACiR | 91 | 175 | 2.7 | 22.1 | 588 | 41.1 | 350 |
| LI1C | Standard | 118 | 238 | 0 | 50.7 | 786 | 50.7 | 425 |
|  | RACiR | 109 | 221 | 0 | 39.8 | 709 | 39.8 | 411 |
| LI2A | Standard | 99 | 256 | 0 | 48.1 | 500 | 48.1 | 410 |
|  | RACiR | 103 | 262 | 0 | 41.8 | 500 | 41.8 | 427 |
| LI2B | Standard | 97 | 232 | 0 | 52.3 | 598 | 52.3 | 417 |
|  | RACiR | 87 | 206 | 0 | 45.2 | 500 | 45.2 | 340 |
| LI2C | Standard | 124 | 233 | 1.1 | 42.5 | 900 | 50.9 | 476 |
|  | RACiR | 118 | 218 | 0 | 42.1 | 884 | 42.1 | 450 |