A test of rapid leaf photosynthesis-intercellular CO₂ concentration response (RACiR) technique for evergreens and herbs

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September 24, 2021

Abstract

Using traditional photosynthesis-intercellular CO₂ concentration ($A-C_i$) response (TACiR) curves to obtain the maximum rates of ribulose-1,5-bisphosphate carboxylase oxygenase carboxylation (V_{cmax}) and electron transport (J_{max}) is time-consuming and labor-intensive. Instead, the rapid $A-C_i$ response (RACiR) technique provides a potential way with high efficiency. However, multiple parameter settings of RACiR technique for different plant life forms remain unclear. Here, we used Li-Cor 6800 to test the applicability and optimum parameter settings of RACiR curves for evergreens and herbs. We set 11 groups of [CO₂], i.e., R1 (400-1500 ppm), R2 (400-200-800 ppm), R3 (420-20-620 ppm), R4 (420-20-820 ppm), R5 (420-20-1020 ppm), R6 (420-20-1220 ppm), R7 (420-20-1520 ppm), R8 (420-20-1820 ppm), R9 (450-50-650 ppm), R10 (650-50 ppm) and R11 (650-50-650 ppm), and made contrasts between TACiR and RACiR curves. We found that V_{cmax} and J_{max} calculated by TACiR and RACiR overall showed no significant differences across 11 [CO₂] gradients ($P < 0.05$). The efficiency and accuracy of R2, R3, R4, R9 and R10 showed higher superiority than others. Moreover, the accuracy of manual empty chamber correction method was higher than the automatic method. In conclusion, the RACiR technique could be generally used to obtain photosynthetic parameters with higher efficiency than traditional methods for various life forms.

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Abstract: Using traditional photosynthesis-intercellular CO₂ concentration ($A - C_i$) response (TACiR) curves to obtain the maximum rates of ribulose-1,5-bisphosphate carboxylase oxygenase carboxylation (V_{cmax}) and electron transport (J_{max}) is time-consuming and labor-intensive. Instead, the rapid $A - C_i$ response (RACiR) technique provides a potential way with high efficiency. However, multiple parameter settings of RACiR technique for different plant life forms remain unclear. Here, we used Li-Cor 6800 to test the applicability and optimum parameter settings of RACiR curves for evergreens and herbs. We set 11 groups of [CO₂], i.e., R1 (400-1500 ppm), R2 (400-200-800 ppm), R3 (420-20-620 ppm), R4 (420-20-820 ppm), R5 (420-20-1020 ppm), R6 (420-20-1220 ppm), R7 (420-20-1520 ppm), R8 (420-20-1820 ppm), R9 (450-50-650 ppm), R10 (650-50 ppm) and R11 (650-50-650 ppm), and made contrasts between TACiR and RACiR curves. We found that V_{cmax} and J_{max} calculated by TACiR and RACiR overall showed no significant differences across 11 [CO₂] gradients ($P < 0.05$). The efficiency and accuracy of R2, R3, R4, R9 and R10 showed higher superiority than others. Moreover, the accuracy of manual empty chamber correction method was higher than the automatic method. In conclusion, the RACiR technique could be generally used to obtain photosynthetic parameters with higher efficiency than traditional methods for various life forms.

Keywords: rapid $A - C_i$ response (RACiR), traditional $A - C_i$ response (TACiR), CO₂ response, V_{cmax} , J_{max} , RuBisCo, photosynthesis

1 Introduction

Plant photosynthesis is an important physiological process underlying primary productivity and plant growth (Peng 2000, Kruger and Volin 2006, Yin and Struik 2009). As one of the limiting factors of photosynthesis (Boretti and Florentine 2019), changes in CO₂ concentration ([CO₂]) may lead to a series of corresponding changes in plant physiology, growth performance and morphology (Mott, 1990; Gunderson and Wullschlegel 1994, Kirschbaum 1994). At the leaf level, plant photosynthetic capacity is quantified by measuring the response of net CO₂ assimilation rate (A) to intercellular CO₂ concentration (C_i) (Lewis et al. 1996, Rey and Jarvis 1998). The resulting functional relationship ($A - C_i$ curves) can be used to estimate parameters that determine assimilation rates in the Farquhar-von Caemmerer-Berry (FvCB) model (Farquhar et al. 1980, von Caemmerer and Farquhar 1981), including the maximum rates of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) carboxylation (V_{cmax}) and electron transport (J_{max}) for ribulose-1,5-bisphosphate (RuBP) regeneration, and the photorespiratory compensation point (Liang et al. 2009, Sun et al. 2014).

A large number of biochemical models (Farquhar et al. 1980, von Caemmerer and Farquhar 1981) and empirical models (Baly 1935, Marshall and Biscoe 1980, Bassman and Zwier 1991, Harley et al. 1992, Ye 2007) have described responses of plant photosynthesis to [CO₂]. Among them, the FvCB model is a mechanistic model for C₃ photosynthesis (Farquhar et al. 1980) that is widely adopted in mechanistic models of terrestrial biosphere and Earth System Models (ESMs) (Wohlfahrt et al. 1999, Medlyn et al. 2002, Han et al. 2004, Verheijen et al. 2013, Feng et al. 2018, An et al. 2020). V_{cmax} and J_{max} are key parameters determining photosynthesis rates by C₃ plants in the FvCB model (Long and Bernacchi 2003, Sharkey et al. 2007, Ali et

al. 2015). Therefore, accurately specifying or simulating of V_{cmax} and J_{max} is of great significance for reliable predictions of global carbon fluxes and plant responses to environmental changes (Rogers 2014, Walker et al. 2014, Burnett et al. 2019).

In traditional $A - C_i$ response (TACiR) measurements, a series of $[CO_2]$ should be set and be matched between the reference and the sample $[CO_2]$ of the open gas exchange system, respectively (Long and Bernacchi 2003, Busch 2018). During each measurement, the monitored leaves usually need a period of time to fully activate RuBisCO until their photosynthesis reaches steady state. This method has two limitations: (1) Time-consuming. A complete TACiR curve takes about 30~60 minutes depending on the species, the time required to reach steady state, and the $[CO_2]$ gradient settings (Stinziano et al. 2017, Urban et al. 2017, Burnett et al. 2019). This may hamper measurements with large sample sizes and replicates. (2) Limited $[CO_2]$ settings are not enough to achieve a large amount of $A - C_i$ data.

Considering these shortages of TACiR, Stinziano et al. (2017) found that leaves could rapidly adapt to changes of $[CO_2]$ in a short time (less than 5 minutes) (Davis et al. 1987, McDermitt et al. 1989, Long and Hällgren 1993, Laisk and Oja 1998), then they proposed the rapid $A - C_i$ response (RACiR) technique for *Populus deltoides* and found that the model parameters (e.g., V_{cmax} and J_{max}) calculated by the RACiR curves were close to values derived by the TACiR curves. As most previous researches only mentioned that the development of this high-throughput gas exchange technique allowed for rapid measurement of $A - C_i$ response curves (Burnett et al. 2019, Sharkey 2019, Furbank et al. 2020, Mason et al. 2020, Silva-Pérez et al. 2020, van Bezouw et al. 2020, von Caemmerer 2020) but seldomly explored its application to plants among different life forms, the usefulness of this method is still being questioned.

For the application of RACiR, the ranges and rates of $[CO_2]$ changes are the most important inputting parameters. Previous studies were mainly conducted in the greenhouse with several change types of $[CO_2]$ for limited species, especially seedlings (Table S1). For example, Pilon et al. (2018) set the RACiR curves of 400-0-1000 ppm (100 ppm min⁻¹, 14 minutes) for *Arachis hypogaea* L; Liu and van Iersel (2020) set $[CO_2]$ at 0-1200 ppm (200 ppm min⁻¹, 6 minutes) for *Lactuca sativa*; McKenzie-Gopsill et al. (2020) set the $[CO_2]$ at 10-500 ppm (98 ppm min⁻¹, 5 minutes) for *Phaseolus vulgaris* L. Despite these attempts, a relative lack of the validation for RACiR technique among different species and in the field of forest ecosystems (Wu et al. 2019), in particular regarding the settings of multiple parameters, limits the practical application of this technique.

In addition, to avoid the measurement lags between the reference and the sample $[CO_2]$ caused by systematic errors, the RACiR curves should be calibrated by running the empty chamber (i.e., no leaves in the leaf chamber) response curves (Stinziano et al. 2017, Lawrence et al. 2018). Stinziano et al. (2018) proposed that any condition change, e.g., flow rate, temperature, ramp range, direction and rate, requires a new empty chamber correction for the response curves. Afterwards, Coursolle et al. (2019) reported that empty chamber correction could be conducted once a day as long as the matched values were checked regularly and the environmental conditions and the matching data were kept stable. Two methods for empty chamber response curves' correction could be used: (1) manual correction: the linear response portion of the curves are manually selected to correct the data for the measurements of empty chambers and with leaf chambers (Stinziano et al. 2017 & 2018). However, this method may not be suitable for bulk data processing; (2) automatic correction: a threshold is set for automatic data scrubbing so that only the quasi-linear part of the data is retained for the following curve fitting. This method can automatically correct multiple files at once, and the leaf areas can be modified and then corrected (Coursolle et al. 2019), while the shortage lies in that specific evaluation of some results might be ignored. Therefore, both manual and automatic correction methods have their own advantages and disadvantages, and their specific application scope remains to be explored.

Aiming to test the applicability of RACiR technique among different life-forms, we conducted a comparative study between TACiR and RACiR curves under multiple $[CO_2]$ gradients for evergreen trees (*Castanopsis eyrei* and *Castanopsis sclerophylla*) in the field, evergreen shrubs (*Viburnum odoratissimum* and *Cestrum nocturnum*) and herbs (*Sphagneticola trilobata*) in the greenhouse. Then, we calculated specific photo-

synthetic parameters (V_{max} and J_{max}) to address the following questions: (1) Can RACiR technique be universally applied to evergreens and herbs? (2) Which $[CO_2]$ gradient is the most efficient one for RACiR measurement for different life-forms? (3) Are there significant differences in the photosynthetic parameters derived from manual and automatic empty chamber correction methods? By answering these scientific questions, we aim to explore the generalizability of the RACiR technique, compare the differences of two empty chamber correction methods, and screen out the applicable $[CO_2]$ gradients for different species. We hope to maximize the measurement and calculation efficiency of the CO_2 response curve, and provide a reference for the subsequent extensive application of RACiR technique.

2 Materials and methods

2.1 Plant materials

Two well-grown trees (*C. eyrei* and *C. sclerophylla*) in the subtropical evergreen broad-leaved forest of Guniujiang in Shitai County, Anhui Province (117°21'23"E, 30deg01'47"N) were randomly selected as the subjects of the field experiment, and three well-grown plants (*V. odoratissimum*, *C. nocturnum* and *S. trilobata*) in the exhibition greenhouse of Beijing Botanical Garden (116deg12'7"E, 39deg59'53"N) were randomly selected as the subjects of the greenhouse experiment. Exhibition greenhouse temperature was maintained at about 25 degC. The morphological characteristics and growth properties of these plants were shown in Table 1, where the leaf area was determined as described in 2.2.3 below.

2.2 Experimental design

2.2.1 RACiR curves

Three mature leaves with similar size were selected from each plant. To avoid photosynthetic siesta during the daily variation of the plants, CO_2 response curves were measured on marked leaves between 9:00-11:00 and 14:00-16:00 daily from December, 2020 to January, 2021 and June, 2021 using the LI-6800 Portable Photosynthesis System (Li-COR Inc., Lincoln NE, USA) (Console version: Bluestem v.1.4.02) with fan speed of 10,000 rpm, flow rate of 500 $\mu mol s^{-1}$, overpressure of 0.1 KPa, chamber air relative humidity of 50%, leaf temperature of 25 , and light of 1000 $\mu mol m^{-2}s^{-1}$.

The RACiR curves had a total of 11 different $[CO_2]$ gradients (R1~R11) with specific parameter settings as shown in Table 2. The 'Autolog' program of LI-6800 was used to record measurements every 2 s, where R2~R9 and R11 were recorded only for the up-ramp phase of $[CO_2]$ to fit curves, and R1 and R10 were CO_2 response curves for monotonic changes of $[CO_2]$. In addition, to avoid the lag of $[CO_2]$ measurements between the reference and sample chambers, the RACiR curves of empty chamber were measured daily under the same environmental conditions with different $[CO_2]$ gradients to synchronously calibrate the RACiR curves of the leaf chamber.

2.2.2 TACiR curves

TACiR curves were performed after finishing all RACiR curves and the position of clamping leaves remained unchanged. Firstly, all environmental conditions were set the same as in the RACiR curves except for the $[CO_2]$. Secondly, the sample $[CO_2]$ was set to 400 ppm, and the leaves were kept to adapt for more than 5 minutes under the set environmental conditions until A becomes steady. Then the sample $[CO_2]$ was set to 400, 300, 250, 200, 150, 100, 50, 400, 400, 500, 650, 800, 1000, 1500 and 2000 ppm with minimum and maximum waiting times of 90 s and 180 s, respectively. Automatic matching was carried out before changing each $[CO_2]$, and the measuring time for each TACiR curve was approximately 30 minutes.

2.2.3 Leaf area measurements

Leaves subjected to the photosynthesis measurements were cut along their lower edges, leaving the petiole untouched as much as possible. The leaves were scanned in grayscale with the CanoScan LIDE 400 scanner (Canon, Hanoi, Vietnam) at a resolution of 300 DPI. Then the leaf area (mean $\pm$ se, cm^2) was calculated using ImageJ software (National Institutes of Health, Bethesda, USA).

2.3 Statistical analysis

All statistical analysis were performed using R v.4.0.3 (R Core Team, 2020). We calibrated the RACiR curves using the improved {racir} R Package (v.1.0.0) based on the {RACiR} R Package (Stinziano et al. 2018) by Jiedong Zhu on Github (<https://github.com/zhujiadong/racir>). All CO₂ response curves were fitted using the fitaci function from the {Plantecophys} R Package with the default settings except for the fit method which was set to bilinear, i.e., V_{cmax} and J_{max} were estimated by fitting the measured data of the RACiR and TACiR curves according to the FvCB model (Duursma, 2015). V_{cmax1} and J_{max1} indicated the parameters fitted by the manual empty chamber response curve correction method of Stinziano et al. (2017); V_{cmax2} and J_{max2} indicated the parameters fitted by the automatic empty chamber response curve correction method of Coursolle et al. (2019); V_{cmax3} and J_{max3} indicated the parameters fitted by the TACiR curves.

The data were tested for normality and homoscedasticity by Shapiro-Wilk test and Bartlett test before all statistics were performed. Paired t -test was used to compare the differences between empty chamber response curve correction methods. One-way analysis of variance (one-way ANOVA) was used to compare the differences between RACiR and TACiR curves as well as between empty chamber response curve measurements at different times. Multiple comparisons were performed with Tukey's honestly significant difference (Tukey's HSD), and differences were significant when $P < 0.05$.

The accuracy of the different empty leaf chamber response curve correction methods of RACiR curves was assessed using the coefficient of variation (CV), which was calculated as:

$$CV = \left(\frac{sd}{mean} \right) \times 100\%;$$

$$\Delta CV = CV_1 - CV_2;$$

where *mean* was the mean value; *sd* was the standard deviation; CV_1 was the coefficient of variation of the manual correction method; CV_2 was the coefficient of variation of the automatic correction method; ΔCV was the difference between these two coefficients of variation. When $\Delta CV > 0$, it was more suitable to use the automatic correction method to correct RACiR curves. When $\Delta CV < 0$, it was more suitable to use the manual correction method to correct RACiR curves. When $\Delta CV = 0$, both methods could be used to correct RACiR curves (Li et al. 2016).

3 Results

3.1 Comparison of TACiR and RACiR curves across [CO₂] gradients

Across the 11 [CO₂] gradients, RACiR curves of *C. eyrei* and *S. trilobata* were not significantly different from TACiR curves for both empty chamber response curve correction methods (Figs. 1a, b, i, j). V_{cmax1} calculated by R1 was lower than the other curves for *C. sclerophylla* (Fig. 1c). In *V. odoratissimum*, J_{max1} calculated by R10 was significantly different from that calculated by the T (TACiR curves) (Fig. 1f). V_{cmax1} and J_{max1} calculated by R1 of *C. nocturnum* were significantly lower than the whole CO₂ response curves (Figs. 1g, h). The results obtained by automatic correction method were basically consistent with those obtained by manual correction method. Overall, V_{cmax} and J_{max} calculated by fitting RACiR and TACiR curves generally showed no significant difference, only with some varied values calculated by specific [CO₂] gradients.

In terms of measurement time, R2, R3, R4, R9 and R10, respectively, took [?]10 minutes (Table 2). Comparing the fitting data of TACiR and RACiR curves, T showed no significant difference with R2, R3, R4, R9, R10 of *C. eyrei*, *C. sclerophylla*, *C. nocturnum* and *S. trilobata*. For *V. odoratissimum*, T showed weak difference from R2, R3, R4 and R9, but was significantly higher than R10.

3.2 Comparison of the empty chamber response curve correction methods for RACiR

Results of the paired t -test showed no significant difference in the photosynthetic parameters obtained by the RACiR curves using two empty chamber response curve correction methods under different [CO₂] gradients (Table 3). Exceptions were found for (i) *C. eyrei*, V_{cmax1} and J_{max1} of R6 and R7 were significantly higher than the corresponding V_{cmax2} and J_{max2} , respectively; V_{cmax1} of R11 was significantly higher than

V_{cmax2} ; (ii) *C. sclerophylla*, J_{max1} of R5 and R6 were significantly higher than J_{max2} ; V_{cmax1} of R9 was significantly higher than V_{cmax2} ; (iii) *V. odoratissimum*, J_{max2} of R11 was significantly higher than J_{max1} ; (iv) *C. nocturnum*, V_{cmax} and J_{max} obtained from R6 and R10 by different correction methods were significantly different; V_{cmax2} of R9 was higher than V_{cmax1} ; (v) *S. trilobata*, V_{cmax1} of R3 was higher than V_{cmax2} .

Further comparison of ΔCV with significant differences in V_{cmax} or J_{max} showed that most RACiR curves were with $\Delta CV < 0$, indicating that these curves were more accurate for V_{cmax1} and J_{max1} measured by the manual correction method. The fitting curves of RACiR and TACiR data also showed similar results (Figs. S1~S5).

4 Discussion

4.1 RACiR technique could be applied to evergreens and herbs

Our study showed that V_{cmax} and J_{max} calculated by RACiR curves under multiple $[CO_2]$ gradients were not significantly different from TACiR curves, indicating that the RACiR technique could be applied to herbs as well as evergreen species (Pilon et al. 2018, Lu et al. 2020, McKenzie-Gopsill et al. 2020, Vincent et al. 2020, Liu and van Iersel 2021) and thus is likely to be generalizable among different plant life forms.

Combined with the accuracy of fitted data and the measurement time, our test of RACiR technique proved its potential in improving measurement efficiency of CO_2 response curves. Moreover, R2, R3, R4, R9 and R10 could be the most efficient $[CO_2]$ gradients for RACiR curves of *C. eyrei*, *C. sclerophylla*, *C. nocturnum* and *S. trilobata*. Similarly, R2, R3, R4 and R9 are suitable for RACiR curves of *V. odoratissimum*, but whether R10 could be applied to *V. odoratissimum* still needs further verification. Therefore, we suggest that R2, R3, R4, R9 and R10 could be preferentially validated for the first-time application of the RACiR technique to specific species in the future.

The V_{cmax} and J_{max} calculated by RACiR curves of monotonic vs down-up ramp of $[CO_2]$ showed significant difference, e.g., R1 of *C. nocturnum* were significantly lower than the other CO_2 response curves, while R7 and R8 showed no significant difference. This result indicated that RACiR curves with high monotonic increase but not down-up ramp $[CO_2]$ (e.g., R1) might be an inadvisable choice for *C. nocturnum* considering that the cuticle of papery leaves was usually thinner with lower stomatal density than those of leathery leaves (Ma 2005). However, Vincent et al. (2020) set $[CO_2]$ at 100-900 ppm (100 ppm min⁻¹, 8 minutes) for *Citrus sinensis* (L.) Valencia, a species with low stomatal conductance, and the V_{cmax} and J_{max} obtained using RACiR curves were not significantly different from TACiR curves. Thus, we speculate that setting monotonically increasing $[CO_2]$ too high (e.g., >1000 ppm) in RACiR curves may not be applicable to low stomatal conductance species, although Sharkey (2019) argued that a continuous and rapid increase of $[CO_2]$ was the best when measuring TACiR curves. In our results, even though the V_{cmax} calculated by R1 of *C. sclerophylla* was significantly lower than the other CO_2 response curves, the difference of J_{max} was not significant. The specific reasons are not clear and need to be further studied. In addition, the RACiR curves of *S. trilobata* were not significant different from TACiR curves, implying that RACiR technique might have more practical application in herbaceous functional groups, especially crops in agricultural ecosystem.

4.2 Manual method has high accuracy while the automatic method has high efficiency for the calibration of empty chamber response curves

For the RACiR curves' calibration, Stinziano et al. (2017 & 2018) removed the points at the beginning and near the end of the A -reference $[CO_2]$ curve mainly by visual observation to retain the linear response portion of the curve, i.e., the most suitable range of the response curve for further calibration. After calibration of the measured data from empty chamber and with leaf chamber, new A and C_i values were obtained and thus the V_{cmax} and J_{max} were calculated by fitting the corrected curves. Coursolle et al. (2019) cleaned the data of empty chamber and with leaf chamber by setting a threshold of ± 0.05 for two adjacent measured A values, in this case only the quasi-linear part of the curve was retained for the correction of the original data.

By comparing different empty chamber calibrating methods for RACiR curves of each species under different $[\text{CO}_2]$ gradients, we found that V_{cmax} and J_{max} derived from manual and automatic method generally showed no significant difference, except some specific cases such as R6 and R7 for *C. eyrei*, R6 and R10 for *C. nocturnum*. In addition, although the calculated V_{cmax} and J_{max} of most RACiR curves by different empty chamber calibrating methods did not show large difference, the data obtained by automatic method did not overlap well with the TACiR curves, especially for R1 and R10.

Therefore, for the processing of a small amount of data, we'd better give priority to the manual calibrating method. Moreover, when we are not yet proficient in using the RACiR technique, especially for some species for the first time, we should also screen the most suitable $[\text{CO}_2]$ gradients and the calibrating method by combining the coincidence degree of RACiR and TACiR data points in addition to comparing the parameters obtained from RACiR and TACiR curves. With proficiency and extensive application of this technique, we can then adopt the automatic calibrating method to greatly improve the efficiency of data processing.

It is worth mentioning that the newly upgraded LI-6800 system version (Console version: Bluestem v.1.5) dynamically corrected the RACiR curves to eliminate the asynchrony between the sample and reference chamber due to volume problems, so there was no need to do the empty chamber calibration after system updating. However, whether there is any difference between the RACiR data of the old and new system versions is unknown. Therefore, although the data measured by the upgraded system is more accurate and convenient, we suggest that if the RACiR curves has been measured by the old system version, the old system version can be used for subsequent measurements to ensure the uniformity of the data measurement method.

5 Conclusion

By comparing TACiR and RACiR curves under multiple $[\text{CO}_2]$ gradients, we tested the generalizability of the RACiR technique among different plant life forms. Our results indicated that for the studied evergreens and herbs, RACiR curves could be efficiently measured using the $[\text{CO}_2]$ gradients of R2, R3, R4, R9 and R10, reducing the measurement time from 30 minutes to 6~10 minutes and greatly improving the measurement efficiency of CO_2 response curves. Moreover, our results demonstrated that the manual and automatic calibrating methods for the RACiR curves of the empty chamber for five species under different $[\text{CO}_2]$ gradients basically showed no significant difference. The calculation accuracy but not the efficiency of the manual calibrating method was higher than the automatic calibrating method, so we should choose the empty chamber calibrating method reasonably according to the actual sample size and the $[\text{CO}_2]$ gradients. In conclusion, compared with TACiR curves, the RACiR technique yielded photosynthetic parameters (e.g., V_{cmax} and J_{max}) with high quality and efficiency. This suggests a potential to greatly enhance the volume of data on photosynthetic efficiencies to further improve the understanding of relationships between plant function traits and the environment, and the accurateness of global model predictions for vegetation dynamics.

Acknowledgements

We would like to thank L. A. Liu (Beijing Botanical Garden) and Y. H. Tang (Peking University) for their help in experiment exploration and data collection. We also appreciate all the staffs of Beijing Botanical Garden Exhibition Greenhouse who provided warm support during the experiment. Thank you also to Z. D. Wang and M. X. Xu for their help in the collection of canopy leaves.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by National Natural Science Foundation of China [No. 31800397], [No. 31901086] and [No. 31988102]. Di Tian was supported by the 2020/2021 Swiss Government Excellence Scholarship. Benjamin D. Stocker was funded by the Swiss National Science Foundation Grant No. PCEFP2_181115.

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Supporting Information

Additional supporting information may be found in the online version of this article at publisher's web-site.

Table 1. Morphological characteristics and growth properties of experimental plants.

Species	Family	Life form	Leaf texture	Leaf shape	Growth characteristics	Leaf area/cm ² (mean $\pm$ se)
<i>C. eyrei</i>	<i>Fagaceae</i>	Evergreen trees	Leather	Ovate, lanceolate or long elliptic	Grows on fertile, moist and acidic soils in warm and rainy climates; highly adaptable	12.774 $\pm$ 0.470
<i>C. sclerophylla</i>	<i>Fagaceae</i>	Evergreen trees	Leather	Long elliptic, ovate-elliptic or both obovate-elliptic	Light-loving, also shade tolerant; likes deep and moist soil, also tolerates drought and infertile	32.475 $\pm$ 0.641
<i>V. odoratisimum</i>	<i>Caprifoliaceae</i>	Evergreen shrubs	Leather	Oval to rectangular	Prefers warmth and slightly cold; tolerates light and slightly shade	38.494 $\pm$ 3.027

Species	Family	Life form	Leaf texture	Leaf shape	Growth characteristics	Leaf area/cm ² (mean $\pm$ se)
<i>C. nocturnum</i>	<i>Solanaceae</i>	Evergreen shrubs	Papery	Rectangular-lanceolate	Prefers warm, moist and sunny environment; adaptable, but not hardy	61.713 $\pm$ 2.565
<i>S. trilobata</i>	<i>Compositae</i>	Herbs	Leather	Ovate-lanceolate	Tolerant of drought, humidity and barrenness; highly adaptable; fast growing	31.738 $\pm$ 2.036

Table 2. Different [CO₂] gradient settings of the RACiR curves.

ID	Curve Type	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp
		Initial [CO ₂] (ppm)	Final [CO ₂] (ppm)	Ramp Rates (ppm min ⁻¹)	Time (min)	Initial [CO ₂] (ppm)	Final [CO ₂] (ppm)	Ramp Rates (ppm min ⁻¹)	Time (min)
R1	400-1500	Null	Null	Null	Null	400	1500	100	11
R2	400-200-800	400	200	200	1	200	800	100	6
R3	420-20-620	420	20	200	2	20	620	100	6
R4	420-20-820	420	20	200	2	20	820	100	8
R5	420-20-1020	420	20	200	2	20	1020	100	10
R6	420-20-1220	420	20	200	2	20	1220	100	12
R7	420-20-1520	420	20	200	2	20	1520	100	15
R8	420-20-1820	420	20	200	2	20	1820	100	18
R9	450-50-650	450	50	200	2	50	650	100	6
R10	650-50	650	50	100	6	Null	Null	Null	Null

ID	Curve Type	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Down-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp	[CO ₂] Up-ramp
R11	650-50-650	650	50	100	6	50	650	100	6

Table 3. Results of the paired t -test for the comparison of V_{cmax} and J_{max} generated by different empty chamber response curve correction methods among five species. R1 represented the [CO₂] gradient in the RACiR curves of 400-1500 ppm, R2 was 400-200-800 ppm, R3 was 420-20-620 ppm, R4 was 420-20-820 ppm, R5 was 420-20-1020 ppm, R6 was 420-20-1220 ppm, R7 was 420-20-1520 ppm, R8 was 420-20-1820 ppm, R9 was 450-50-650 ppm, R10 was 650-50 ppm, and R11 was 650-50-650 ppm ($n = 3$). $P < 0.05$ indicated that V_{cmax} and J_{max} obtained by different empty chamber response curve correction methods were significantly different. The significant difference with $P < 0.05$ was labeled in bold. $V_{cmax1} - V_{cmax2}$ denoted the paired t -test between V_{cmax1} derived using the manual correction method and V_{cmax2} derived using the automatic correction method, and $J_{max1} - J_{max2}$ denoted the paired t -test between J_{max1} derived using the manual correction method and J_{max2} derived using the automatic correction method. ΔCV represented the difference between CV_1 derived by the manual calibration method and CV_2 derived by the automatic calibration method.

Species	Curve Type	df	$V_{cmax1} - V_{cmax2}$	$V_{cmax1} - V_{cmax2}$	$V_{cmax1} - V_{cmax2}$	$J_{max1} - J_{max2}$	$J_{max1} - J_{max2}$	$J_{max1} - J_{max2}$
			t	P	ΔCV (%)	t	P	ΔCV (%)
<i>C. eyrei</i>	R1	2	0.335	0.769	11.69%	0.273	0.811	7.1%
	R2	2	2.000	0.184	-1.30%	2.233	0.155	-1.3%
	R3	2	2.583	0.123	-0.32%	-2.262	0.152	1.3%
	R4	2	3.035	0.094	0.47%	3.522	0.072	0.4%
	R5	2	2.144	0.165	0.81%	2.736	0.112	0.8%
	R6	2	5.532	0.031	0.28%	4.732	0.042	0.2%
	R7	2	8.053	0.015	-0.51%	5.652	0.030	-0.5%
	R8	2	3.918	0.059	-1.54%	2.579	0.123	-1.5%
	R9	2	3.144	0.088	-0.20%	-1.390	0.299	1.3%
	R10	2	-0.275	0.809	-7.97%	0.707	0.553	5.5%
	R11	2	20.853	0.002	-0.63%	-0.580	0.620	0.6%
<i>C. sclerophylla</i>	R1	2	2.707	0.114	-23.38%	2.421	0.137	-1.3%
	R2	2	2.874	0.103	2.55%	0.925	0.453	2.5%
	R3	2	2.572	0.124	0.72%	0.437	0.705	0.7%
	R4	2	2.775	0.109	1.22%	2.471	0.132	1.2%
	R5	2	2.097	0.171	1.26%	5.357	0.033	1.2%
	R6	2	2.596	0.122	0.90%	6.343	0.024	0.9%
	R7	2	1.641	0.243	-0.04%	2.520	0.128	-0.0%
	R8	2	-0.512	0.660	-1.66%	-0.392	0.733	1.6%
	R9	2	4.712	0.042	0.31%	0.380	0.740	0.3%
	R10	2	-2.756	0.110	6.60%	1.212	0.349	6.6%
	R11	2	1.270	0.332	-5.12%	1.110	0.383	-5.1%
<i>V. odoratissimum</i>	R1	2	2.083	0.173	-12.52%	2.003	0.183	-12.5%
	R2	2	1.556	0.260	4.18%	1.130	0.376	4.1%
	R3	2	-0.633	0.592	0.12%	-0.749	0.532	0.1%
	R4	2	0.943	0.445	-0.49%	-0.573	0.625	-0.4%
	R5	2	2.920	0.100	-1.86%	0.199	0.861	-1.8%
	R6	2	-2.378	0.141	0.52%	-2.374	0.141	0.5%
	R7	2	-3.342	0.079	-0.02%	-0.692	0.560	-0.0%

Species	Curve Type	df	$V_{cmax1} - V_{cmax2}$	$V_{cmax1} - V_{cmax2}$	$V_{cmax1} - V_{cmax2}$	$J_{max1} - J_{max2}$	$J_{max1} - J_{max2}$	$J_{max1} - J_{max2}$
<i>C. nocturnum</i>	R8	2	2.155	0.164	0.41%	1.767	0.219	0.
	R9	2	-1.375	0.303	-8.15%	-1.351	0.309	-1.
	R10	2	0.501	0.666	11.34%	2.668	0.117	2.
	R11	2	-0.366	0.750	-4.52%	-6.061	0.026	-0.
	R1	2	-2.516	0.128	-0.21%	-3.589	0.070	0.
	R2	2	-0.042	0.970	5.89%	0.410	0.722	2.
	R3	2	-1.524	0.267	-1.18%	2.306	0.148	0.
	R4	2	1.873	0.202	2.59%	2.727	0.112	1.
	R5	2	2.004	0.183	1.49%	1.968	0.188	0.
	R6	2	5.428	0.032	-1.18%	5.820	0.028	-0.
	R7	2	-0.881	0.471	1.36%	-0.613	0.602	1.
<i>S. trilobata</i>	R8	2	-0.393	0.733	-1.26%	0.194	0.864	-1.
	R9	2	-31.759	0.001	0.25%	0.345	0.763	-4.
	R10	2	7.066	0.019	-4.04%	12.718	0.006	-2.
	R11	2	1.042	0.407	-1.43%	2.373	0.141	-1.
	R1	2	-0.681	0.566	-7.28%	-0.907	0.460	-1.
	R2	2	0.905	0.461	0.85%	0.509	0.661	-0.
	R3	2	8.692	0.013	-1.21%	-2.971	0.097	0.
	R4	2	1.076	0.395	-2.74%	-1.119	0.380	0.
	R5	2	2.250	0.153	-1.11%	0.091	0.936	-0.
	R6	2	2.471	0.132	-0.98%	2.233	0.155	-0.
	R7	2	-0.398	0.729	1.02%	-0.509	0.661	0.
	R8	2	-0.828	0.495	-0.87%	-0.049	0.965	-0.
	R9	2	2.058	0.176	-3.12%	-0.537	0.645	-0.
	R10	2	3.309	0.080	1.68%	4.058	0.056	1.
	R11	2	0.791	0.512	2.36%	-1.237	0.342	2.

Fig.1 V_{max} and J_{max} generated by TACiR and RACiR fitting curves for five species in different life forms. (a) represented V_{max} of *C. eyrei* , (b) representd J_{max} of *C. eyrei* , (c) representd V_{max} of *C. sclerophylla* , (d) representd J_{max} of *C. sclerophylla* , (e) represented V_{max} of *V. odoratissimum* , (f) represented J_{max} of *V. odoratissimum* , (g) representd V_{max} of *C. nocturnum* , (h) represented J_{max} of *C. nocturnum* , (i) representd V_{max} of *S. trilobata* , (j) represented J_{max} of *S. trilobata* . R1 represented the $[\text{CO}_2]$ gradient in the RACiR curves of 400-1500 ppm, R2 was 400-200-800 ppm, R3 was 420-20-620 ppm, R4 was 420-20-820 ppm, R5 was 420-20-1020 ppm, R6 was 420-20-1220 ppm, R7 was 420-20-1520 ppm, R8 was 420-20-1820 ppm, R9 was 450-50-650 ppm, R10 was 650-50 ppm, and R11 was 650-50-650 ppm ($n = 3$). Different lowercase letters indicated significant differences (Tukey's HSD) in photosynthetic parameters derived with manual correction method, while different capital letters indicated significant differences in photosynthetic parameters derived

with automatic correction method. The difference was insignificant without letter markers.