Deriving C₄ photosynthesis parameters by fitting intensive A/C_i curves

Haoran Zhou¹, Erol Akçay¹ and Brent R. Helliker¹

¹ Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

Correspondence: Haoran Zhou

Address: 433 S University Ave.

314 Leidy Labs

Philadelphia, PA 19104

Phone: 1-215-808-7042

Email: haoranzh@sas.upenn.edu

Brent R. Helliker: helliker@sas.upenn.edu

Erol Akçay: ekacay@sas.upenn.edu

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ABSTRACT

Measurements of photosynthetic assimilation rate as a function of intercellular CO₂ (A/C_i curves) are widely used to estimate photosynthetic parameters for C₃ species, yet few parameters have been reported for C₄ plants, because of a lack of estimation methods. Here, we extend the framework of widely-used estimation methods for C₃ plants to build estimation tools by exclusively fitting intensive A/C_i curves (6-8 more sampling points) for C₄ using three versions of photosynthesis models with different assumptions about carbonic anhydrase processes and ATP distribution. We use simulation-analysis, out-of-sample tests, existing in vitro measurements and chlorophyll-fluorescence-measurements to validate the new estimation methods. Of the five/six photosynthetic parameters obtained, sensitivity analyses show that maximal-Rubisco-carboxylation-rate, electron-transport-rate, maximal-PEP-carboxylation-rate and carbonic-anhydrase were robust to variation in the input parameters, while day-respiration and mesophyll-conductance varied. Our method provides a way to estimate carbonic anhydrase activity, a new parameter, from A/C_i curves, yet also shows that models that do not explicitly consider carbonic anhydrase yield approximate results. The two photosynthesis models, differing in whether ATP could freely transport between RuBP and PEP regeneration processes yielded consistent results under high light, but they may diverge under low light intensities. Modeling results show selection for Rubisco of low specificity and high catalytic rate, low leakage of bundle sheath and high PEPC affinity, which may further increase C₄ efficiency.

Key words: A/C_i curves, C₄, estimation method, nonlinear curve fitting, photosynthesis parameters, V_{cmax} , electron transport, PEP carboxylation rate, carbonic anhydrase

38 **Abbreviations:** a , light absorptance of leaf; A_c , Rubisco carboxylation assimilation rate; AEE ,
39 RuBP carboxylation and PEPc carboxylation limitation assimilation; AET , RuBP
40 regeneration and PEP carboxylation limitation assimilation; A_g , gross CO_2 assimilation rate
41 per unit leaf area; A_j , RuBP regeneration assimilation rate; A_n , net CO_2 assimilation rate per
42 unit leaf area; ATE , RuBP carboxylation and PEPc regeneration limitation assimilation; ATT ,
43 RuBP regeneration and PEPc regeneration limitation assimilation; α , the fraction of O_2
44 evolution occurring in the bundle sheath; c , scaling constant for temperature dependence for
45 parameters; CaL , Lower boundary CO_2 under which assimilation is limited by RuBP
46 carboxylation and PEPc carboxylation; CaH , Higher boundary CO_2 above which
47 assimilation is limited by RuBP regeneration and PEPc regeneration; C_{bs} , bundle sheath
48 CO_2 concentration; C_i , intercellular CO_2 concentration; C_m , mesophyll CO_2 concentration;
49 ΔH_a , energy of activation for temperature dependence for parameters; ΔH_d , energy of
50 deactivation for temperature dependence for parameters; ΔS , entropy for temperature
51 dependence for parameters; ϕ_{PSII} , quantum yield; $\gamma^*(25)$, the specificity of Rubisco at $25^\circ C$;
52 g_{bs} , bundle sheath conductance for CO_2 ; g_{bso} , bundle sheath conductance for O_2 ; g_m ,
53 mesophyll conductance for CO_2 ; I , light intensity; $J_{max}(25)$, maximum rate of electron
54 transport at $25^\circ C$; $K_c(25)$, Michaelis-Menten constant of Rubisco activity for CO_2 at $25^\circ C$;
55 $K_o(25)$, Michaelis-Menten constants of Rubisco activity for O_2 ; $K_p(25)$, Michaelis-Menten
56 constants of PEP carboxylation for CO_2 ; O_{bs} , O_2 concentration in the bundle sheath cells;
57 Q_{10} for K_p , temperature sensitivity parameter for K_p ; R , the molar gas constant; R_d , daytime
58 respiration; R_{dbs} , daytime respiration in bundle sheath cells; R_{dm} , daytime respiration in
59 mesophyll cells; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP,
60 ribulose-1,5-bisphosphate; T_k , leaf absolute temperature; V_c , velocity of Rubisco

61 carboxylation; $V_{\text{cmax}}(25)$, maximal velocity of Rubisco carboxylation at 25°C; V_p , PEP
62 carboxylation; V_{pc} , PEPc reaction rate; $V_{\text{pmax}}(25)$, maximal PEP carboxylation rate at 25°C;
63 V_{pr} , PEP regeneration rate; x , the maximal ratio of total electron transport could be used for
64 PEP carboxylation.
65

1. INTRODUCTION

Key photosynthetic parameters allow for the assessment of how biochemical and biophysical components of photosynthesis affect net carbon assimilation in response to environmental changes, phenotypic/genotypic differences, and genetic modification. The changes in net assimilation (A_n) that occur along with the changes of intercellular CO_2 concentration (C_i) —or A/C_i curves— are widely used to estimate photosynthetic parameters for C_3 species. In particular, the method by Sharkey et al. (2007), based on the C_3 photosynthesis model of Farquhar et al. (1980; FvCB model), has been one of the most widely used tools since it is based exclusively on A/C_i curves, which are easy to measure in both lab and field conditions.

Fewer estimates of photosynthetic parameters have been reported for C_4 species, as there has been a lack of accessible C_4 estimation methods. Several recent studies, however, used A/C_i curves to estimate photosynthesis parameters based on the C_4 photosynthesis model of von Caemmerer (2000) (Ubierna et al., 2013; Bellasio et al., 2015). These studies use partial A/C_i curves; measuring assimilation rates for only a few CO_2 concentrations coupled with ancillary measurements of chlorophyll fluorescence and/or 2% O_2 . While these estimation methods lead to estimates of photosynthetic parameters, the additional measurements they require make estimation more cumbersome for field work or large-scale sampling. Theoretically, it is possible to estimate photosynthetic parameters by exclusively fitting A/C_i curves to a C_4 photosynthesis model. In this paper, we propose the method to estimate C_4 photosynthesis parameters using only A/C_i curves.

There are several potential problems with A/C_i –based estimation methods for C_3 plants that carry over to existing C_4 methods (Gu et al. 2010); it is therefore important to develop a C_4 estimation method with improvements to solve the general problems and drawbacks outlined below. First, the structure of the FvCB model makes it easy to be over-parameterized. Second, a general shortcoming for the estimation methods is that they require an artificial assignment of the RuBP regeneration and Rubisco carboxylation limitation states to parts of the A/C_i curves (Xu and Baldocchi, 2003; Ethier et al., 2006; Ubierna et al., 2013; Bellasio et al., 2015), which has turned out to be problematic (Type I methods) (Gu et al. 2010). These methods assume constant transition points of limitation states for different species. Furthermore, Type I methods tend to minimize separate cost functions of different limitation states instead of minimizing a joint cost function. Some recent estimation methods for C_3 species ameliorate these problems by allowing the limitation states to vary at each iterative step of minimizing the cost function (Type II methods; Dubois et al., 2007; Miao et al., 2009; Yin et al., 2009; Gu et al., 2010). However, for these type II methods, additional degrees of freedom in these “auto-identifying” strategies can lead to over-parameterization if limitation states are allowed to change freely for all data points. Gu et al. (2010) also pointed out that existing Type I and Type II methods fail to check for inadmissible fits, which happen when estimated parameters lead to an inconsistent identification of limitation states from the formerly assigned limitation states. More specifically to C_4 , the recently developed C_4 estimation methods artificially assign limitation states for A/C_i curves (Ubierna et al., 2013; Bellasio et al., 2015) and also did not check for inadmissible fits.

We developed methods to estimate photosynthetic parameters for C_4 species based solely on

fitting intensive A/C_i curves to a C_4 photosynthesis model (von Caemmerer, 2000). The intensive A/C_i curves (A/C_i curves with 6-8 more sampling points than the common A/C_i for C_3 species) are important for two reasons: First, at low C_i , the slope of A/C_i is very steep and the assimilation rate saturates quickly. Second, C_4 species have more photosynthetic parameters as the carbon concentrating mechanism adds complexity. Additionally, carbonic anhydrase catalyzes the first reaction step for C_4 photosynthesis (Jenkins et al., 1989), and it has been commonly assumed to not limit CO_2 uptake in estimation methods and C_4 models (von Caemmerer, 2000; Yin et al., 2011b). Recent studies, however, showed evidence of potential limitation by carbonic anhydrase (von Caemmerer et al., 2004; Studer et al., 2014; Boyd et al., 2015; Ubierna et al., 2017).

Therefore, first, we built estimation methods using two different fitting procedures of Sharkey et al. (2007) and Yin et al. (2011b) without considering carbonic anhydrase activity. Then, we add carbonic anhydrase limitation into the estimation method. We can also use this approach to examine how the carbonic-anhydrase-limitation assumption impacts parameter estimation, and whether the modeling of C_4 photosynthesis can be simplified by omitting it. All together, our method estimates five to six photosynthesis parameters: (1) maximum carboxylation rate allowed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (V_{cmax}), (2) rate of photosynthetic electron transport (J), (3) day respiration (R_d), (4) maximal PEP carboxylation rate (V_{pmax}), (5) mesophyll conductance (g_m), and optionally (6) the rate constant for carbonic anhydrase hydration activity (k_{CA}). These approaches yield the following improvements to eliminate common problems occurring in the previous C_3 and C_4 estimation methods: avoiding over-parameterization, maximizing joint cost function, freely

determining transition points instead of assigning in advance, and checking for inadmissible fits. Second, since both RuBP regeneration and PEP regeneration need ATP (Hatch, 1987), we also examine two different assumptions about ATP distribution between RuBP regeneration and PEP regeneration in C_4 photosynthesis models. Third, we validate the estimation methods in four independent ways, using: (i) simulation tests using A/C_i curves generated using our model with known parameters and adding random errors, (ii) out of sample test, (iii) existing in vitro measurements and (iv) Chlorophyll fluorescence measurement. Finally, we used the C_4 photosynthesis model to perform sensitivity analyses and simulation analyses for important physiological input parameters. These analyses allow us to illustrate the underlying physiological significance of these parameters to the ecology and evolution of the C_4 photosynthesis pathway.

2. MATERIALS and METHODS

2.1 C_4 Mechanism

The CO_2 concentrating mechanism of C_4 pathway increases CO_2 in the bundle sheath cells to eliminate photorespiration. Like the C_3 pathway, the diffusion of CO_2 starts from the ambient atmosphere through stomata into intercellular spaces, and then into the mesophyll cells. In the mesophyll cells, the first step is the hydration of CO_2 into HCO_3^- by carbonic anhydrase. PEPC, then, catalyze HCO_3^- and PEP into C_4 acids and the C_4 acids are transported to the bundle sheath cells. In the bundle sheath cell, C_4 acids are decarboxylated to create a high CO_2 environment for the C_3 photosynthetic cycle, and PEP is regenerated. All the modeling equations and mechanistic processes used for our estimation method are from von Caemmerer (2000), Hatch and Burnell (1990), Boyd et al. (2015) and Ubierna et

al. (2017) (Supplementary Methods).

Given the two limitation states of C_4 cycle (PEP carboxylation (V_{pc}) and PEP Regeneration (V_{pr})), and two limitation states of C_3 cycle (RuBP carboxylation (A_c) and RuBP Regeneration (A_j)) in the C_4 photosynthesis model, there are four combinations of limitation states (as Yin et al., 2011b, Fig. 1): RuBP carboxylation and PEP carboxylation limited assimilation (AEE), RuBP carboxylation and PEP regeneration limited assimilation (ATE), RuBP regeneration and PEP carboxylation limited assimilation (AET) and RuBP regeneration and PEP regeneration limited assimilation (ATT). Since the C_4 cycle operates before the C_3 cycle and provides substrates for the C_3 cycle, the determination process of A_n is as follows:

$$\text{If } (V_{pc} < V_{pr}), A_c = AEE, A_j = AET, \text{ otherwise } A_c = ATE, A_j = ATT \quad (1)$$

$$A_n = \min(A_c, A_j), \quad (2)$$

which we used for our estimation method.

2.2 Plant Material

We performed intensive A/C_i curves on nine different C_4 species to develop and examine the efficacy of our estimation tools: *Zea mays* L., *Eragrostis trichodes* (Nutt.) Alph. Wood, *Andropogon virginicus* L., *Schizachyrium scoparium* (Michx.) Nash, *Panicum virgatum* L., *Panicum amarum* Elliott, *Setaria faberi* Herrm., *Sorghastrum nutans* (L.) Nash and *Tripsacum dactyloides* (L.) L. The intensive A/C_i curves contain more sample points under more CO_2 concentrations than the default curve used for C_3 species. Here we set the CO_2 concentrations as 400, 200, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325,

350, 400, 500, 600, 700, 800, 1000, 1200, 1400 ppm under light intensity of $1500 \mu\text{molm}^{-2}\text{s}^{-1}$ (light intensity encountered by the plants in greenhouse). At each point, data was recorded when the intercellular CO_2 concentration equilibrated within 2-5 minutes. The datasets were obtained using a standard $2 \times 3 \text{ cm}^2$ leaf chamber with a red/blue LED light source of LI-6400 (LI-COR Inc., Lincoln, NE, USA). If the stomatal conductance of a species does not decrease quickly at high CO_2 , then the sample points at the high CO_2 level can be increased. Fluorescence was measured along with A/C_i curves for seven C_4 species (CO_2 concentration is similar with above). After each change of CO_2 concentration and A reached steady state, the quantum yield was measured by multiphase flash using a 2 cm^2 fluorescence chamber head (Bellasio et al., 2014). All the measurements are conducted at 25°C and VPD is controlled at 1-1.7kPa. The cuvette was covered by Fun-Tak to avoid and correct for the leakiness (Chi et al., 2013).

2.3 Estimation Protocol

We implemented the estimation methods using the non-linear curve-fitting routine in MS Excel (Supplementary Material I, II, III) and independently in R (“C4Estimation”) to get solutions that minimize the squared difference between observed and predicted assimilation rates (A). Five (or six when considering carbonic anhydrase) parameters will be estimated by fitting the A/C_i curve: V_{cmax} , J , R_d , V_{pmax} , g_m , and k_{CA} . Other input parameters for C_4 are in Table S1.

Input data sets and preliminary calculations. The input data sets are the leaf temperature during measurements, atmosphere pressure, two CO_2 bounds (CaL and CaH discussed in the

following section), and the assimilation rates (A) and the C_i s (in ppm) in the A/C_i curve.

Also, reasonable initial values of output parameters need to be given in the output section to initiate the non-linear curve fitting (Supplementary Material IV). C_i will be adjusted from the unit of ppm to the unit of Pa inside the program as suggested by Sharkey et al. (2007).

Estimating limitation states. We set upper and lower limits to the value of C_i between which the assimilation rates are freely determined by limitation states. Also, we can avoid over-parameterization by pre-assigning limitation states at the lower and upper ends of the C_i range. We assumed that under very low C_i (CaL), CO_2 is the limiting substrate; thus, V_p is limited by V_{pc} and A is given by A_c (AEE); under very high C_i (CaH) electron transport is limiting, thus, V_p is limited by V_{pr} and A is given by A_j (ATT) (Fig. 1). The points between CaL to CaH are freely determined by AEE, ATE, AET or ATT from eq. (16) and (17) to minimize the cost function. We suggest setting CaL as 10 Pa initially, then adjusting based on the preliminary results. The points of constant A at high C_i end can initially be set as being limited by ATT primarily (based on the three points, we can CaH) or use 65 Pa as the first trial. The range of freely determined points can be adjusted by users by setting appropriate CaL and CaH . In the column of “Estimate Limitation”, whether the data points are limited by AEE (represented by “1”), ATT (represented by “4”) or freely vary (represented by “0”), all the assignments of “1”, “4” and “0” are determined automatically by the given values of CaL and CaH . One can input “-1” to disregard a data pair. Users can adjust limitation states according to how many points and the range of C_i they have in their A/C_i curves.

227 We assume different processes in the C_4 photosynthesis are coordinated with each other and
 228 co-limit the assimilation rate (Sharkey et al., 2007; Yin et al., 2011b; Ubierna et al., 2013;
 229 Bellasio et al., 2015). Thus, the estimation parameters allow the limitation states to be
 230 compactly clustered with each other (Fig. 1). However, if there were only a few points under
 231 CaL , the estimation results will depend heavily on the given initial values and unbalanced
 232 results would be more likely. Fig. S1 shows an example of unbalanced estimation results by
 233 deleting some points under 10 Pa or setting a very low CaL : in the estimation results, A_n is
 234 limited by AEE at very low C_i and is mostly limited by A_j (shown by AET and ATT) in the
 235 C_3 cycle. In this case, A_c (shown by AEE and ATE) has a clear redundancy at higher C_i .
 236 Unbalanced results happened when there are not enough constraints points under CaL or
 237 above CaH . Such results explain why intensive A/C_i curves are preferred, especially more
 238 measuring points under the lower end and higher end of C_i . However, existing A/C_i data
 239 with 14 points might be used in the current estimation method if there are at least four points
 240 below CaL and three points above CaH .

Fig. 1 An introduction of how our estimation methods assign transition points between limitation states. AEE represents RuBP carboxylation, and PEP carboxylation limited assimilation rate, ATT represents RuBP regeneration and PEP regeneration limited assimilation rate. Transition states indicate assimilation could be limited by AEE, ATT, ATE (RuBP carboxylation and PEP regeneration) and AET (RuBP regeneration and PEP carboxylation). Our algorithm allows the transition states to be freely limited by the above four conditions from a lower bound (CaL , 10 Pa for instance) and a higher bound (CaH , 65 Pa for example), indicated by the dashed vertical lines in the figure.

Estimation algorithm and fitting procedures. The objective of our estimation methods is to minimize the following joint cost function (eq. 3 and 4) by varying the above five or six output parameters (V_{cmax} , J , R_d , V_{pmax} , g_m , and k_{CA}):

$$f = \sum_{i=1}^n (A_i - A_{mi})^2. \quad (3)$$

$$A_i = [If(C_i \leq CaL), AEE; If(C_i \geq CaH), ATT; If(CaL \leq C_i \leq CaH), \min(A_{ci}, A_{ji})] \quad (4)$$

n is the total number of observations, A_{ci} is determined by AEE and ATE and A_{ji} is determined by AET and ATT from eq. (1), A_{mi} is the observed net assimilation rate. In this calculation, we take Michaelis-Menten constant of Rubisco activity for CO_2 (K_c), Michaelis-Menten constant of Rubisco activity for O_2 (K_o), the specificity of Rubisco (γ^*), Michaelis-Menten constants of PEP carboxylation for CO_2 or HCO_3^- (K_p), the fraction of O_2 evolution occurring in the bundle sheath (α) and bundle sheath conductance (g_{bs}) as given (input parameters), similar to Sharkey et al. (2007). We conduct further sensitivity analyses in the following section to determine the effects of variability of these inputs parameters on the estimation results.

We used two fitting procedures in the current study: one was from Sharkey et al. (2007), which is an implicit minimization of error (Supplementary Material I, III), and the other one was based on the explicit calculations given by Yin et al. (2011b) (Supplementary Material II). For the method of Sharkey et al. (2007), "estimated" A_n was calculated using the above equations and observed A_n values. We call them "estimated", because when we calculate A_n , observed A_n is used to calculate intermediate parameters, for example, the CO_2 concentration in mesophyll cells (C_m), the CO_2 concentration in bundle sheath (C_{bs}), which we then use to calculate A_c and A_j . The objective function is to minimize the sum of square errors between "estimated" A_n and observed A_n (Simulation Error in Supplementary Material I, III). For the model without carbonic anhydrase, Yin et al. (2011b) gave explicit solutions for AEE, ATE, AEE, and ATT). "Explicit" here means the assimilation rates are totally calculated by the estimated parameters without calculating the intermediates with observed A_n . These calculations give us the real estimation error of our fitting procedure for models without carbonic anhydrase and thus provide a validation for the goodness of fit ("True Error" in Supplementary Material I-III).

Checking inadmissible fits. We made it possible to check the inadmissible fits for limitation states in our estimation method. After the estimation process finishes, the limitation states based on the estimated parameters will be calculated in the last column. If the calculated limitation states are inconsistent with the assigned ones in the estimation method, one needs to readjust the assignment of the "Estimate Limitation" (adjust CaL or CaH) and rerun the estimation method, until they are consistent with each other.

288

289 **3. RESULTS**

290 **3.1 Estimation results and assumptions**

291 Estimation methods based on assumptions with and without carbonic anhydrase yield
 292 similar results (Supplementary material V). In Supplementary material III, carbonic
 293 anhydrase indeed shows limitation to V_{pc} , which confirms its potential role as a limiting step
 294 in the C_4 cycle. However, V_{pc} calculated from CO_2 are only a little higher than V_{pc} calculated
 295 from HCO_3^- , which resulted in the similar estimation results. In addition, the estimation
 296 errors and true errors from Yin's equations are quite small (average<1), and also similar
 297 between models with and without carbonic anhydrase.

298

299 Estimation methods based on the two equations of different assumptions about electron
 300 transport between RuBP regeneration and PEP regeneration yield consistent parameter
 301 estimates and assimilation- CO_2 response curves (Fig. 2), but there were minor differences.
 302 The second assumption that ATP, resulting from electron transport, is freely allocated
 303 between PEP carboxylation-regeneration and RuBP regeneration leads to a bump at low
 304 CO_2 when estimating ATE. The two assumptions produce different ATE under low CO_2 ; but
 305 this is largely inconsequential because, under low CO_2 , assimilation is usually limited by
 306 AEE.

Fig. 2 Assimilation- CO_2 response curves (A/C_i) generated using C_4 photosynthesis of two different assumptions about electron transport. Photosynthetic parameters (V_{cmax} , J , R_d , V_{pmax} , and g_m) are the same for both assumptions. AET_{e^-} Assumption1 and ATT_{e^-} Assumption1 represent results of the assumption that no matter how much electron transport is used for PEP carboxylation/regeneration, a certain amount (xJ) is confined for this use. AET_{e^-} Assumption2 and ATT_{e^-} Assumption2 represent results of the assumption that electron transport can be freely distributed between PEP carboxylation/regeneration and RuBP regeneration. Parameters are estimated from A/C_i curve of *T. dactyloides* under the light intensity of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. AEE and ATE are the same for both assumptions.

3.2 Sensitivity analysis

The parameters K_c , K_o , γ^* , K_p , α , and g_{bs} can vary among species in nature (Cousins et al., 2010) and it is therefore important to know how sensitive our results are to variation in these parameters. We conducted a sensitivity analysis for variation in these parameters on the

estimated V_{cmax} , J , R_d , V_{pmax} , g_m and k_{CA} (Fig. 3). This analysis shows all the estimated parameters are robust under the variation of α (Fig. 3A) and showed little variation responding to the change of γ^* (Fig. 3E) and K_o (Fig. 3C); however, the estimated parameters are dependent on the other input parameters to different extents (Fig. 3B, D, F). We calculate the average percentage change of estimated parameters along with the 50 % decrease and 100 % increase of the input parameters. V_{cmax} showed some medium extent of sensitivity for K_c , K_p , and g_{bs} with the average percentage change of 23.11, 7.54 and 17.69 % respectively. J is robust in the variations of K_c , and g_{bs} (the average change is less than 2%) and with a medium 6.96 % change for K_p . k_{CA} is robust in the variations of K_c , K_p , and g_{bs} (average change less than 5%). V_{pmax} is sensitive for K_p with the average change of 27.34%, moderately sensitive to the change of g_{bs} with 4.01 % and 13.38% change and is robust for K_c . R_d is sensitive to K_c , K_p , and g_{bs} with the change of 6.73, 43.88 and 13.38%. g_m is strongly sensitive to K_c , K_p , and g_{bs} with the average percentage changes of 22.95, 107.04 and 23.19 %. This results suggest that V_{cmax} , J , V_{pmax} , and k_{CA} estimated using our method are relatively robust.

Fig. 3 Sensitivity analysis of six estimation parameters to the variation in six input parameters using the model with carbonic anhydrase. Relative changes in the estimated V_{cmax} , J , R_d , V_{pmax} , g_m and k_{CA}

in response to the relative change of six input parameters [(A) α , (B) K_p , (C) K_o , (D) K_c , (E) γ^* and (F) g_{bs}] from the initial values in Table S1. The relative change of estimated parameters refers to the ratio of estimated values at a changed input parameter to the estimated value at the initial value of that input parameter. The symbols represent the average change of the nine C_4 species and error bars represent standard error.

3.3 Physiological significance for assimilation rate of the input parameters

In addition to the sensitivity analysis, we performed a simulation analysis to illustrate the physiological importance of input parameters further, and to indicate further the importance of physiological properties in maintaining the efficiency of C_4 photosynthesis pathway. We chose the estimation parameter set of *T. dactyloides* as an example, held photosynthetic parameters constant V_{cmax} ($28 \mu\text{mol m}^{-2} \text{s}^{-1}$), J ($134 \mu\text{mol m}^{-2} \text{s}^{-1}$), R_d ($0.78 \mu\text{mol m}^{-2} \text{s}^{-1}$), g_m ($30.00 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$) and V_{pmax} ($41.91 \mu\text{mol m}^{-2} \text{s}^{-1}$), while changing the values of α , γ^* , g_{bs} , and K_p (as half or twice of the original parameters) to see their effects on the assimilation rate, C_{bs} and the O_2 concentration in bundle sheath (O_{bs}) (Fig. 4, Table 1). Using photosynthetic parameter sets of other species to perform the simulation analysis yielded similar results (data not shown). The change of α did not lead to changes in assimilation rate (Fig. 4A) and led to small changes in O_{bs} (Table 1). The decrease of γ^* to half of the current value led to a small change of C_{bs} and assimilation rate (less than $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) while doubling γ^* led to a larger, but still not significant, change (less than $1 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Fig. 4B, Table 1). Importantly, the changes of assimilation rates were less than $0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ when C_i was less than 20 Pa, which is the regular range of C_i under current ambient CO_2 . However, the change of g_{bs} significantly changed the assimilation rate

and C_{bs} (Fig. 4C, Table 1). The change of K_p significantly affected the assimilation rate and C_{bs} to a large degree under low C_i (Fig. 4D, Table 1).

Table 1 The average change of percentage of CO_2 concentration (C_{bs}) and O_2 concentration at bundle sheath (O_{bs}) compared to the reference value of $\alpha 0$, $\gamma^* 0$, $g_{bs} 0$ and K_p . Simulation results are obtained by using the original parameter set of *T. dactyloides* with $V_{cmax} = 28 \mu\text{mol m}^{-2} \text{s}^{-1}$, $J = 134 \mu\text{mol m}^{-2} \text{s}^{-1}$, $R_d = 0.78 \mu\text{mol m}^{-2} \text{s}^{-1}$, $g_m = 30.00 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ and $V_{pmax} = 41.91 \mu\text{mol m}^{-2} \text{s}^{-1}$. The values represent average change of percentage of 21 values from 0-120 Pa of intercellular CO_2 (C_i) (data show mean (standard error)).

Parameters	$\alpha = 0$	$\alpha = 2 \alpha 0$	$\gamma^* = 0.5 \gamma^* 0$	$\gamma^* = 2 \gamma^* 0$
Chang of C_{bs} (%)	-0.91(0.06)	0.97(0.06)	-2.96(0.28)	5.05(0.49)
Chang of O_{bs} (%)	-6.07(0.30)	6.01(0.30)	0.07(0.01)	-0.21(0.02)
Parameters	$g_{bs} = 0.5 g_{bs} 0$	$g_{bs} = 2 g_{bs} 0$	$K_p = 0.5 K_p 0$	$K_p = 2 K_p 0$
Chang of C_{bs} (%)	56.99(3.03)	-29.48(0.41)	43.12(10.75)	-36.57(4.07)
Chang of O_{bs} (%)	6.77(0.29)	-3.41(0.16)	0.91(0.18)	-1.18(0.14)

Fig. 4 Simulation results of assimilation rate along with different intercellular CO₂ concentration (C_i) with the known photosynthetic parameters, but with the change of (A) α , (B) γ^* , (C) g_{bs} and (D) K_p . The original data set are $V_{cmax} = 28 \mu\text{mol m}^{-2} \text{s}^{-1}$, $J = 134 \mu\text{mol m}^{-2} \text{s}^{-1}$, $R_d = 0.78 \mu\text{mol m}^{-2} \text{s}^{-1}$, $g_m = 30.00 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ and $V_{pmax} = 41.91 \mu\text{mol m}^{-2} \text{s}^{-1}$. The reference value of changing parameters at 25°C: $\alpha_0(25) = 0.15$, $\gamma^*_0(25) = 0.000244$, $g_{bs0}(25) = 0.0295$ and $K_p(25) = 8.55 \text{ Pa}$.

3.4 Validating the estimation methods

In order to test our estimation methods, we first conducted a simulation test with manipulated error terms. We use the estimated results of the nine species as known parameters (the known values in Fig. 5) to generate new datasets using the C₄

photosynthesis equations based the first assumption of electron transport and adding error terms to the assimilation rates. The error terms were randomly drawn from a normal distribution of mean zero and standard deviation of 0.1 or 0.2 in an effort to simulate the inevitable random errors in the real measurements. Estimating simulated data sets gave us an idea about how likely we can capture the real parameters of the species given unavoidable errors in measurements. The results show that robust estimation results for V_{cmax} , J , V_{pmax} , and R_d can be obtained (Fig. 5A, B, C, D). However, some estimation results of g_m and k_{CA} show some deviation from the real values (Fig. 5E, F).

To test whether our estimation method could give accurate predictions across typical prediction scenarios, (CO_2 ranging from 20 Pa to 60 Pa), we performed out of sample tests for our nine target species. To perform these tests, we removed five points of CO_2 concentrations between 20 and 60 Pa range out of the A/C_i curves and used the rest of the A/C_i curves to estimate parameters. And then we used these parameters to predict the assimilation rate under the CO_2 concentrations we took out before and calculated the estimation errors. In general, the estimation errors for all our species were small (Table 2).

Table 2 Out of sample test results. Five measured points from 20 Pa-60 Pa were taken out when we conducted the estimation process. Then the calculated assimilation rates under these five CO_2 concentrations were compared with the measured ones. The data shows estimated error between the calculated and measured assimilation rates (data show mean (standard error)).

Species	<i>A. virginicus</i>	<i>Z. mays</i>	<i>E. trichodes</i>	<i>P. virgatum</i>	<i>P. amarum</i>
Model without CA	0.069(0.036)	0.150(0.056)	0.035 (0.017)	0.193(0.063)	0.055(0.034)
Model with CA	0.066(0.043)	0.154(0.057)	0.111 (0.058)	0.195 (0.061)	0.054(0.033)

Species	<i>S. scoparium</i>	<i>S. faberi</i>	<i>S. nutans</i>	<i>T. dactyloides</i>
Model without CA	0.023(0.010)	0.114(0.055)	0.258(0.080)	0.199(0.090)
Model with CA	0.105(0.034)	0.068(0.040)	0.263(0.133)	0.200(0.090)

Fig. 5 Simulation tests for the estimated parameters ((A) V_{cmax} , (B) J , (C) V_{pmax} , (D) R_d , (E) g_m and (F) k_{CA}) using estimation methods with and without carbonic anhydrase reaction (With CA and Without CA). Datasets are generated by adding random errors for the modeling results using the known photosynthesis parameters of nine species. These known photosynthesis parameters are the true values in the x-axis and are used to compare with the newly estimation parameters. The small error refers to error term randomly chosen with mean 0 and standard deviation of 0.1 and the bigger error refers to error term with randomly chosen mean 0 and standard deviation of 0.2.

We tried to compare our estimation methods with in vitro measurements or other estimation methods using isotopic analysis, especially for *Zea*. Our estimation results for *Zea* obtained similar V_{cmax} with the in vitro estimated Rubisco activity of Pinto et al. (2014); however, the estimated value for V_{pmax} is a little lower than the in vitro PEPC activity measurement with a difference of around $10 \mu\text{mol m}^{-2} \text{s}^{-1}$. For species of the Panicum family with NAD-ME subtype, *P. virgatum* and *P. amarum* in the current study and *P. coloratum* in Pinto et al. (2014), the estimated V_{cmax} and V_{pmax} are quite consistent with the in vitro measurements. Ubierna et al. (2017) reported the g_m for *Zea* ranged from 1.69 ± 0.17 to $8.19 \pm 0.80 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ using ^{18}O and in vitro V_{pmax} . Our estimation method fitted a g_m for *Zea* of $7.34 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$, which falls into the range of their measurements. Barbour et al. (2016) reported a little lower mesophyll conductance for *Zea* using ^{18}O measurements.

3.5 Validating transition point range

We used chlorophyll fluorescence measurements from seven C_4 species to test whether the upper and lower boundary CO_2 concentrations, CaL and CaH , are reasonable (Table 3). The apparent quantum efficiency of PSII electron transport was calculated with $\Delta F/F_m' = (F_m' -$

F_s) F_m' (Genty, Briantais & Baker 1989). Fluorescence analysis (Baker et al. 2007) is a powerful tool for identifying the limitation states of C_3 species (Sharkey et al. 2007). If Chlorophyll fluorescence is increasing with increasing CO_2 , A_n is limited by Rubisco carboxylation limited; when Chlorophyll fluorescence stays constant with increasing CO_2 , A_n is limited by RuBP regeneration. For C_4 species, however, the situation is more complicated. Since V_p could be limited by V_{pr} and V_{pc} (eq. (9)). Part of the RuBP carboxylation limited condition and RuBP regeneration limited condition for the C_3 cycle will mix together, leading to a linear increase of fluorescence with increasing of CO_2 , but of a small slope (Fig. S2). Thus, we can only obtain two boundaries of CO_2 concentrations. Below the lower boundary, A and fluorescence increases with increasing C_i with a steep slope and A is RuBP carboxylation and PEP carboxylation limited (AEE); above the higher boundary, A and fluorescence is relatively constant along with the increase of C_i and A is RuBP regeneration and PEP regeneration limited (ATT). We measured fluorescence to test whether the upper and lower boundary CO_2 concentrations, CaL and CaH , are reasonable. It seems all the CaL are above 14 Pa and all the CaH are below 65 Pa (Table 3). These results suggest that 10Pa-65Pa is a reasonable range for the transitional point.

Table 3 CO_2 concentration boundaries result for assimilation-limited conditions from fluorescence measurements for seven species. Low: CO_2 concentration under which assimilation rate increases greatly with increasing CO_2 (potentially assimilation is limited by PEP carboxylation and RuBP carboxylation). High: CO_2 concentration above which assimilation rate no longer increases with increasing CO_2 (potentially assimilation is limited by PEP regeneration and RuBP regeneration). Data show the mean (standard error).

Species	<i>P. virgatum</i>	<i>P. amarum</i>	<i>S. scoparium</i>	<i>S. nutans</i>
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Low(Pa)	14.1(1.12)	18.0(1.09)	17.8(1.09)	17.6(0.28)
High(Pa)	34.1(1.78)	55.5(1.40)	53.1(1.10)	63.1(2.07)
Species	<i>T. dactyloides</i>	<i>T. flavus</i>	<i>B. mutica</i>	
Low(Pa)	13.8(0.35)	14.9(2.35)	15.8(1.13)	
High(Pa)	46.1(0.20)	41.4(1.73)	42.3(1.24)	

4. DISCUSSION

The photosynthetic parameters from the estimation method are good indicators for the biochemical and biophysical mechanisms underlying the photosynthesis processes of plants. Together with photosynthesis models, they can provide powerful information for evolutionary and ecological questions in both physiological and ecosystem response to natural environmental variation and climate change, to illustrate evolutionary trajectory of C_4 pathway, as well as in efforts to improve crop productivity (Osborne & Beerling, 2006; Osborne & Sack, 2012; Heckmann et al., 2013). Photosynthetic parameters represent different physiological traits, and comparison of these parameters within a phylogenetic background could help us to understand the further divergence of lineages and species through evolutionary time. Additionally, the response of productivity and carbon cycle of vegetation towards the future climate change depends heavily on photosynthesis parameter estimation as input parameters.

Each of the two different fitting procedures has advantages and disadvantages. Yin's method (Supplementary material II) uses the explicit calculation of assimilation rate and consequently gives lower estimation error. However, it needs a more accurate assignment of limitation states, especially at the lower end. Thus, Yin's method will be preferable if one

has additional support (e.g. fluorescence measurement) to define the limitation states; otherwise, the Yin's method may give unbalanced results (Fig. 3). However, Sharkey's method (Supplementary material I) usually can avoid unbalanced results even without ancillary measurements. Thus, it is better to use both procedures to support each other to find more accurate results. For example, one can first use Sharkey's method to get estimation results and limitation states, and then use them as initial values for Yin's method.

Our estimation methods yielded similar results when using models with and without carbonic anhydrase reaction processes. Although carbonic anhydrase activity may well be a limiting step for C_4 cycle (von Caemmerer et al., 2004; Studer et al., 2014; Boyd et al., 2015; Ubierna et al., 2017), its limitation did not greatly affect assimilation rates in this study. Including the carbonic anhydrase reaction makes the model more complex and difficult to get an explicit solution; therefore, the model without carbonic anhydrase could be used as a simplified form yielding flawed but 'nearly correct' predicted values as a part of larger models. However, carbonic anhydrase limitation of C_4 photosynthesis needs the further assessment from physiological or biochemical perspectives, and our estimation method provides another way to derive carbonic anhydrase parameters, which were comparable with *in vitro* measurements (Boyd et al., 2015). In addition, our results for models with and without carbonic anhydrase activity support the proposition of Cousins et al. (2007) that carbonic anhydrase activity may not be a limiting factor for A/C_i curves of C_4 plants.

Our results show that despite a clear difference between the assumptions of how the

products of electron transport are distributed, the results were similar and comparable with studies using different models under measurements of high light intensity. The bump in the second model happens in AET. In AET, assimilation is limited by RuBP regeneration and PEP carboxylation; therefore, PEP regeneration is not reaching V_{pr} , and the extra electron transport in PEP regeneration could be freely assigned to RuBP regeneration. This effect will weaken as PEP carboxylation increases. However, under lower photosynthetic photon flux density, assimilation rate will be limited more by electron transport, and the separate assumptions concerning electron transport may start to show divergent results.

The photosynthetic parameters from the estimation method used together with photosynthesis models can provide information and inspiration about the evolutionary and physiological importance of different aspects of the C_4 syndrome (Osborne & Sack, 2012; Heckmann et al., 2013), which can be investigated by empirical measurements. Several examples emanate from our simulation analysis: (1) α represents the fraction of O_2 evolution from photosynthesis occurring in the bundle sheath cells (eq. (4)) and any $\alpha > 0$ means that O_2 will accumulate in the bundle sheath cells, due to low g_{bs} . Both the sensitivity analysis and the simulation analysis showed the change of α did not affect the estimated parameters and assimilation rates, because the high C_{bs} created by C_4 carbon concentrating mechanism overcame any increase of O_{bs} and did not lead to high photorespiration. Thus, the compartmentation of O_2 evolution may not have played an important role in the evolution of C_4 photosynthesis. (2) A lower Rubisco specificity factor (γ^* ; eq. (11)) means lower specificity for O_2 , higher specificity for CO_2 , and lower photorespiration. In C_3 species, selection for Rubisco with lower specificity to O_2 and high specificity of CO_2 can

increase the carbon gain. However, there is a trade-off between the specificity of Rubisco for CO₂ and its catalytic rate (Savir et al., 2010; Studer et al., 2014). Based on this trade-off, we can hypothesize that since C₄ elevates CO₂ around Rubisco relative to the O₂ concentration, maintaining low specificity might be optimal, in order to get high catalytic rate of the enzyme to reach higher assimilation rate as shown by the empirical measurements of Sage (2002) and Savir et al. (2010). Our simulation analysis showed the increase of specificity for CO₂ (decrease of γ^*) did not increase the assimilation rate much, which indicates the selection upon Rubisco specificity in C₄ plants should be relaxed. (3) g_{bs} represents CO₂ leakage from bundle sheath to the mesophyll cell, and changes in g_{bs} significantly change the assimilation rate and C_{bs}. Therefore, avoiding CO₂ leakage was of great importance for the evolution and efficiency of C₄ photosynthesis pathway (Brown and Byrd, 1993; Ubierna et al., 2013; Kromdijk et al., 2014).

Although we have shown that parameter estimation can be achieved solely with A/C_i curves, it is easy to combine our methods with ancillary measurements to yield more accurate estimation results by defining the parameters as estimated or known or add additional constraints (Supplementary Material IV). Yin et al. (2011b) proposed a method to obtain R_d from the fluorescence-light curve, since the method used for C₃ species, the Laisk method, is inappropriate (Yin et al., 2011a). Additional measurement of dark respiration could be an approximation for R_d or could help to provide a constraint for estimating R_d in our estimating method. Ubierna et al. (2017) discussed the estimation method of g_m using instantaneous carbon isotope discrimination. With external measurement results, one can change estimated parameters (such as R_d, g_m and J) as input parameters, instead of output

parameters, in this curve fitting method (Supplementary material IV). Additional methods, such as in vitro measurements (Boyd et al., 2015; Pedomo et al., 2015) and membrane inlet mass spectrometry (Cousins et al., 2010) of V_{cmax} , V_{pmax} , and carbonic anhydrase activity can also provide potential parameter values. Furthermore, if some output parameters are determined in the external measurements, one can also relax the input parameters (such as g_{bs}) and make them estimated parameters (Supplementary material IV).

5. Conclusion

We have developed new, accessible estimation tools for extracting C_4 photosynthesis parameters from intensive A/C_i curves. Our estimation method is based on an established estimation protocol for C_3 plants and makes several improvements upon C_4 photosynthesis models. External measurements for specific parameters will increase the reliability of estimation methods and are summarized independently. We developed estimation methods with and without carbonic anhydrase activity. The comparison of these two methods allows for an estimation of carbonic anhydrase activity, and further shows that the method that did not consider carbonic anhydrase activity was a sufficient simplification for C_4 photosynthesis. We tested two assumptions related to whether the electron transport is freely distributed between RuBP regeneration and PEP regeneration or certain proportions are confined to the two mechanisms. They show similar results under high light, but they may diverge under low light intensities. Simulation test, out of sample test, fluorescence analysis, and sensitivity analysis confirmed that our methods gave robust estimation especially for V_{cmax} , J , and V_{pmax} .

Author contributions

HZ, EA and BH conceived the ideas, designed methodology, analyzed the data and led the writing of the manuscript; HZ collected the data; HZ and BH coordinate the study. All the authors contributed to the critical review of the manuscript and approved its final version.

ACKNOWLEDGMENT

We thank Dr. Jesse Nippert, Kansas State University, for providing the fluorometer chamber.

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