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Using the rapid A-C_i response (RACiR) in the Li-Cor 6400 to measure developmental gradients of photosynthetic capacity in poplar

Erica H. Lawrence[1†] | Joseph R. Stinziano[2†] | David T. Hanson[2]

[1] Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania

[2] The Department of Biology, The University of New Mexico, Albuquerque, New Mexico

Correspondence
Erica H. Lawrence, Department of Biology, University of Pennsylvania, 433 S. University Ave, Philadelphia, PA 19104.
Email: lawrenceericah@gmail.com

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Abstract

The rapid A-C_i response (RACiR) technique alleviates limitations of measuring photosynthetic capacity by reducing the time needed to determine the maximum carboxylation rate (V_{cmax}) and electron transport rate (J_{max}) in leaves. Photosynthetic capacity and its relationships with leaf development are important for understanding ecological and agricultural productivity; however, our current understanding is incomplete. Here, we show that RACiR can be used in previous generation gas exchange systems (i.e., the LI-6400) and apply this method to rapidly investigate developmental gradients of photosynthetic capacity in poplar. We compared RACiR-determined V_{cmax} and J_{max} as well as respiration and stomatal conductance (g_s) across four stages of leaf expansion in *Populus deltoides* and the poplar hybrid 717-1B4 (*Populus tremula* × *Populus alba*). These physiological data were paired with leaf traits including nitrogen concentration, chlorophyll concentrations, and specific leaf area. Several traits displayed developmental trends that differed between the poplar species, demonstrating the utility of RACiR approaches to rapidly generate accurate measures of photosynthetic capacity. By using both new and old machines, we have shown how more investigators will be able to incorporate measurements of important photosynthetic traits in future studies and further our understanding of relationships between development and leaf-level physiology.

KEYWORDS

leaf development, photosynthesis, photosynthetic capacity, poplar, rapid A-C_i response (RACiR)

1 | INTRODUCTION

Photosynthesis significantly impacts plant traits that are central to agricultural and natural ecosystem productivity (e.g., biomass; Ainsworth & Long, 2005; Long, Zhu, Naidu, & Ort, 2006; McAllister, Knapp, & Maragni, 1998; Quick et al., 1991). It is generally accepted that photosynthesis increases during leaf development until full expansion and later declines through senescence. This relationship is confounded by environmental factors such as light, temperature, and resource availability (Šesták, 1985). Photosynthetic traits regarding leaf development have been extensively studied using instantaneous photosynthetic rates (Constable & Rawson, 1980; Escudero & Mediavilla, 2003; Intrieri, Poni, Silvestroni, & Filippetti, 1992; Morecroft, Stokes, & Morison, 2003; Peat, 1970; Slatyer, 1970; Wilson & Ludlow, 1970). However, far fewer studies have investigated this relationship using photosynthetic capacity (Adam et al., 2000; Morgan, Bernacchi, Ort, & Long, 2004; Osborne et al., 1998; Xu & Baldocchi, 2003; Zhang, Hu, & Li, 2008), determined using the response of net CO_2 assimilation (A_{net}) to intercellular CO_2 concentration (A-C_i response curves). A-C_i responses and the parameters generated from them are much less dependent on immediate environmental conditions and describe leaf biochemistry (Farquhar, Von Caemmerer, & Berry, 1980).

Previously, the time required to determine photosynthetic capacity (i.e., maximum rate of Rubisco carboxylation, V_{cmax}, and maximum

[†] These authors contributed equally to the present work.

rate of electron transport, J_{max}) made it difficult to collect enough measurements for leaf developmental studies. However, Stinziano et al. (2017) recently developed a rapid A-C_i response (RACiR) method that continuously changes [CO_2] across the leaf to yield A-C_i responses in a fraction of the time needed for steady-state A-C_i approaches. Although Stinziano et al. (2017) used a new gas exchange system, it is unclear whether other gas exchange systems can be programmed to run sufficiently dynamic CO_2 ramps (i.e., changing [CO_2] at a constant rate), and if so, whether RACiR gives meaningful data on older machines. Here, we assess the validity of RACiR in the Li-6400 Portable Photosynthesis System (Li-Cor, Inc. Lincoln, NE, USA) and use the technique to investigate relationships between leaf developmental traits and photosynthetic capacity in two poplar species.

Leaf morphological and biochemical traits including specific leaf area (SLA), nitrogen content, and chlorophyll content are closely related to photosynthetic capacity (Croft et al., 2017; Evans & Poorter, 2001; Hanson, 1917; Reich, Walters, & Ellsworth, 1997). SLA, the projected leaf area per unit leaf dry mass, describes the thickness or density of a leaf. Leaves with low SLA often have additional or longer palisade cells containing higher numbers of chloroplasts and photosynthetic enzymes and thus increased photosynthetic capacity per unit leaf area (Hanson, 1917). Due to the cell arrangement, leaves with low SLA absorb fewer photons per unit leaf mass than those with higher SLA, resulting in lower photosynthetic capacity per mass (Evans & Poorter, 2001). This phenomenon is considered universal of plants and was observed in 111 species across 6 different biomes (Reich et al., 1997). Additionally, Reich et al. (1997) observed a consistent positive relationship between leaf nitrogen and photosynthesis across this diverse group of plants. This relationship exists due to the large proportion of leaf nitrogen devoted to the photosynthetic apparatus (Chapin, Matson, & Vitousek, 2011; Niinemets & Tenhunen, 1997). Croft et al. (2017) found leaf chlorophyll is closely related to both V_{cmax} and J_{max} at 25°C in multiple tree species and could be used as a proxy for photosynthetic capacity. Using these relationships between leaf nitrogen, chlorophyll, and photosynthetic capacity, we can further evaluate the validity of RACiR in our study.

Here, we validate RACiR in the LI-6400 and use the technique to elucidate developmental trends in photosynthetic capacity in two poplar species. Further, we connect photosynthetic capacity measurements with leaf biochemistry and morphology to better understand the mechanisms behind the developmental changes and assess the ability of RACiR to capture classical relationships between leaf biochemistry and photosynthetic capacity. Using modifications to the methods described in Stinziano et al. (2017), we show that RACiR can be used in older portable photosynthesis instruments and that RACiR provides the ability to rapidly increase our understanding of plant and ecosystem productivity by allowing us to quickly collect data on photosynthetic capacity.

2 | MATERIALS AND METHODS

2.1 | Plant material

Poplar (*Populus deltoides* Barr. S7c8 East Texas day neutral clone) were grown from cuttings in a greenhouse at the University of New Mexico (35.0843°N, 106.6198°W, 1587 m a.s.l.) at 18.3 to 21.1/15.6 to 21.1°C day/night temperature with daily irradiances of 600 to 1,200 μmol m^{-2} s^{-1}. Initially, cuttings were grown for 2 months in Metro-Mix 300 potting soil (Sun Gro Horticulture, Seba Beach, AB, Canada). Cuttings were then transplanted to 3.8 L pots with 2:1 mixture of vermiculite and perlite (Therm-O-Rock West Inc., Chandler, AZ, USA) with <5% Agrosoke crystals (Agrosoke International, Arlington, TX, USA). Seedlings were then transplanted into 12.5-L pots with Metro-Mix 300 potting soil after 6 months and subsequently into 23-L pots, then 28-L pots after 6 months of growth in each pot size. Plants were fertilized twice weekly with Peters 20-20-20 (Scotts Miracle-Gro, Marysville, OH, USA) and once weekly with chelated liquid iron (Ferti-Lome, Bonham, TX, USA). Plants had been transferred to new soil and pots 16 weeks prior to this experiment and had initiated approximately 120 leaves.

Poplar 717-1B4 (*Populus tremula* × *Populus alba*) were obtained by in vitro propagation and hardening of the poplar clone on propagation media as described in Meilan and Ma (2006). Plants were then transplanted to Fafard-2 growing mix (Sangro Horticulture, Massachusetts, USA) in 0.64-L pots in the greenhouse at the University of Pennsylvania (39.9493°N, 75.1995°W, 22.38 m a.s.l.) and kept in plastic bags for increased humidity for 1 week. Plants were then transferred to 4.2-L pots with Fafard-52 growing mix 3 weeks later and fertilized with osmocote classic 14-14-14 (The Scotts Company, Marysville, OH, USA). Plants were additionally fertilized once a week with Peters 20-10-20 (ICL Fertilizers, Dublin, OH, USA). Greenhouse conditions consisted of a 16-hr photoperiod with temperatures between 22 and 27°C. Light levels were based on natural light and supplemented with 400-W metal halide lamps (P.L. Light Systems, Ontario, Canada) with daily irradiances of 300 to 1,500 μmol m^{-2} s^{-1}. All settings controlled by Priva (Ontario, Canada) and Microgrow (Temecula, Canada) greenhouse systems. Plants had been transferred to soil 16 weeks prior to this experiment and had initiated approximately 40 leaves.

2.2 | Principles of RACiR

The RACiR is achieved by continually changing the [CO_2] entering the leaf chamber of a gas exchange instrument instead of using step changes in [CO_2]. Because each infrared gas analyser (IRGA) has a unique calibration function, offsets can accumulate between pairs of IRGAs in a machine. Offsets are usually accounted for by matching IRGA outputs to read the same value at a specific [CO_2]. Matching requires that the system be in a stable state (i.e., incoming [CO_2] is constant); thus, matching is precluded when [CO_2] is continuously changing. To account for offsets in IRGAs under continuously changing [CO_2], we can measure cumulative offsets between IRGAs by running an empty cuvette response and account for time lags within the system and match offsets. A matching function can then be derived using a first- to fifth-order polynomial (Stinziano et al., 2017; see Stinziano, 2018 for an R-based implementation)—we emphasize this point: The correction factor is a *function* not a *constant*. IRGAs must be matched prior to running RACiR, and match values must be similar between empty cuvette and sample runs—any large deviation in match values necessitates a new empty cuvette response. In practice, a new empty cuvette response is needed when there are large changes in

water concentrations and possibly temperature. In theory, it should be possible to use RACiR with any programmable gas exchange system—the challenge lies in programming a continuous ramp in systems with step changes (e.g., the Li-6400), and this can affect the potential speed and repeatability of the measurements.

RACiR must be validated in each species for which it is used, which requires initial screening where RACiR is run across a broad range of $[CO_2]$ (e.g., reference concentrations ranging from 300 to over 1,000 μmol mol^{-1}). Initial screening is crucial so that RACiR data can be obtained in the region of co-limitation by $[CO_2]$ and RuBP-regeneration, the key region for obtaining accurate V_{cmax} and J estimates from Gu-type curve fitting (Gu, Pallardy, Tu, Law, & Wullschleger, 2010). Although Stinziano et al. (2017) used 300 to 800 μmol mol^{-1}, other species may require a wider range in $[CO_2]$ or a different start and end point (400 to 1,200 μmol mol^{-1} for example)—this may be particularly relevant for species with low stomatal and/or mesophyll conductance.

2.3 | Validation of RACiR in the LI-6400

Using a Licor 6400XT equipped with the fluorescence head (Licor Biosciences, Lincoln, NE, USA), a program was written to obtain nearly continuous changes in $[CO_2]$, changing the reference $[CO_2]$ by 10 μmol mol^{-1} every 10 s (60 μmol mol^{-1} min^{-1}; our preliminary tests suggested that this was the best compromise between speed and data density, Figure S1). Continuous changes in $[CO_2]$ cause an apparent A_{net} value, so the program was run with an empty chamber to correct leaf sample measurements as per Stinziano et al. (2017). The RACiR program was run from 300 to 800 μmol mol^{-1} logging every 10 s (collecting 50 data points over 8.3 min, see supplementary file "LI-6400 RACiR Program") and compared with a steady-state approach, measuring A_{net} at reference $[CO_2]$ values of 400, 300, 200, 150, 100, 50, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,500, and 1,800 μmol mol^{-1}. Measurements were performed using the poplar hybrid 717-1B4 after leaf stabilization under chamber conditions with $[CO_2]$ set to that of the first measurement (i.e., 300 or 400 μmol for RACiR and steady-state approaches, respectively) with a flow rate of 600 μmol air s^{-1} at 25°C under saturating irradiance (1800 μmol m^{-2} s^{-1}).

2.4 | Gas exchange measurements

We measured photosynthetic capacity using RACiRs from a $[CO_2]$ of 300 to 800 μmol mol^{-1} at 60 μmol mol^{-1} min^{-1} CO_2 logging every 10 s and 25°C under previously determined saturating irradiance (1,000 μmol m^{-2} s^{-1} for *P. deltoides*, 1,800 μmol m^{-2} s^{-1} for 717-1B4). Prior to measurements, leaves were given time to acclimate to chamber conditions under their respective saturating irradiances and $[CO_2]$ of 300 μmol mol^{-1}. Photosynthetic capacity was measured across four developmental stages: old fully expanded leaves (OF) (~20–25 leaves down from the youngest leaf measured and with minimal signs of senescence), new fully expanded leaves (NF) (~10 leaves down from the youngest leaf measured), youngest leaves that would fill the leaf cuvette (Y, ~4 leaves from the leaf producing meristem and 15–25% of NF leaf area), and midway between the smallest leaf

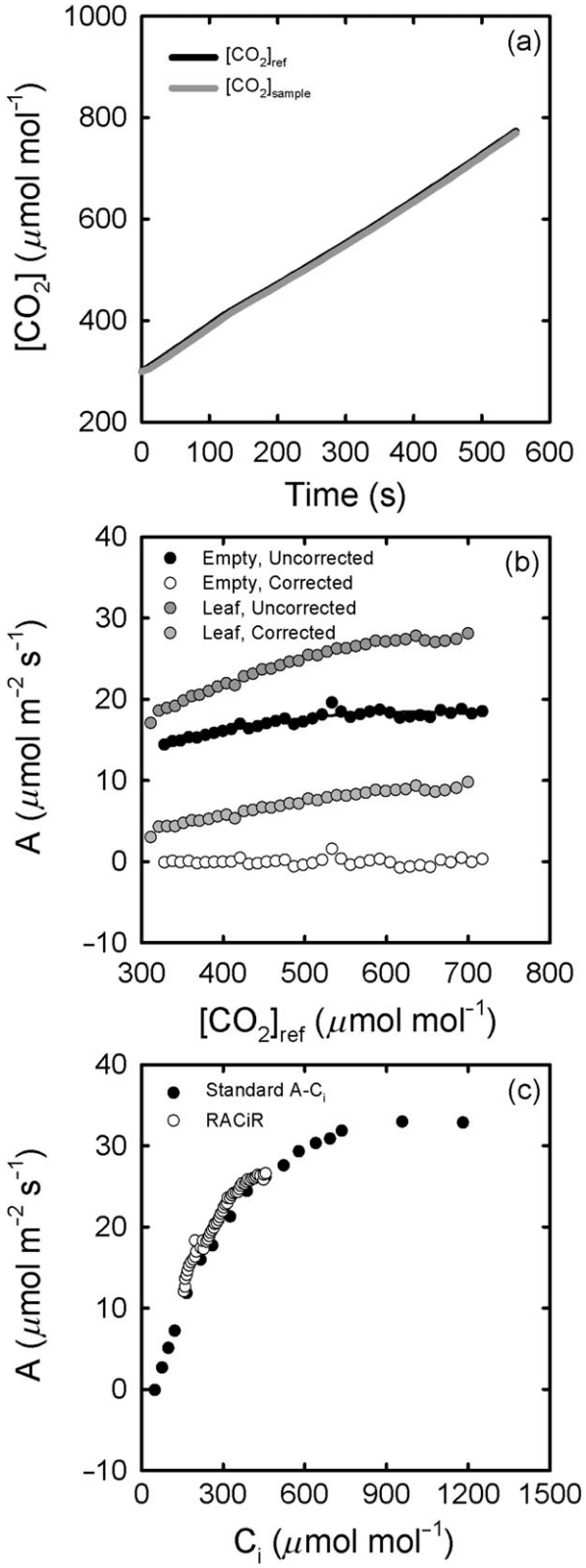

FIGURE 1 Dynamically changing the reference CO_2 concentration ($[CO_2]_{ref}$) causes a lag between $[CO_2]_{ref}$ and the sample CO_2 concentration ($[CO_2]_{sample}$) (a), leading to an apparent CO_2 assimilation rate (A) that can be corrected in an empty chamber response (b), and can be used for a rapid A-C_i response (RACiR) that yields similar CO_2 response data to the traditional steady-state approach (standard A-C_i) (c)

size and new fully expanded leaves (EX; ~3–4 leaves down from Y and 50–70% of NF leaf area). For the fully expanded leaves, we used RACiR on three locations from most proximal to most distal to the leaf petiole (P, proximal; M, middle; D, distal). For the mid-way developed leaves, we measured two locations P and D in *P. deltoides*, or three P, M, and D, in 717-1B4.

Leaf respiration (R) and minimum g_s (g_{smin}) was measured at each location by incubating the leaf spot in complete darkness for 30 min at 25°C, reference [CO_2] of 400 μmol mol^{-1}, and a flow rate of 600 μmol air s^{-1} prior to measurement.

2.5 | Leaf nitrogen and chlorophyll

Leaf tissue was sampled after gas exchange; one subsample for each leaf was dried at 60°C until constant mass to determine SLA. Dried tissues were ground using a mortar and pestle. Leaf nitrogen was measured in the dried samples using an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies INC, Valencia, CA, USA) for both species.

A second subsample was used to for chlorophyll quantification. Chlorophyll was extracted using 100% methanol and quantified using a spectrophotometer according to the equations found in Wellburn (1994) for *P. deltoides* samples or with 80% acetone using equations in Porra, Thompson, and Kriedemann (1989) for 717-IB4 samples.

2.6 | Curve fitting

The {plantecophys} package in R (Duursma, 2015) was used for fitting A-C_i curves to determine apparent V_{cmax} and apparent J_{max}. We used the bilinear function from the package as it is similar to the A-C_i curve fitting approach and philosophy of Gu et al. (2010), in that co-limited regions of the A-C_i curve provide more statistical power for curve-fitting and that the model of Farquhar et al. (1980) needs to be fit as a change point model.

2.7 | Data analysis

All statistical analyses were performed in R v. 3.3.3 (R Core Development Team, 2017). The steady-state A-C_i data were compared with the RACiR data using a paired *t* test. Intraleaf variation was compared using repeated measures linear models in R using the {nlme} (Pinheiro et al., 2018) and {multcomp} (Hothorn, Bretz, & Westfall, 2008) packages for each OF, NF, and EX leaves. Developmental stages were compared using repeated measures linear models on the grand mean values of the leaf spots for each developmental stage.

3 | RESULTS

3.1 | RACiR in the LI-6400

As with RACiR in the LI-6800, rapid changes in [CO_2] create differences in sample and reference [CO_2] resulting in apparent CO_2 assimilation values in an empty chamber (Figures 1a,b). Following the methods of Stinziano et al. (2017), this is corrected for in the LI-6400 using a regression-based approach allowing RACiR data to match steady-state A-C_i response data accurately (Figure 1c). Corrected RACiR and steady-state A-C_i curves measured with the LI-6400 overlay with one another well (Figure 1), and when analysed, apparent V_{cmax} and apparent J_{max} values between the two methods have nonsignificant mean differences of 0.6 and 8.2 μmol m^{-2} s^{-1}, respectively (Table 1). Furthermore, RACiR has much reduced variance in g_s over the measurement period than the steady-state A-C_i curves (Table 1; Figure 2).

3.2 | Leaf physiology across a developmental gradient

P. deltoides and the 717-1B4 hybrid showed developmentally dependent variation in leaf physiology (Table S1). Both species showed variation in R and g_{smin} across leaf expansion (Table S1). R decreased by 69% and 74% in *P. deltoides* and the poplar hybrid, respectively, and g_{smin} decreased by 65% and 64% between very young and new fully expanded leaves, respectively (Figure 3). No significant differences in these traits were observed between new and old fully expanded leaves. Further, the poplar hybrid showed significant changes in apparent V_{cmax} (but not apparent J_{max}) on both area and mass bases with a 52% difference on an area basis between very young and new fully

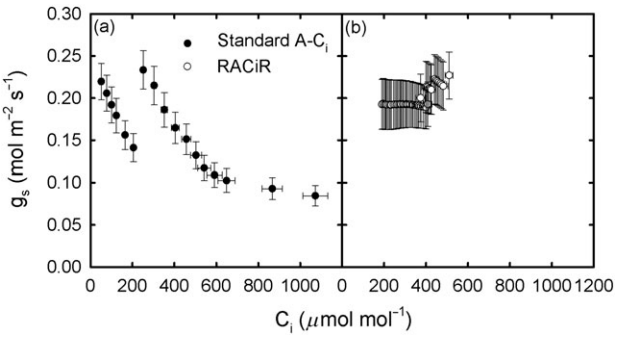

FIGURE 2 Stomatal conductance (g_s) shows greater variation across the range of intercellular CO_2 concentration (C_i) used for (a) the standard A-C_i approach compared to (b) the rapid A-C_i response (RACiR). Data presented as means ± s.e.m. N = 18

TABLE 1 Validation statistics

Curve type	V_{cmax} (μmol m^{-2} s^{-1})	J_{max} (μmol m^{-2} s^{-1})	g_s variance (mol m^{-2} s^{-1})
Standard A-C_i	73.5 ± 2.3	128.7 ± 4.2	0.00317 ± 0.00055
RACiR	74.1 ± 6.3	136.9 ± 14.4	0.00020 ± 0.00007
t	0.094	0.627	5.974
d.f.	17	17	17
P	0.926	0.539	<0.001

Note. V_{cmax}: maximum rate of Rubisco carboxylation capacity; J_{max}: maximum rate of electron transport; g_s: stomatal conductance.

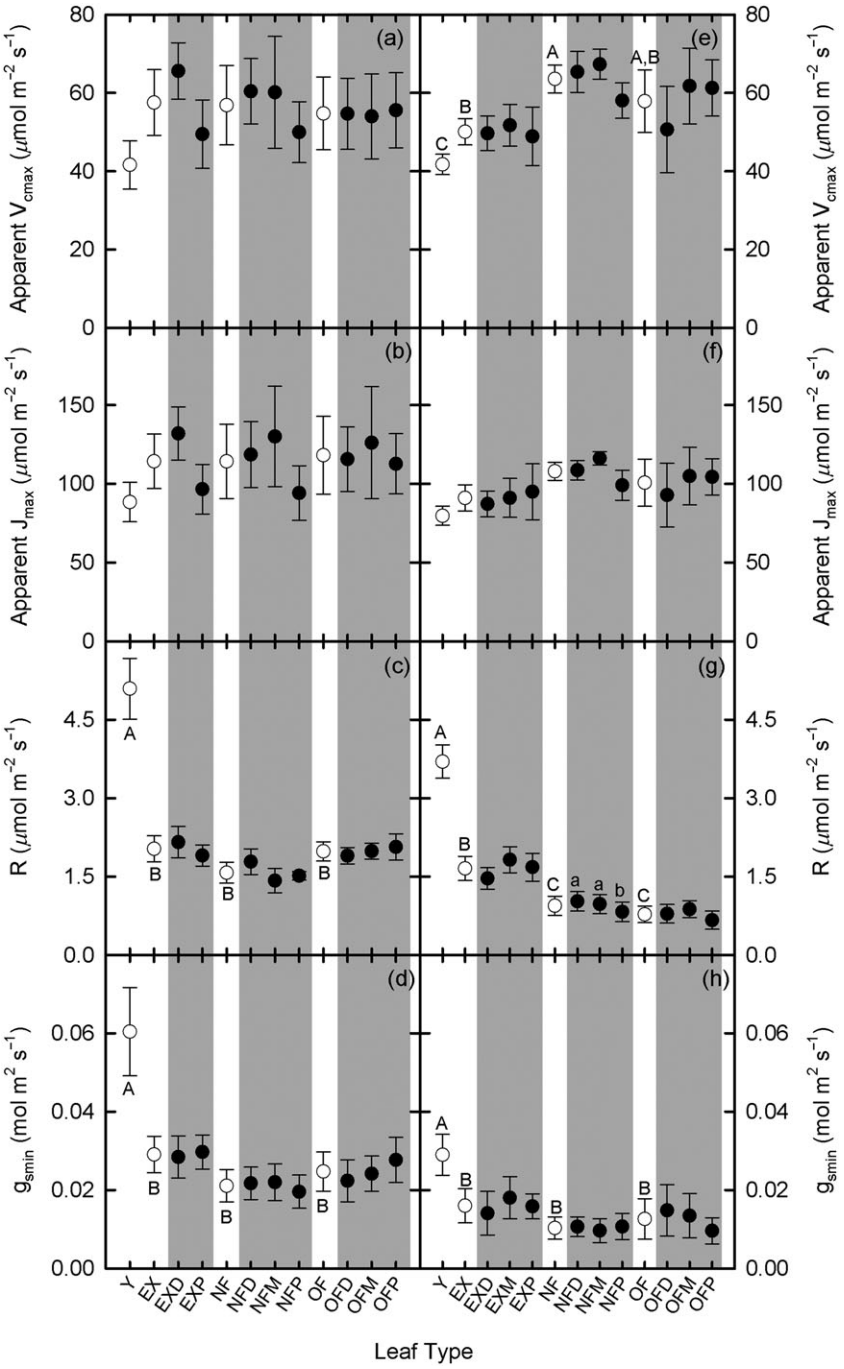

FIGURE 3 Gas exchange properties within leaves and across developmental stages for *Populus deltoides* (a, b, c, d) and poplar 717-1B4 (*Populus tremula* × *Populus alba*; e, f, g, h). Y: young leaf; EX: expanding leaf; NF: new fully expanded leaf; OF: old fully expanded leaf; D: distal position to petiole; M: middle position to petiole; P: proximal position to petiole. Data presented as means ± s.e.m. Different capital letters indicate means of the developmental stage are significantly different according to Tukey's HSD ($P < 0.05$), whereas different lowercase letters indicate means of the leaf position are significantly different according to Tukey's HSD ($P < 0.05$)

expanded leaves, whereas *P. deltoides* did not show any significant differences in apparent V_{cmax} or apparent J_{max} across the developmental gradient (Figures 3).

3.3 | Foliar biochemistry and morphology across a developmental gradient

Developmental variation in leaf biochemical and morphological traits was also present in both poplar species. Both species showed developmentally dependent variation in chlorophyll content and nitrogen concentration (%N) such that chlorophyll *a* and *b* and %N increased by 77, 58, and 120%, respectively, as the leaves expanded in the poplar hybrid, with chlorophyll *a* and *b* both continuing to increase by 24% between new and old fully expanded leaves, whereas only chlorophyll *b* increased (266%) and nitrogen decreased (45%) as leaves expanded in *P. deltoides* (Table S1; Figures 4 and 5). Carbon concentration (%C) decreased with development in *P. deltoides* by 16% but not in the hybrid, leading to an increase in carbon to nitrogen ratio (C:N) in *P. deltoides* of 49% and a decrease of 55% in the hybrid with development (Figures 4 and 5). *P. deltoides* showed decreasing SLA with development (by 16%), whereas SLA remained stable in the hybrid (Table S1; Figures 4 and 5). Nitrogen concentrations were correlated with SLA in *P. deltoides* but not the hybrid (Figure S2).

3.4 | Within-leaf physiological and biochemical variation

Neither species of poplar showed consistent variation in foliar traits among distal, middle, and proximal regions of leaves across different

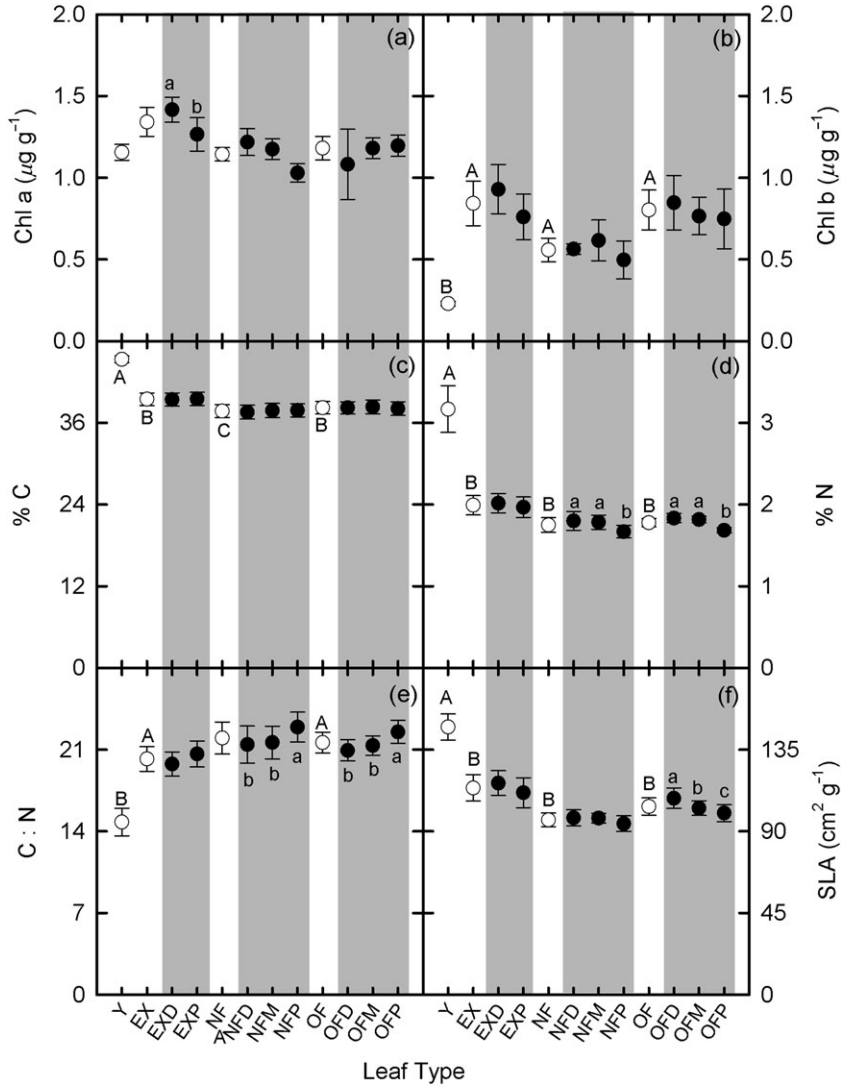

FIGURE 4 Leaf chlorophyll concentration (a, chlorophyll *a*; b, chlorophyll *b*), carbon/nitrogen composition (c, carbon concentration, %C; d, nitrogen concentration, %N; e, carbon to nitrogen ratio, C:N) and specific leaf area (f, SLA) across leaf developmental stages (white points) and within-leaf positions (black points) in *Populus deltoides*. Lettering and symbols are the same as Figure 3

leaf expansion stages (Table S2). In the hybrid, R was slightly lower in the proximal region of new fully expanded leaves (Figure 3), but there were no other significant differences within leaves (Table S2; Figures 4 and 5). In *P. deltoides*, both V_{cmax} and J_{max} by mass were ~50% lower in the proximal region compared to the distal region of expanding leaves (Table S2) corresponding with the proximal region having a lower chlorophyll *a* concentration (Figure 4). The proximal region of new and old fully expanded leaves showed lower %N leading to an increased C:N relative to the middle and distal regions, whereas only the old fully expanded leaves had a gradient of increasing SLA from proximal to distal regions of the leaf (Table S2; Figure 4).

3.5 | Correlations between photosynthetic capacity and leaf traits

The poplar hybrid, but not *P. deltoides*, showed a significant positive correlation between photosynthetic capacity (both apparent V_{cmax} and apparent J_{max}) and foliar nitrogen concentration on both area and mass bases when all developmental stages were pooled. On a per mass basis, 71% of the variation in V_{cmax} and 58% of the variation in J_{max} can be attributed to changes in nitrogen content in the hybrid (Figure 6). In relation to chlorophyll, there was a significant correlation between photosynthetic capacity on a mass basis and total chlorophyll, but not on an area basis in the poplar hybrid, whereas *P. deltoides* did not show a significant correlation between photosynthetic capacity and total chlorophyll (Figure S3). Meanwhile, *P. deltoides*, but not the hybrid, showed a significant negative correlation between photosynthetic capacity and SLA. SLA was responsible for 30% of the variation in V_{cmax} and 22% of the variation in J_{max} in *P. deltoides* (Figure 6). In regard to photosynthetic nitrogen-use-efficiency (PNUE), there were no significant differences between leaf developmental stages in *P. deltoides* for PNUE calculated on either a V_{cmax}- or J_{max}-basis, whereas the poplar hybrid showed a decline in both measures of PNUE from the youngest leaf to mid-expanding leaves (Table S1; Figure 7).

4 | DISCUSSION

We validated RACiR in the LI-6400 through comparison with steady-state A-C_i curves and support of physiological and biochemical relationships. As shown in Stinziano et al. (2017) with the LI-6800 and here with the LI-6400, a lack of significant differences between steady-state A-C_i and RACiR techniques implies that leaf carbon metabolism reaches near-steady-state conditions as fast as changes

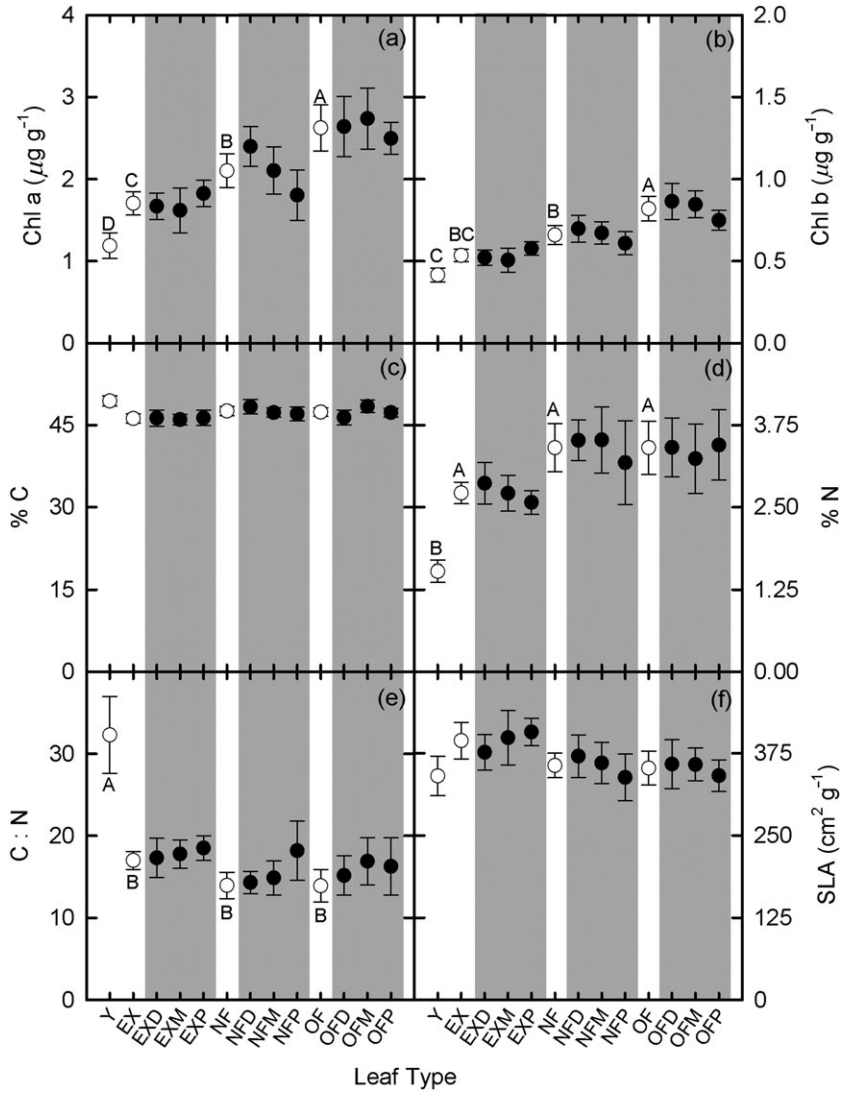

FIGURE 5 Leaf chlorophyll concentration (a, chlorophyll *a*; b, chlorophyll *b*), carbon/nitrogen composition (c, carbon concentration, %C; d, nitrogen concentration, %N; e, carbon to nitrogen ratio, C:N) and specific leaf area (f, SLA) across leaf developmental stages (white points) and within-leaf positions (black points) in *Populus tremula* × *Populus alba*. Lettering and symbols are the same as Figure 3

in chamber [CO_2] allowing for accurate high-speed measurements of the A-C_i response.

Photosynthetic capacity determined using RACiR was supported by its relationships with morphology and biochemistry. Apparent V_{cmax} increased as the leaf expanded in the poplar 717-1B4 hybrid, corresponding with an increase in chlorophyll and nitrogen content. Although *P. deltoides* showed no change in apparent V_{cmax} with development despite decreasing nitrogen content, there was a decrease in SLA (resulting in increased photosynthetic machinery per area) and contrasting increase in chlorophyll *b* with leaf expansion. This relationship between SLA and photosynthetic capacity, along with increases in chlorophyll, is likely responsible for the consistencies in V_{cmax} across leaf expansion in *P. deltoides* despite changes in leaf nitrogen (Figure 6). Although this data set is limited to just two species, we found that even closely related species can have different developmental patterns in leaf physiology and biochemistry, although we cannot rule out differences in growth environment as driving these differences. Furthermore, in both poplar species RACiR-determined V_{cmax} and J_{max} coupled well with one another (Figure 8). This relationship has been previously observed across many environments and species and is considered a fundamental feature of photosynthetic trait relationships, further supporting the accuracy of RACiR (Walker et al., 2014).

4.1 | Caveats for RACiR in the LI-6400

The LI-6400 system design, with a linear flow path between analysers, causes larger offsets to accumulate during RACiR compared with the LI-6800, which splits the air stream before the IRGAs (Stinziano et al., 2017). We reiterate our point above and from Stinziano et al. (2017): RACiR offsets must be corrected for using a *function* rather than a *constant*. The function itself will be related to IRGA calibration differences within an instrument, the CO_2 range used, the directionality of RACiR, and the rate of change. Our program differs from the LI-6800 in that the step changes in [CO_2] and time per step ensure that the LI-6400 never quite reaches the CO_2 set point, causing a predictable and continuous change in [CO_2]. This limited the effective rate of RACiR to 60 μmol mol^{-1} min^{-1} in the LI-6400. It may be possible to increase the rate with further tweaking of the program times and step changes, although if the time steps are too short, or if the step change

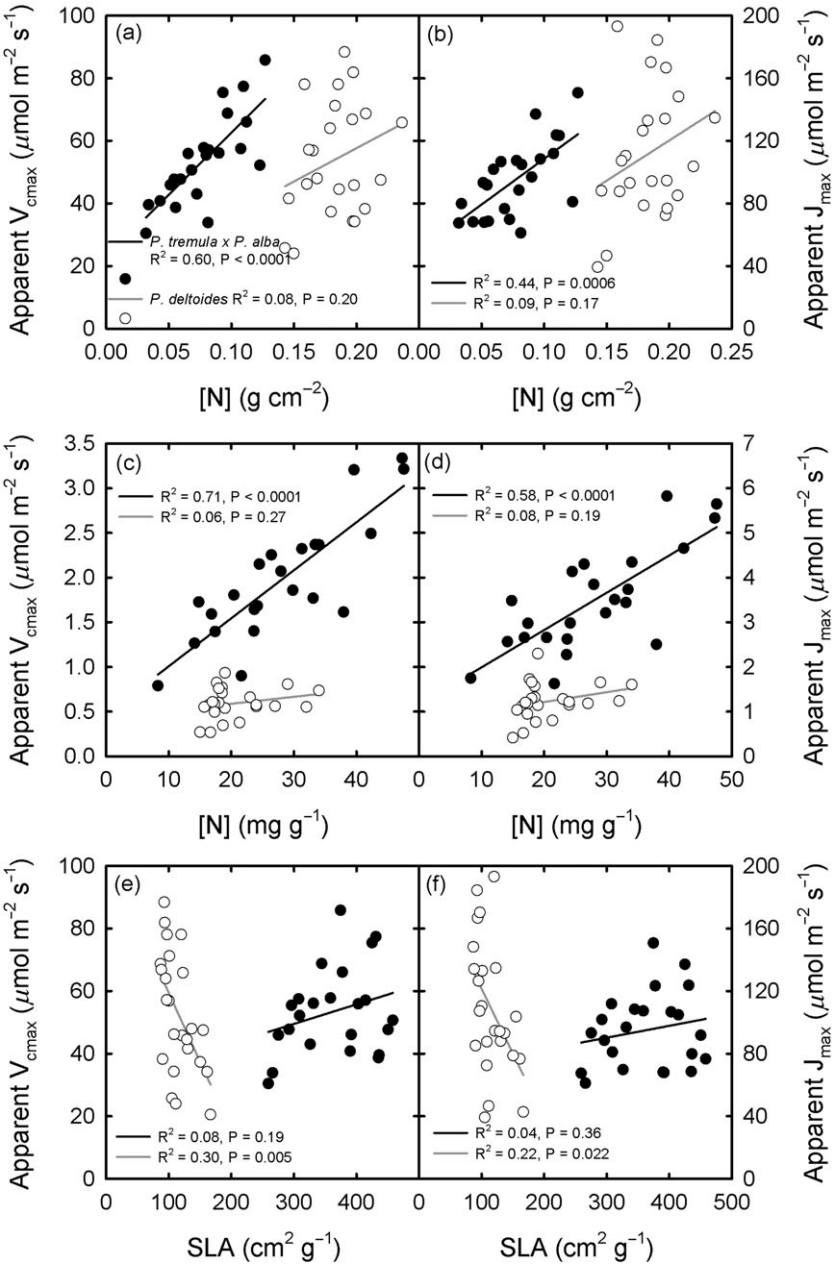

FIGURE 6 Apparent photosynthetic capacity (V_{cmax}, a, c, e; J_{max}, b, d, f) is significantly correlated with foliar nitrogen concentration ([N]) in *Populus tremula* × *Populus alba* (black points and line) but not *Populus deltoides* (white points and grey line) on an area (a, b) and mass basis (c, d), whereas it is significantly correlated with specific leaf area (SLA) in *P. deltoides* but not *P. tremula* × *P. alba* (e, f). Data presented as means of each leaf type for each individual

is too large, the CO_2 injector tends to overcompensate rendering RACiR less predictable. When possible, we would still recommend using the LI-6800 for RACiR approaches because of the smaller corrections, faster ramping speeds, faster chamber stabilization, greater programming ease, greater IRGA precision, and better control of environmental conditions (especially the vapour pressure deficit).

It is important to consider the implications of g_s for RACiR and A/C_i measurements. Several studies (Boyer, 2015a, 2015b; Tominaga & Kawamitsu, 2015; Tominaga, Shimada, & Kawamitsu, 2018) show that C_i is overestimated due to cuticular conductance to water. Due to the rate of RACiR, the bias in C_i due to cuticle conductance should be less variable relative to standard A/C_i measurements, because g_s is less variable. The relatively constant g_s across the C_i range during RACiR would keep the ratio of g_s to cuticular conductance relatively constant, simplifying estimates of C_i overestimation relative to that of a standard A/C_i. This implies that cuticle conductance, while still an issue in estimating C_i, is *less* of an issue for RACiR than for standard A/C_i approaches.

4.2 | Importance of studying photosynthetic capacity during leaf development

There have been varying patterns of PNUE with leaf development observed across species (Escudero & Mediavilla, 2003; Field & Mooney, 1983; Hom & Oechel, 1983; Kitajima, Mulkey, & Wright, 1997; Mooney, Field, Gulmon, & Bazzaz, 1981; Sobrado, 1994). Although few studies examine components of photosynthetic capacity (V_{cmax} and J_{max}) exist to fully understand their relationship with leaf development, varying patterns seen in PNUE suggest there is variability in photosynthetic capacity (Poorter & Evans, 1998). Here, we show that PNUE based on photosynthetic capacity shows different patterns with leaf development in two poplar species. PNUE is maintained across development in *P. deltoides* and declines with development in the poplar hybrid (Figure 7), supporting the interspecies variability in PNUE based on A_{net} (Escudero & Mediavilla, 2003; Field & Mooney, 1983; Hom & Oechel, 1983; Kitajima et al., 1997; Mooney et al., 1981; Sobrado, 1994).

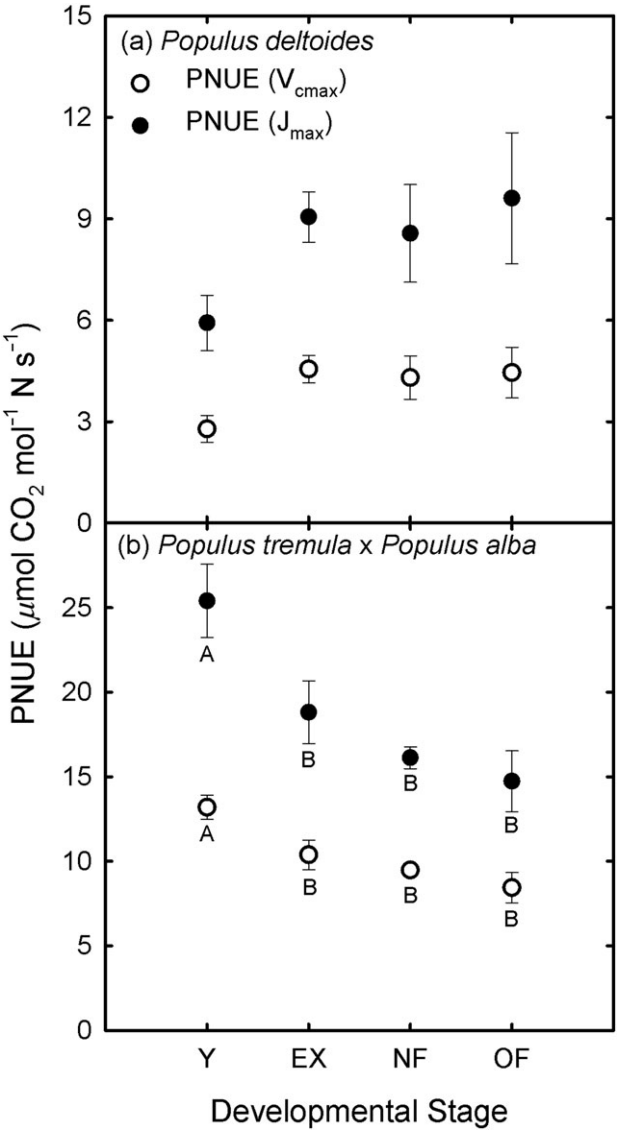

FIGURE 7 Photosynthetic nitrogen use efficiency (PNUE) on a V_{cmax}-basis (white points) and J_{max}-basis (black points) across leaf developmental stages in (a) *Populus deltoides* and (b) *Populus tremula* × *Populus alba*. Y: young leaf; EX: expanding leaf; NF: new fully expanded leaf; OF: old fully expanded leaf. Data presented as means ± s.e.m. Different capital letters indicate means of the developmental stage are significantly different according to Tukey's HSD ($P < 0.05$)

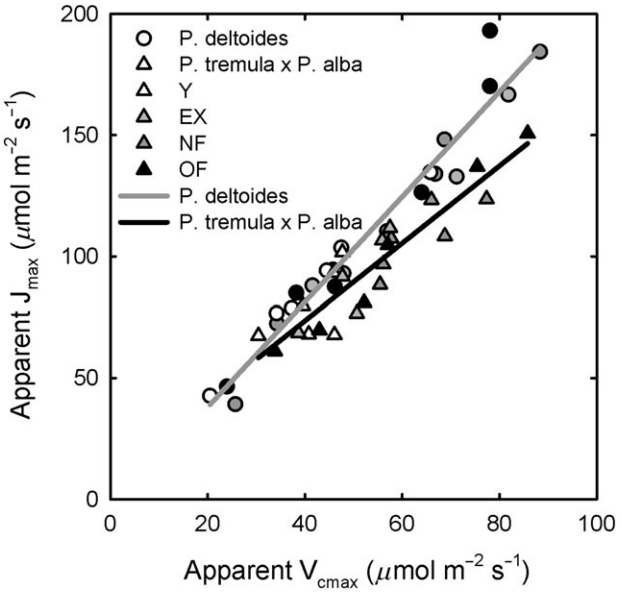

FIGURE 8 Apparent maximum electron transport rates (J_{max}) and Rubisco carboxylation rates (V_{cmax}) are tightly coupled across developmental stages in both *Populus deltoides* and *Populus tremula* × *Populus alba*. *P. deltoides*: $R^2 = 0.96$, $P < 0.0001$, $J_{max} = 2.16 \times V_{cmax} - 5.28$; *P. tremula* × *P. alba*: $R^2 = 0.85$, $P < 0.0001$, $J_{max} = 1.59 \times V_{cmax} + 9.82$

Developmental changes in photosynthetic capacity contribute to fluctuations in ecosystem productivity, even in year-round warm tropical evergreen forests where the extent and magnitude of seasonality was previously believed to be minimal (Barnes et al., 2017; Kim et al., 2012; Parazoo et al., 2008; Wu et al., 2016). Wu et al. (2016) found that in the Amazon evergreen forests, changes in photosynthetic capacity related to leaf development and demography explained large photosynthetic increases of approximately 27% observed through remote sensing. Wu et al. (2016) suggests that leaf phenology is sufficient to drive seasonal patterns in ecosystem productivity and highlights the importance of including such data in future ecological studies. Leaf biochemical traits such as [N] and [chlorophyll] used for modelling and estimating vegetative carbon fluxes on large spatial scales are often built with midseason data on new, fully developed leaves (Rogers, 2014). Given that these scaling relationships exclude leaf phenology and development when leaf nitrogen and chlorophyll vary, current data used to scale photosynthetic capacity likely represents its maximum potential. We may therefore be overestimating photosynthetic capacity during parts of the year for most species when modelling or using remote sensing to estimate vegetative carbon fluxes due to leaf development.

Understanding developmental patterns of photosynthesis has significant implications for future ecological models, agricultural development, and inherently our understanding of plant development (Long et al., 2006; Wu et al., 2016). Here, we show that RACiR provides data that match established relationships among photosynthetic capacity, nitrogen content, and SLA and can be used in other gas exchange systems, providing further support for the use of the technique in physiological studies. Because of its efficiency, RACiR provides accessibility to future large scale developmental studies that would have previously been prohibitively time intensive.

AUTHOR CONTRIBUTIONS

E. H. L. and J. R. S. carried out the research, designed the study, and wrote the paper with input from all authors. D. T. H. provided advice on experimental design.

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ORCID

Erica H. Lawrence http://orcid.org/0000-0002-0220-3210
Joseph R. Stinziano http://orcid.org/0000-0002-7628-4201
David T. Hanson http://orcid.org/0000-0003-0964-9335

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