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Activation of CO₂ assimilation during photosynthetic induction is slower in C₄ than in C₃ photosynthesis in three phylogenetically controlled experiments

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Introduction: Despite their importance for the global carbon cycle and crop production, species with C₄ photosynthesis are still somewhat understudied relative to C₃ species. Although the benefits of the C₄ carbon concentrating mechanism are readily observable under optimal steady state conditions, it is less clear how the presence of C₄ affects activation of CO₂ assimilation during photosynthetic induction.

Methods: In this study we aimed to characterise differences between C₄ and C₃ photosynthetic induction responses by analysing steady state photosynthesis and photosynthetic induction in three phylogenetically linked pairs of C₃ and C₄ species from *Alloteropsis*, *Flaveria*, and *Cleome* genera. Experiments were conducted both at 21% and 2% O₂ to evaluate the role of photorespiration during photosynthetic induction.

Results: Our results confirm C₄ species have slower activation of CO₂ assimilation during photosynthetic induction than C₃ species, but the apparent mechanism behind these differences varied between genera. Incomplete suppression of photorespiration was found to impact photosynthetic induction significantly in C₄ *Flaveria bidentis*, whereas in the *Cleome* and *Alloteropsis* C₄ species, delayed activation of the C₃ cycle appeared to limit induction and a potentially supporting role for photorespiration was also identified.

Discussion: The sheer variation in photosynthetic induction responses observed in our limited sample of species highlights the importance of controlling for evolutionary distance when comparing C_3 and C_4 photosynthetic pathways.

KEYWORDS

C_4 photosynthesis, C_3 photosynthesis, photosynthetic induction, CO_2 assimilation, photorespiration, non-steady state, light response

Introduction

Photosynthesis is the foundation of life on earth, the source of food, oxygen, and most of our energy. A particularly successful adaptation to the ancestral form is C_4 photosynthesis, which despite its complexity has independently arisen in at least 66 lineages of angiosperms and appeared in 19 unrelated plant families (Sage, 2004; Kellogg, 2013). Although only 3% of flowering species use the C_4 pathway, C_4 species represent 23% of global carbon fixation (Still et al., 2003), and maize and sugar cane are two of the four crops that account for half of the world's crop production (FAO, 2020). Despite the undeniable importance of C_4 species, there is comparatively less focus on improving C_4 performance – photosynthetic induction has consistently been flagged as a source of inefficiency in C_4 species relative to C_3 ones (Sage and McKown, 2006; Slattery et al., 2018; Sales et al., 2021), yet a lack of knowledge on the specifics of the C_4 induction response persists due to limited understanding of variation in photosynthetic induction between C_3 and C_4 photosynthesis, as well as across different C_4 species.

Most C_4 species display 'Kranz' anatomy, in which mesophyll (M) and bundle sheath (BS) cells are arranged concentrically around the leaf veins. Unlike in C_3 species, where initial CO_2 fixation and assimilation processes occur within the same cell, in C_4 species these activities are typically partitioned between M and BS cells. In the cytosol of M cells, equilibrium between CO_2 and bicarbonate is rapidly established by carbonic anhydrase. Bicarbonate is then fixed by phosphoenolpyruvate carboxylase (PEPC) into 4-carbon molecules that are further reduced before diffusing into the BS, where they are decarboxylated to release CO_2 around ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco), the central enzyme in carbon fixation (Leegood, 2002). The C_4 carbon concentrating mechanism (CCM) thus enhances photosynthesis and suppresses Rubisco's oxygenase activity and resulting photorespiration – the phospho-glycolate salvaging pathway that consumes energy and reducing equivalents, and releases CO_2 . However, the operation of the CCM has an energetic cost, and C_4 species require additional ATP

for PEP regeneration on top of the energetic demands of the C_3 cycle (Yin and Struik, 2018).

The increased efficiency of the C_4 pathway relative to the C_3 ancestral state is especially apparent under constant high light (Wang et al., 2012). However, during changes in light intensity, some C_4 species display impaired carbon assimilation in comparison to C_3 species (Kubásek et al., 2013; Slattery et al., 2018; Li et al., 2021). Decreases in photosynthetic efficiency during light induction (in response to an increase in light intensity) occur irrespective of photosynthetic pathway and are often explained by lags in regeneration of ribulose-1,5-biphosphate (RuBP) within the C_3 cycle, Rubisco activation, and stomatal opening (Percy and Seemann, 1990; Percy, 1990; Sassenrath-Cole and Percy, 1992; Mott and Woodrow, 2000). In addition, C_4 photosynthesis requires synchronous operation of C_3 and C_4 cycles and any loss of coordination during induction could lead to reduced efficiency of carbon fixation in C_4 species. Faster activation of the CCM relative to C_3 cycle activation in the BS may result in diffusional leakage of highly concentrated CO_2 out of the permeable BS back into M cells and a raised energetic cost for CO_2 assimilation, as recently suggested by Wang et al. (2022) and Lee et al. (2022). A 30–60% increase in BS leakiness of CO_2 during photosynthetic induction relative to steady state photosynthesis has been identified in maize and sorghum (Wang et al., 2022). This suggests that the C_3 cycle in some C_4 species is slower to activate than the C_4 CCM. Alternatively, the C_4 cycle could be the limiting factor during induction due to the need to build up metabolite gradients for the shuttling of CCM intermediates between M and BS cells. If so, the reduced supply of CO_2 to the BS would lead to weaker suppression of photorespiration, and a temporary disconnect between photosynthetic electron transport and CO_2 fixation (Sage and McKown, 2006; Kromdijk et al., 2010; Slattery et al., 2018). Although incomplete suppression of photorespiration can reduce photosynthetic efficiency, photorespiratory metabolite pools have also been suggested to help prime the C_4 cycle (Kromdijk et al., 2014; Stitt and Zhu, 2014; Schlüter and Weber, 2020; Medeiros et al., 2022). Higher relative

photorespiratory rates appear to occur under low light and during photosynthetic induction (Kromdijk et al., 2010; Medeiros et al., 2022); the resulting photorespiratory intermediates could act as a carbon reservoir from which to build C₃ and C₄ metabolite pools (Fu and Walker, 2022). The presence of an endogenous source of carbon is supported by the inability to account for the net increase in C₃ and C₄ cycle intermediates during light induction based on rates of CO₂ assimilation alone (Leegood and Furbank, 1984; Usuda, 1985).

Whilst the experimental evidence and putative mechanisms detailed above may indeed suggest that C₄ species could be more affected by transient decreases in photosynthetic efficiency during induction relative to steady state than C₃ species, most of the work does not directly compare C₃ and C₄ species in a common experiment, but instead often focuses on a single species, such as maize (Zelitch et al., 2009; Kromdijk et al., 2010; Medeiros et al., 2022). Some direct comparisons between C₃ and C₄ photosynthesis have been made in sets of contrasting grass species (Lee et al., 2022) and species within the same genus (Kubásek et al., 2013), but so far the only C₃ and C₄ species studied that share a relatively recent common ancestor are *Flaveria* (Li et al., 2021). Phylogenetic distance can strongly confound the apparent photosynthetic differences observed (Taylor et al., 2010) and to confirm whether observed differences are due to photosynthetic pathway or evolutionary variation, studies conducted on phylogenetically linked C₃ and C₄ species are necessary. Furthermore, despite striking similarities in the anatomy and biochemistry of C₄ species from diverse evolutionary origins, there is still great diversity amongst species. Some common variations are the main decarboxylases utilised to release CO₂ in BS cells: nicotinamide adenine dinucleotide-malic enzyme (NAD-ME), nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME), and phosphoenolpyruvate carboxykinase (PEPCK). Although C₄ subtypes had initially been defined by these main decarboxylases (Hatch et al., 1975), more recent work suggests there is a greater degree of nuance than traditional C₄ classifications connote, as NADP-ME and NAD-ME often operate alongside a PEPCK auxiliary pathway, and the energetic requirements of C₄ photosynthesis render a pure PEPCK subtype unlikely (Wang et al., 2014a). Variations across C₄ species and phylogenetic distance are thus important considerations when trying to derive generic differences between C₃ and C₄ photosynthesis.

In this study we analysed steady state photosynthesis and photosynthetic induction in three phylogenetically linked pairs of C₃ and C₄ species from *Alloteropsis*, *Flaveria*, and *Cleome* genera, representative of monocots and dicots, and all three C₄ decarboxylation enzymes. Photosynthetic gas exchange was measured in response to a step-change to moderate and strongly saturating light intensities to characterise differences

in photosynthetic induction rates. Experiments were conducted at both 21% and 2% O₂ concentration to evaluate the role of photorespiration during induction. Activation of CO₂ assimilation at the start of light induction was slower in all C₄ species compared to their C₃ counterparts although the mechanism of impairment varied across genera. Furthermore, although both C₃ and C₄ *Flaveria* had greater CO₂ assimilation under 2% O₂, assimilation in C₃ and C₄ *Alloteropsis* species as well as C₃ *T. hassleriana* was negatively impacted by low O₂. The variation in responses highlights the natural diversity of C₄ species, and the importance of controlling for phylogenetic distance in comparisons between C₃ and C₄ photosynthesis.

Materials and methods

Plant materials

Three pairs of phylogenetically linked *Alloteropsis*, *Flaveria* and *Cleome* C₃ and C₄ species were selected to decrease evolutionary variation within each pair (see Figure 1) (Lyu et al., 2015) but maintain significant evolutionary distance between the three genera, as C₄ origins arose ~ 17 million years ago (Ma) in *Cleome*, ~ 2 Ma in *Flaveria*, and even earlier in *Alloteropsis* (Christin et al., 2011; Lundgren et al., 2015). The selected species include monocots (C₃ *Alloteropsis semialata* subspecies *semialata* accession GMT and C₄ *Alloteropsis semialata* subspecies *eckloniana* accession MDG), dicots (C₃ *Flaveria cronquistii*, C₄ *Flaveria bidentis*, C₃ *Tarenaya hassleriana* and C₄ *Gynandropsis gynandra*), and the three major decarboxylase enzymes of the C₄ pathway, which have been suggested to be dominant in different C₄ species: PEPCK in mixed NADP-ME-PEPCK pathway in *A. semialata* MDG (Ueno and Sentoku, 2006), NADP-ME in *F. bidentis* (Gowik et al., 2011), and NAD-ME in *G. gynandra* (Bräutigam et al., 2010).

Plant growth and propagation

All plants were measured during the vegetative growth phase. Plants were grown in Levington Advance M3 compost (Scotts, Ipswich, UK) mixed with Miracle-Gro All Purpose Continuous Release Osmocote (Scotts Miracle-Gro Company, Marysville, OH, USA; 4 L compost: 25g Osmocote). Medium vermiculite was added to the *Alloteropsis* soil mix (4 L compost: 1 L vermiculite: 25g Osmocote) to prevent waterlogging.

The *Alloteropsis* GMT and MDG accessions were vegetatively propagated and grown on 2 L pots under well-watered conditions in a glasshouse at 18–25°C, 40–60% relative humidity (RH), with supplemental lightning provided to ensure at least 140–160 μmol m⁻² s⁻¹ photon flux density (PFD) across a

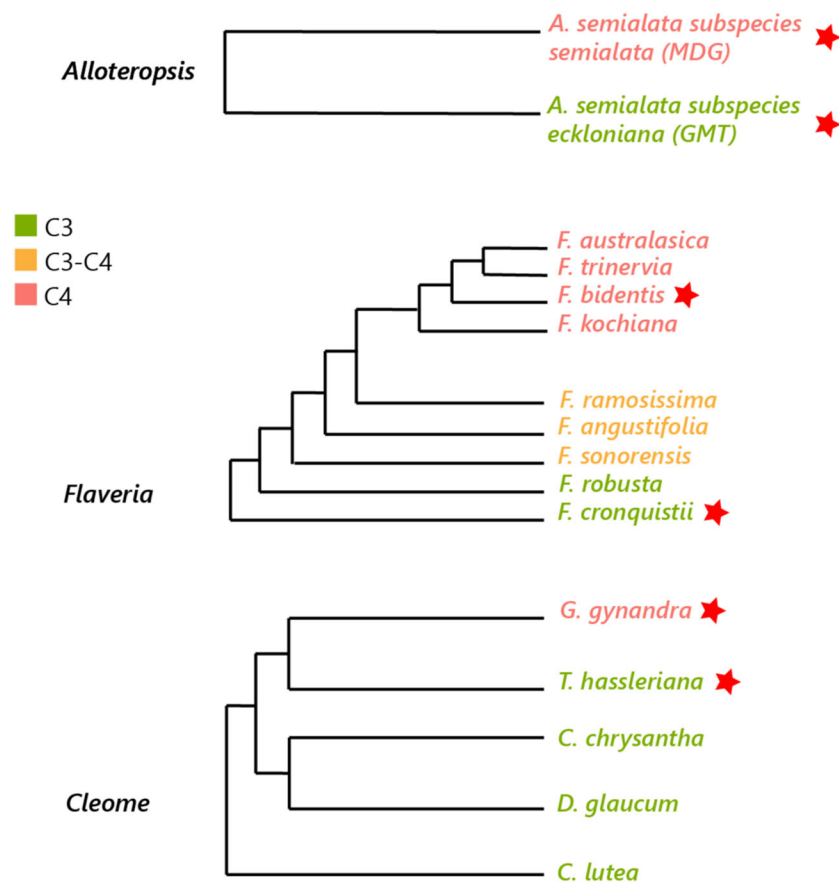


FIGURE 1

Phylogenetic tree of *Alloteropsis*, *Flaveria* and *Cleome* genera. Species used in the study are marked with a red star (adapted from Ibrahim et al., 2009; Lyu et al., 2015; Shi et al., 2021).

16-hour photoperiod. Plants were measured two weeks after propagation.

The *Flaveria* and *Cleome* species were grown under well-watered conditions in a Conviron growth room (Conviron Ltd., Winnipeg, MB, CA) at 20°C temperature, 60% RH, and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD over a 16-hour photoperiod. Because *F. cronquistii* requires vegetative propagation, plants from both *Flaveria* species were propagated from lateral shoot cuttings – *F. bidentis* plants were initially grown from seed and then propagated. Cuttings were dipped in Doff Hormone Rooting Powder (Doff Portland Ltd., Hucknall, UK) to induce root development, and *Flaveria* cuttings were grown on 0.25 L pots and measured after 8–10 weeks.

Cleome germination was induced under sterile conditions at 30°C/20°C day/night cycle for *T. hassleriana*, and at 30°C for *G. gynandra*. Germinated seeds were initially sown in 24-cell seed trays before transfer to 0.25 L pots. As *G. gynandra* has a lower development rate than *T. hassleriana*, germination was staggered so both species could be measured at approximately the same

developmental stage, after 8–10 weeks for *G. gynandra* and 4–6 weeks for *T. hassleriana*.

Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured simultaneously using an open gas exchange system (LI-6400XT, LI-COR, Lincoln, NE, USA) with an integrated leaf chamber fluorometer (6400-40 LCF). Leaves were measured in a 2 cm^2 chamber at 25°C block temperature, 410 ppm sample CO_2 concentration, and 50–65% RH with flow of 300 $\mu\text{mol s}^{-1}$. Average leaf VPD was 1.1 ± 0.1 kPa at the start and 1.35 ± 0.1 kPa at the end of the light treatment. Actinic light was provided by the LCF and composed of 10% blue (470 nm) and 90% red light (630 nm).

The LCF used a 0.25 Hz modulated measuring light and a multiphase flash (Loriaux et al., 2013) to measure steady (F') and maximal (F_m') fluorescence to derive the quantum yield of

Photosystem II (Φ PSII) (Genty et al., 1989).

For experiments using 2% O₂, a pre-mixed 2% O₂ and 98% N₂ gas mixture was supplied to the LI-6400XT through the air inlet, using a mass flow controller (EL-FLOW, Bronkhorst High-tech BV, Ruurlo, NL) and an open T-junction to regulate constant surplus flow. Infrared Gas Analyzer (IRGA) calibration was adjusted to the O₂ gaseous composition in the instrument settings prior to measurement.

Leaf absorbance

Light absorbance of the plants used in experiments was measured with an integrating sphere (LI-1800-12, LI-COR) optically connected to a miniature spectrometer (Li-Cor, 1988). Incident PFD was converted to absorbed photon flux density (PFD_{abs}) using the measured leaf absorbance of the emission wavelengths of the 6400-40 LCF light source. The specific absorbance values can be found in [Supplementary Material 1](#).

Steady state light response curves

Steady state light response curves of photosynthetic gas exchange were measured for all species at both 21% and 2% O₂. Leaves were light-adapted at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD and once CO₂ assimilation and stomatal conductance reached steady state, gas exchange and chlorophyll fluorescence parameters were measured in a descending gradient of light intensity: 2000, 1700, 1500, 1200, 1000, 800, 600, 400, 300, 200, 100, 75, 30, and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD. Gas exchange and chlorophyll fluorescence parameters were logged after 120–240 s, when leaf intracellular CO₂ concentration (C_i) and CO₂ assimilation were stable.

To analyse steady state responses, a non-rectangular hyperbola was fitted to the light response curves (Stinziano et al., 2021). The quantum yield of assimilation (α) was derived from the initial slope, and the light-saturated photosynthetic rate ($A_{\max}$) from the asymptote of the curve. C_i values obtained above 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD were averaged to estimate light-saturated C_i ($C_{i \max}$).

Approximate light intensities at the inflection point (600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD) and in the saturating part of the response (1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD) were used in the light induction experiments. Respiration in the light (R_d) was estimated for all species at each O₂ concentration as the y-intercept using a linear regression of the initial light response curve slope. To account for the Kok effect, measurements in darkness and at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity were not included in the regression (Kok, 1949).

Light induction experiments and analysis of lag in carbon assimilation

Leaves were dark-adapted until stomatal conductance reached constant levels (between 30–60 minutes depending on the species), illuminated with 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ or 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD for 1 hour, and then returned to darkness for another half hour. Starting from the last 5 minutes of initial dark adaption, gas exchange parameters were logged every minute, and chlorophyll fluorescence parameters at 5, 15, 25, 35, 45, and 60 minutes after starting light exposure. Light induction experiments at both light intensities were conducted at 21% and 2% O₂. Carbon assimilation was corrected for respiration to determine net photosynthetic CO₂ assimilation (A_{CO_2}) using the R_d obtained from light response curves.

To analyse photosynthetic responses across the induction period, the trapezoidal rule (Jawieñ, 2014) was used to integrate the area under the curve (AUC) (Makowski et al., 2019) of A_{CO_2} during the 0–5, 5–10, and 10–60 minute phases of light exposure.

Alternative electron sinks

The electron cost of assimilation can be approximated by the $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio (Genty et al., 1989; Oberhuber and Edwards, 1993), with ΦCO_2 being the quantum yield of CO₂ assimilation (Equation 1). Lower ratios are associated with greater coupling as more electrons captured by PSII go towards CO₂ assimilation (Kral and Edwards, 1990). For light response curves the $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio was calculated for the values obtained at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD (600 $\Phi\text{PSII}/\Phi\text{CO}_2$) and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD (1500 $\Phi\text{PSII}/\Phi\text{CO}_2$). During light induction $\Phi\text{PSII}/\Phi\text{CO}_2$ values were taken from across the light period at each intensity. Data points were excluded if calculated ΦCO_2 showed negative values.

$$\Phi\text{CO}_2 = \frac{(A_{\text{CO}_2} + R_d)}{PFD_{abs}} \quad \text{Equation 1}$$

Statistical analysis

All statistical analyses were conducted separately on paired *Alloteropsis*, *Flaveria*, and *Cleome* light response curves, light induction at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD, and light induction at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD. Mean and standard error of the mean of light response curve parameters ($A_{\max}$, α , $C_{i \max}$, 600 $\Phi\text{PSII}/\Phi\text{CO}_2$ and 1500 $\Phi\text{PSII}/\Phi\text{CO}_2$), A_{CO_2} AUC at different phases of induction, and $\Phi\text{PSII}/\Phi\text{CO}_2$ across light induction were

calculated. Linear mixed models (LMMs) were fitted to the light response curve parameters and A_{CO_2} AUC at different phases of induction using photosynthetic pathway, O_2 concentration and their interaction as fixed effects; and to $\Phi PSII/\Phi CO_2$ across light induction using photosynthetic pathway, O_2 concentration, time, and their interactions as fixed effects. Time of day and measured plant were included as random effects in all models. Two and three-way ANOVA tables for the fixed effects were generated from the LMMs using the Satterthwaite's approximation method (Kuznetsova et al., 2017). The data was independent and assumptions of normality, homogeneity of variance and sphericity were satisfied.

All data analysis and plot generation was done on RStudio 1.3 (RStudio Team, 2022) with R 4.1.1 (R Core Team, 2021) using the tidyverse (Wickham et al., 2019), RColorBrewer (Neuwirth, 2014), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017) and bayestestR libraries (Makowski et al., 2019).

Results

Steady state measurements confirm canonical differences in CO_2 assimilation between C_3 and C_4 species

Light response curves were used to first characterise C_3 and C_4 responses under steady state at 21% (Figures 2A–C, 3A–C and Table 1). The responses of C_4 species in comparison to their C_3 phylogenetic pairs were genus specific – C_4 *F. bidentis* had

higher maximum rates of net carbon assimilation (A_{max}) than C_3 *F. cronquistii* ($P = 0.03$; C_3 12.5 ± 1.3 vs C_4 $16.7 \pm 1.0 \mu mol m^{-2} s^{-1}$), but A_{max} values in C_4 *G. gynandra* were similar to those found in C_3 *T. hassleriana* ($P = 0.24$; C_3 15.5 ± 2.0 vs C_4 $16.7 \pm 0.7 \mu mol m^{-2} s^{-1}$), and A_{max} values in C_4 *A. semialata* MDG also were similar to C_3 *A. semialata* GMT ($P = 0.02$; C_3 11.8 ± 1.1 vs C_4 $10.2 \pm 2.7 \mu mol m^{-2} s^{-1}$). A two-way ANOVA (Table 2) showed photosynthetic pathway had a significant effect on C_i during light saturation in all genera. However, whilst the C_4 pathway was associated with lower $C_{i max}$ in *Flaveria* ($P \leq 0.001$; C_3 238 ± 19 vs C_4 $86 \pm 27 \mu mol mol^{-1}$) and *Cleome* ($P \leq 0.001$; C_3 310 ± 8 vs C_4 $125 \pm 27 \mu mol mol^{-1}$), in *Alloteropsis* the C_4 association was instead with higher $C_{i max}$ ($P \leq 0.01$; C_3 242 ± 9 vs C_4 $285 \pm 21 \mu mol mol^{-1}$). Figure 2C shows that the lower C_i of C_4 species corresponded to lower stomatal conductance, excepting C_4 *A. semialata* MDG, where stomatal conductance to water vapour (g_{sw}) was similar to C_3 *A. semialata* GMT across the light response.

$\Phi PSII$ decreased exponentially with higher light intensities. Although $\Phi PSII$ values were very similar across C_3 and C_4 pairs in *Flaveria* and *Cleome*, more pronounced decreases were observed in C_4 *A. semialata* MDG than in C_3 *A. semialata* GMT (Figure 3A). ΦCO_2 was also lower at higher light intensities, following a similar pattern to A_{max} across the light response, as ΦCO_2 was similar between C_3 and C_4 species in *Alloteropsis* and *Cleome*, but higher in C_4 *F. bidentis* compared to C_3 *F. cronquistii* (Figure 3B). These differences across genera were also apparent for the observed $\Phi PSII$ and ΦCO_2 ratios, but not always significantly so. C_4 *F. bidentis* had lower $\Phi PSII/\Phi CO_2$ than C_3 *F. cronquistii* ($P = 0.16$; C_3 14.0 ± 0.3 vs C_4 9.0 ± 2.4 at $PFD = 600 \mu mol m^{-2} s^{-1}$, and $P = 0.56$; C_3 12.2 ± 0.6 ,

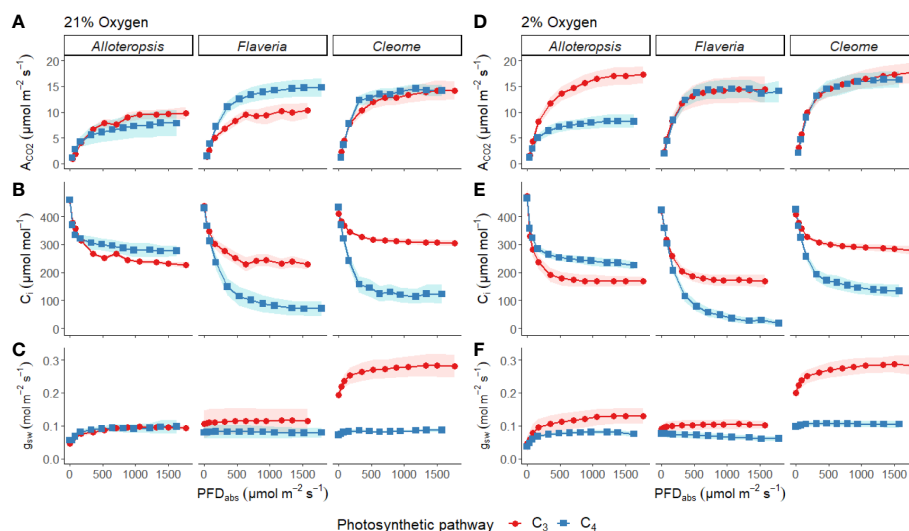


FIGURE 2

Measurements of gas exchange traits during light response curves for phylogenetically linked C_3 and C_4 *Alloteropsis*, *Flaveria* and *Cleome* species under 21% and 2% O_2 . Plots show net CO_2 assimilation (A_{CO_2} , A, D), intercellular CO_2 concentration (C_i , B, E), and stomatal conductance to water vapour (g_{sw} , C, F) as a function of absorbed light intensity. Ribbons represent standard error of the mean ($n=5$).

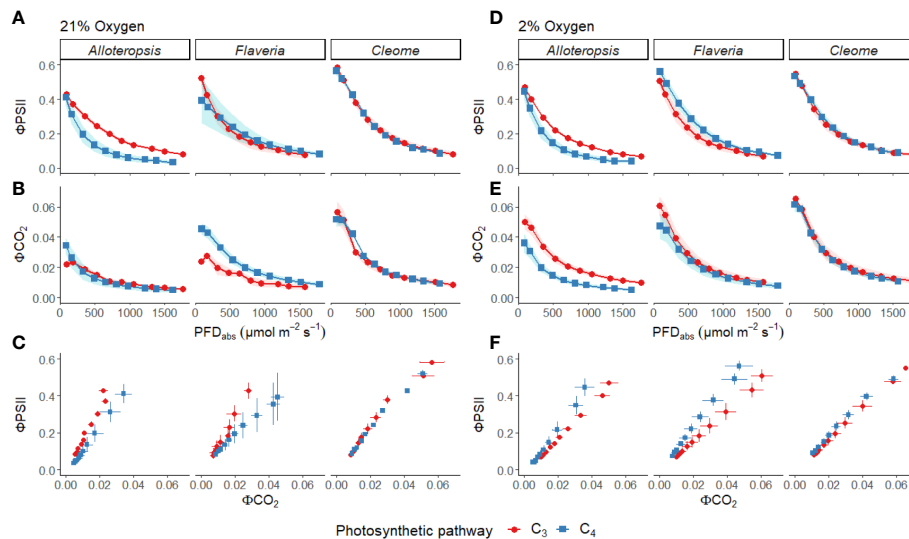


FIGURE 3

Measurements of gas exchange and chlorophyll fluorescence traits during light response curves for phylogenetically linked C₃ and C₄ *Alloteropsis*, *Flaveria* and *Cleome* species under 21% and 2% O₂. Plots show quantum yield of PSII (ΦPSII, A, D), and quantum yield of CO₂ assimilation (ΦCO₂, B, E) as a function of absorbed light intensity. Ribbons represent standard error of the mean (n=5). Plots (C, F) display the relationship between ΦPSII and ΦCO₂. Error bars represent standard error of the mean for both parameters.

C₄ 9.5 ± 2.2 at PFD = $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the same was observed for C₄ *A. semialata* MDG compared to C₃ *A. semialata* GMT ($P = 0.03$; C₃ 16.7 ± 1.8 vs C₄ 11.3 ± 2.4 at PFD = $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $P \leq 0.001$; C₃ 16.2 ± 1.6 vs C₄ 8.6 ± 2.1 at PFD = $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$), suggesting that in these C₄ species less electron transfer through PSII is needed per CO₂ fixed. Figure 3C shows that the lower ratio of ΦPSII to ΦCO₂ in C₄ *Flaveria* and *Alloteropsis* in relation to their C₃ counterparts was observed across most light intensities, although the difference appeared to be marginal at higher light intensities. At 21% O₂, both C₃ and C₄ *Cleome* species had very similar ΦPSII/ΦCO₂ ($P = 0.84$; C₃ 12.3 ± 0.8 vs C₄ 11.7 ± 1.1 at PFD = $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $P = 0.78$; C₃ 10.5 ± 0.5 vs C₄ 9.8 ± 1.0 at PFD = $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Light response curves were also performed at 2% O₂ to minimize photorespiration (Figures 2D-F, 3D-F and Table 1). All three C₃ species had substantially higher CO₂ assimilation rates under low O₂ – A_{max} was around 75% higher in C₃ *A. semialata* MDG, 25% higher in C₃ *F. cronquistii*, and 35% higher in C₃ *T. hassleriana* than under 21% O₂. 2% O₂ also led to a decrease in C_{i max} in *Alloteropsis* ($P \leq 0.01$ in C₃ 171 ± 19 vs C₄ $240 \pm 14 \mu\text{mol mol}^{-1}$) and *Flaveria* ($P = 0.02$; C₃ 174 ± 18 vs C₄ $43 \pm 15 \mu\text{mol mol}^{-1}$) but no significant change in either *Cleome* species ($P = 0.93$; C₃ 290 ± 11 vs C₄ $149 \pm 21 \mu\text{mol mol}^{-1}$), where A_{CO2} and g_{sw} appeared tightly coordinated. The increase in A_{max} was not mirrored in C₄ species, as evidenced by significant interactions (Table 2) between photosynthetic pathway and oxygen in *Alloteropsis* ($P = 0.03$) and *Flaveria* ($P = 0.01$), where low O₂ concentrations were associated with

higher A_{max} on C₃ but not C₄ species. (Table 2). Similar patterns were observed for the two *Cleome* species, however the increase in A_{max} at 2% O₂ for C₃ *T. hassleriana* was less pronounced than for the other C₃ species and instead the initial slope α appeared to be subject to a significant interaction between effects of photosynthetic pathway and O₂ ($P = 0.03$). The different effects on assimilation at low O₂ between C₃ and C₄ species were also reflected in the changing relationship between ΦPSII and ΦCO₂ (Figures 3C, F) – photosynthetic pathway and O₂ concentration were found to have significant interactions on ΦPSII/ΦCO₂ in all three genera (Table 2, $P = 0.05$ in *Flaveria* and *Cleome*, $P \leq 0.01$ in *Alloteropsis*), due to decreases in ΦPSII/ΦCO₂ in C₃ species at 2% O₂ not observed in C₄ species. This data confirms that photorespiration is a significant electron sink under steady state for all three C₃ species, whereas the steady state suppression of photorespiration at 21% O₂ in the C₄ species is sufficient to prevent any significant further decreases in ΦPSII/ΦCO₂ under 2% O₂.

Substantial differences in photosynthetic traits exist between C₃ and C₄ species during light induction

Photosynthetic induction rates were measured in leaves exposed to $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ or $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD from darkness (Figures 4A-C, G-I). The light induction response across

TABLE 1 Light response curve parameters estimated from steady state light response curves under 21% and 2% O₂ on phylogenetically linked C₃ and C₄ *Alloteropsis*, *Flaveria* and *Cleome* species.

Genus	Parameter	21% Oxygen		2% Oxygen	
		C ₃	C ₄	C ₃	C ₄
<i>Alloteropsis</i>	A _{max} (μmol m ⁻² s ⁻¹)	11.8 ± 1.1	10.2 ± 2.7	20.9 ± 2.4	10.3 ± 1.4
	α	0.04 ± 0.00	0.10 ± 0.02	0.08 ± 0.01	0.09 ± 0.01
	C _{i max} (μmol mol ⁻¹)	242 ± 9	285 ± 21	171 ± 19	240 ± 14
	600 ΦPSII/ΦCO ₂	16.7 ± 1.8	11.3 ± 2.4	8.9 ± 1.7	10.0 ± 2.4
	1500 ΦPSII/ΦCO ₂	16.2 ± 1.6	8.6 ± 2.1	7.5 ± 1.2	6.8 ± 2.6
	Rd	0.9 ± 0.2	1.4 ± 0.6	1.5 ± 0.3	1.5 ± 0.1
<i>Flaveria</i>	A _{max} (μmol m ⁻² s ⁻¹)	12.5 ± 1.3	16.7 ± 1.0	15.6 ± 1.7	15.3 ± 1.8
	α	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
	C _{i max} (μmol mol ⁻¹)	238 ± 19	86 ± 27	174 ± 18	43 ± 15
	600 ΦPSII/ΦCO ₂	14.0 ± 0.3	9.0 ± 5.2	7.6 ± 0.3	12.6 ± 3.2
	1500 ΦPSII/ΦCO ₂	12.2 ± 0.6	9.5 ± 2.2	8.1 ± 0.6	11.1 ± 3.2
	Rd	0.9 ± 0.3	1.0 ± 0.4	0.6 ± 0.2	0.4 ± 0.2
<i>Cleome</i>	A _{max}	15.5 ± 2.0	16.7 ± 0.7	20.9 ± 2.5	17.7 ± 1.5
	α	0.07 ± 0.01	0.09 ± 0.02	0.15 ± 0.03	0.08 ± 0.01
	C _{i max} (μmol mol ⁻¹)	310 ± 8	125 ± 27	290 ± 11	149 ± 21
	600 ΦPSII/ΦCO ₂	12.3 ± 0.8	11.7 ± 1.1	7.7 ± 1.0	9.4 ± 0.5
	1500 ΦPSII/ΦCO ₂	10.5 ± 0.5	9.8 ± 1.0	8.7 ± 0.7	8.8 ± 0.3
	Rd	0.3 ± 0.2	1.8 ± 0.7	2.1 ± 0.7	0.8 ± 0.5

The light-saturated photosynthetic rate (A_{max}) and the quantum yield of assimilation (α) were calculated by fitting the light response curves with a non-rectangular hyperbola. The C_i at light saturation point (C_{i max}) is the average C_i at PFD ≥ 600 μmol m⁻² s⁻¹. Values for 600 ΦPSII/ΦCO₂ and 1500 ΦPSII/ΦCO₂ were taken at PFD = 600 μmol m⁻² s⁻¹ and PFD = 1500 μmol m⁻² s⁻¹. Means and standard error of the mean are shown (n = 5).

all species and light intensities generally consisted of gradual stomatal opening in line with a rise in A_{CO2} towards steady state, and a sharp drop in C_i at the start of induction, followed by a gradual recovery.

In addition to these general patterns, several differences were observed across genera and between paired species. Stomata tended to open more quickly in the C₃ species than in their respective C₄ counterparts. Furthermore, the speed of A_{CO2} induction appeared to vary between some C₃ and C₄ pairs; where carbon assimilation was notably slower to induce in C₄ *G. gynandra* compared to C₃ *T. hassleriana* under both light intensities, with more subtle differences observed in the *Alloteropsis* and *Flaveria* C₃ and C₄ pairs.

Reductions in assimilation of CO₂ at the start of induction in C₃ and C₄ species vary across genera

In order to systematically explore C₃ and C₄ differences in the activation of CO₂ assimilation, the induction time-series were subdivided into three periods, 0 – 5 min, 5 – 10 min, and the

remaining 10 – 60 min, and integrated carbon assimilation (AUC) was calculated for each period (Figure 5). During the 0 – 5 min period, C₄ *F. bidentis* had lower AUC than C₃ *F. cronquistii* under both PFD = 600 μmol m⁻² s⁻¹ ($P = 0.09$; C₃ 12.4 ± 1.7 vs C₄ 5.8 ± 2.0 μmol m⁻²) and PFD = 1500 μmol m⁻² s⁻¹ ($P = 0.02$; C₃ 14.8 ± 1.8 vs C₄ 8.0 ± 1.8 μmol m⁻²), with the difference becoming significant under higher light (Table 3). The difference in assimilated CO₂ between C₃ and C₄ species at the start of induction was even more pronounced in *Cleome*, where the AUC of C₄ *G. gynandra* was significantly lower than that of C₃ *T. hassleriana* under both light intensities ($P \leq 0.01$; C₃ 12.6 ± 2.0 vs C₄ -1.4 ± 1.9 μmol m⁻² at PFD = 600 μmol m⁻² s⁻¹, and $P \leq 0.01$; C₃ 13.8 ± 3.2 vs C₄ -3.1 ± 1.8 μmol m⁻² at PFD = 1500 μmol m⁻² s⁻¹). The AUC in C₄ *G. gynandra* continued to be significantly lower than in C₃ *T. hassleriana* under both light intensities during the following two periods of induction analysed (Table 3). In contrast, the significant difference in cumulative CO₂ uptake between *Flaveria* species was only significant during the first five minutes of induction (Figure 5A). Thus, there was a more pronounced lag in CO₂ assimilation during induction in C₄ photosynthesis in *Flaveria* and *Cleome* than in C₃ photosynthesis in the same genera. This was

TABLE 2 ANOVA table of modelled light response curve parameters for phylogenetically linked *C*₃ and *C*₄ *Alloteropsis*, *Flaveria* and *Cleome* species.

Parameter		<i>Alloteropsis</i>			<i>Flaveria</i>			<i>Cleome</i>		
		PP	[O ₂]	PP:[O ₂]	PP	[O ₂]	PP:[O ₂]	PP	[O ₂]	PP:[O ₂]
<i>A</i> _{max}	df; <i>F</i> - value; <i>P</i> -value	1,15; 7.40; 0.02	1,15; 4.47; 0.06	1,15; 5.90; 0.03	1,15; 5.81; 0.03	1,15; 1.40; 0.26	1,15; 7.73; 0.01	1,15; 0.29; 0.59	1,15; 3.19; 0.09	1,15; 1.51; 0.24
α	df; <i>F</i> - value; <i>P</i> -value	1,15; 3.89; 0.06	1,15; 1.85; 0.20	1,15; 3.69; 0.07	1,15; 1.38; 0.26	1,15; 0.03; 0.87	1,15; 0.26; 0.62	1,15; 1.45; 0.24	1,15; 3.77; 0.07	1,15; 5.58; 0.03
<i>C</i> _{imax}	df; <i>F</i> - value; <i>P</i> -value	1, 16; 11.06; ≤0.01	1, 16; 12.00; ≤0.01	1, 16; 0.54; 0.45	1, 16; 49.07; ≤0.001	1, 16; 6.75; 0.02	1, 16; 0.27; 0.61	1, 16; 70.23; ≤0.001	1, 16; 0.00; 0.93	1, 16; 1.27; 0.27
600 ΦPSII/ΦCO ₂	df; <i>F</i> - value; <i>P</i> -value	1,16; 6.05; 0.03	1,16; 24.29; ≤0.001	1,16; 12.28; ≤0.01	1,16; 2.01; 0.16	1,16; 5.31; 0.05	1,16; 5.95; 0.03	1,16; 0.05; 0.84	1,16; 30.01; ≤0.01	1,16; 5.39; 0.05
1500 ΦPSII/ ΦCO ₂	df; <i>F</i> - value; <i>P</i> -value	1,16; 22.75; ≤0.001	1,16; 35.56; ≤0.001	1,16; 15.67; ≤0.01	1,16; 0.37; 0.56	1,16; 1.55; 0.26	1,16; 6.68; 0.05	1,16; 0.08; 0.78	1,16; 15.61; 0.01	1,16; 5.57; 0.05

Photosynthetic pathway, PP. O₂ concentration, [O₂]. Interaction effect, PP:[O₂]. Table shows degrees of freedom; *F*-value; and *P*-value. Significant ($\alpha < 0.05$) *P*-values are shown in bold.

especially apparent in relation to the steady state comparison between both species-pairs (Figure 2A). In *Alloteropsis*, the *C*₄ *A. semialata* MDG also started at lower AUC than *C*₃ *A. semialata* GMT during the 0 – 5 min period under both light intensities ($P = 0.36$; *C*₃ -4.4 ± 0.5 vs *C*₄ -6.22 ± 0.39 at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and $P = 0.07$; *C*₃ -4.2 ± 0.2 , vs *C*₄ -6.8 ± 0.6 at PFD = 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$), but the difference in AUC between pathways was only found to be significant for the final 10 – 60 min ($P \leq 0.01$; *C*₃ 194.8 ± 34.3 vs *C*₄ 142.2 ± 101.0 at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and $P = 0.03$; *C*₃ 403.3 ± 59.5 vs *C*₄ 241.5 ± 115.6 at PFD = 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

CO₂ assimilation during induction is enhanced under 2% O₂ in some species but suppressed in others

In order to test whether the presence or absence of photorespiration affected the activation of CO₂ assimilation, both light treatments were also conducted under 2% O₂ (Figures 4D–F, J–L). In *Flaveria*, the decrease in O₂ concentration significantly increased the AUC of both *C*₃ *F. cronquistii* and *C*₄ *F. bidentis* during the first five minutes of induction (Table 3 and Figure 5A), under both light intensities ($P \leq 0.01$; *C*₃ 26.1 ± 5.4 vs *C*₄ 19.9 ± 2.6 $\mu\text{mol m}^{-2}$ at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and $P \leq 0.001$; *C*₃ 33.8 ± 6.3 vs *C*₄ 23.1 ± 1.6 at PFD = 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Interestingly, although the stimulating effect of 2% O₂ on *C*₄ *F. bidentis* was less pronounced for 5 – 10 min and 10 – 60 min, the effect was still significant across both periods under both light intensities, except for 5 – 10 min at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ($P = 0.08$, Table 3). This suggests that photorespiration is insufficiently suppressed during

induction in *C*₄ *F. bidentis*, whereas in contrast, no change was observed for CO₂ assimilation in steady state *C*₄ *F. bidentis* under 2% O₂ (Figures 2A, D). In *Cleome* no such enhancement of the photosynthetic response was observed in *C*₄ *G. gynandra*. Instead, the significant interaction between photosynthetic pathway and O₂ concentration from 0 – 5 min was primarily associated with a decrease in assimilated CO₂ in *C*₃ *T. hassleriana* and only a marginal increase in *C*₄ *G. gynandra* AUC under 2% O₂ compared to under 21% O₂ ($P \leq 0.001$). The negative effect of 2% O₂ on AUC in *C*₃ *T. hassleriana* was transiently observed from 0 – 10 min at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and only from 0 – 5 min at PFD = 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. From 10 – 60 min AUC at 2% O₂ in *C*₃ *T. hassleriana* was similar to the AUC at 21% O₂ under both light intensities. Thus, the stimulation of steady state CO₂ assimilation by 2% O₂ in this species was not observed under any of the transient conditions (Table 3 and Figure 5). Suppression of carbon assimilation by low O₂ was also observed during the start of induction in both *C*₃ and *C*₄ *Alloteropsis* species. The AUC from 0 – 5 min was reduced in *C*₃ *A. semialata* GMT and *C*₄ *A. semialata* MDG compared to AUC in 21% O₂ under both light intensities, a significant effect ($P \leq 0.001$) that persisted well into the 5 – 10 min period for PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ($P \leq 0.02$). However, by 10 – 60 min the effect of O₂ was reversed in *C*₃ *A. semialata* GMT, with AUC for this period being significantly higher than for 21% O₂ ($P = 0.03$). For this period *C*₃ *A. semialata* GMT also had a significantly higher AUC in 2% O₂ than *C*₄ *A. semialata* MDG ($P \leq 0.01$; *C*₃ 504.4 ± 57.5 vs *C*₄ 116.4 ± 84.7 $\mu\text{mol m}^{-2}$ at PFD = 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and $P = 0.03$; *C*₃ 558.71 ± 94.6 vs *C*₄ 285.2 ± 108.7 $\mu\text{mol m}^{-2}$ at PFD

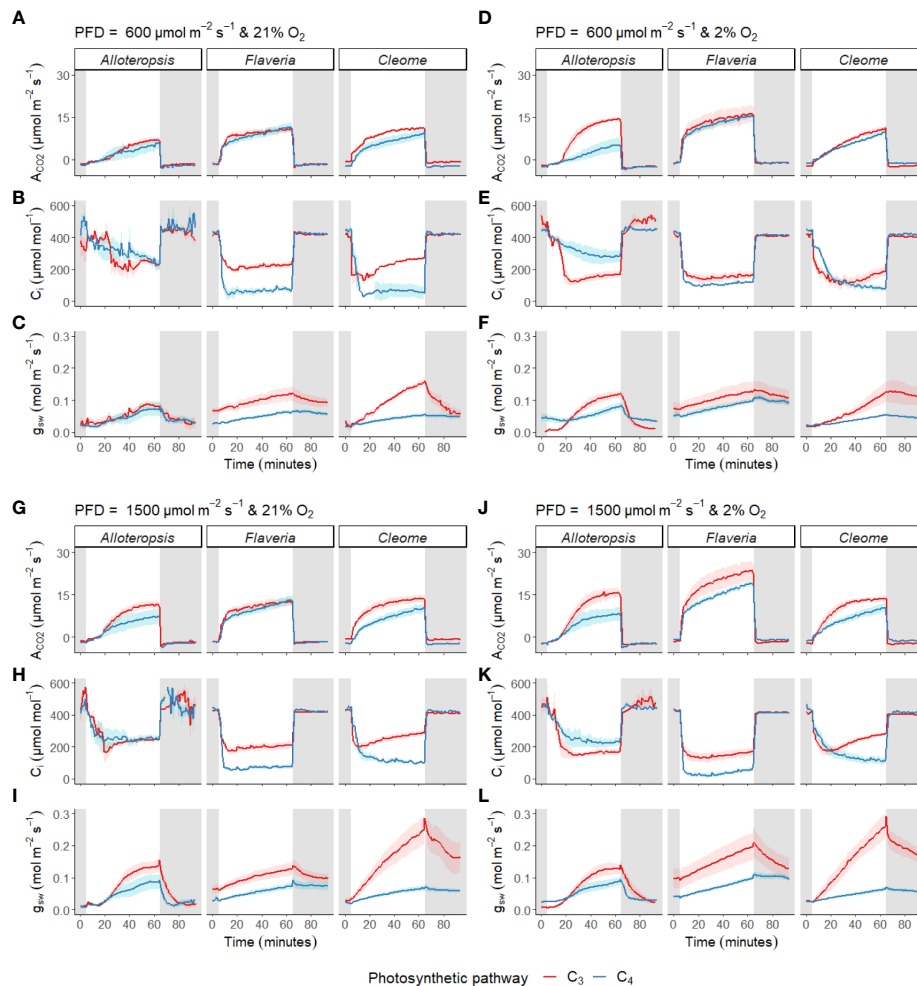


FIGURE 4

Measurements of gas exchange during light induction in phylogenetically linked C_3 and C_4 *Alloteropsis*, *Flaveria* and *Cleome* species. Leaves were acclimated to darkness, exposed to 1 hour of light, and returned to darkness for another half hour. Plots show net CO_2 assimilation (A_{CO_2} , A, D, G, J), intercellular CO_2 concentration (C_i , B, E, H, K) and stomatal conductance to water vapour (g_{sw} , C, F, I, L) across the light induction experiment, at PFD = $600 \mu mol m^{-2} s^{-1}$ and PFD = $1500 \mu mol m^{-2} s^{-1}$, in 21% and 2% O_2 . Ribbons represent standard error of the mean ($n = 5$).

= $1500 \mu mol m^{-2} s^{-1}$). Whereas the stimulating effect of 2% O_2 on transient CO_2 assimilation may be indicative of photorespiration as a negative factor during photosynthetic induction, the suppression of carbon assimilation found under 2% O_2 for both *Alloteropsis* species as well as the *Cleome* C_3 *T. hassleriana* could indicate photorespiration is not always detrimental to photosynthetic efficiency and may indeed support the activation of CO_2 assimilation in some C_3 and C_4 species.

Transient decoupling between electron transport and carbon fixation during induction is more pronounced in C_4 species and ameliorated by 2% O_2

During activation of CO_2 assimilation, a temporary decoupling between the electron transport chain and

photosynthetic carbon fixation in C_4 species could occur due to the time needed to activate the C_3 cycle, incomplete suppression of photorespiration due to an inactive CCM, or because of an increase in the energetic cost of carbon fixation via BS CO_2 leakage. To look for evidence of transient decoupling during induction, $\Phi PSII/\Phi CO_2$ ratios across the light induction period under each light and O_2 condition were further analysed within each genus (Figure 6 and Table 4).

The effect of time was significant for all genera. All the C_4 species showed higher $\Phi PSII/\Phi CO_2$ at the start of induction under 21% O_2 , with values gradually decreasing as the leaves became more acclimated to the light conditions. The average $\Phi PSII/\Phi CO_2$ ratio during induction was also noticeably higher than the steady state $\Phi PSII/\Phi CO_2$ for all species, which ranged between 8–12 e^-/CO_2 depending on the species, indicating a

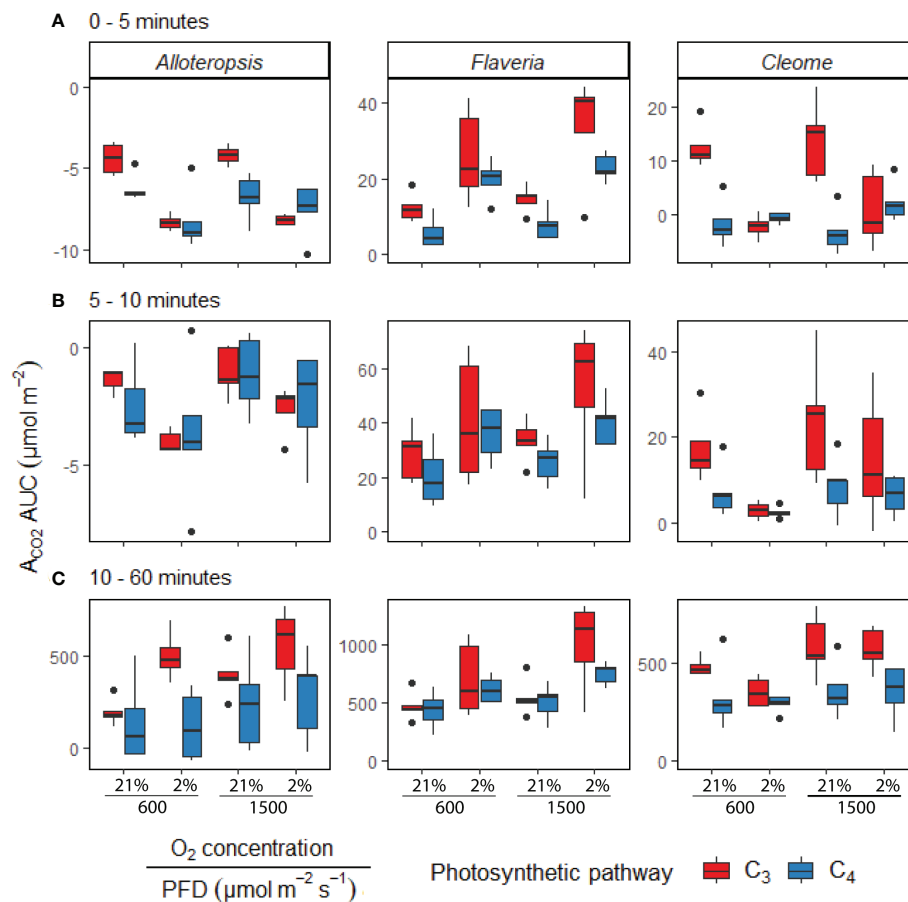


FIGURE 5

Boxplots of cumulative CO_2 assimilation over different phases of light induction in phylogenetically linked C_3 and C_4 *Alloteropsis*, *Flaveria* and *Cleome* species, under different light and O_2 treatments ($n = 5$ for each combination of species/measurement condition). Box edges represent first and third quartiles, the solid line indicates the median, and points represent outliers beyond 1.5 times the interquartile range. The area under the curve (AUC) was calculated from the A_{CO_2} of light induction experiments where plants at 21% or 2% O_2 concentrations were dark-adapted and exposed to $\text{PFD} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ or $\text{PFD} = 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 hour. Plots show the AUC of induction during 0 – 5 minutes (A), 5 – 10 minutes (B), and 10 – 60 minutes (C). Two-way ANOVAs (Table 3) were used to test the effect of photosynthetic pathway, O_2 concentration and their interaction on A_{CO_2} AUC at different phases of light induction in *Alloteropsis*, *Flaveria*, and *Cleome*.

significant transient decoupling during induction compared to steady state. In *Flaveria*, C_4 *F. bidentis* had higher $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio than C_3 *F. cronquistii* under all light and oxygen conditions, the complete opposite of steady state. This suggests the C_4 pathway in *Flaveria* does have some features that make the activation of photosynthesis more energetically demanding at the onset of light induction. Notably, the interaction of O_2 concentration and time also had a significant effect on $\Phi\text{PSII}/\Phi\text{CO}_2$ ($P = 0.01$ at $\text{PFD} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P = 0.01$ at $\text{PFD} = 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$), since in 2% O_2 the ratio decreased at an earlier point of induction than in 21% O_2 . During induction at $\text{PFD} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$, the three-way interaction was significant ($P = 0.04$), reflecting the strong decrease in $\Phi\text{PSII}/\Phi\text{CO}_2$ over time observed at 21% O_2 in C_4 *F. bidentis*. This

decrease may reflect the progressive suppression of photorespiration by activation of the C_4 CCM, since the same trend in $\Phi\text{PSII}/\Phi\text{CO}_2$ was not present in C_3 *F. cronquistii*, nor under 2% O_2 when photorespiration would have been negligible.

In *Cleome*, C_3 *T. hassleriana* also had a lower $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio than C_4 *G. gynandra*, in another reversal of differences observed during steady state conditions. $\Phi\text{PSII}/\Phi\text{CO}_2$ was significantly affected by time ($P \leq 0.01$ at $\text{PFD} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P = 0.01$ at $\text{PFD} = 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$) as well as O_2 concentration ($P \leq 0.001$ at both light intensities), but in contrast to *Flaveria* no significant interaction was found between O_2 and time ($P = 0.21$ at $\text{PFD} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P = 0.47$ at $\text{PFD} = 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Instead, in *Cleome* $\Phi\text{PSII}/\Phi\text{CO}_2$ was marginally lower at 2% O_2 than at 21% O_2 across the

TABLE 3 ANOVA table of the carbon assimilation AUC of different phases of light induction for phylogenetically linked C₃ and C₄ *Alloteropsis*, *Flaveria* and *Cleome* species.

Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Induction phase (minutes)		<i>Alloteropsis</i>			<i>Flaveria</i>			<i>Cleome</i>		
			PP	[O ₂]	PP:[O ₂]	PP	[O ₂]	PP:[O ₂]	PP	[O ₂]	PP:[O ₂]
600	0 – 5	df; <i>F</i> -value; <i>P</i> -value	1,16; 1.34; 0.26	1,16; 27.85; ≤0.001	1,16; 3.13; 0.10	1,16; 3.18; 0.09	1,16; 15.08; ≤0.01	1,16; 0.00; 0.95	1,16; 15.56; ≤0.01	1,16; 15.12; ≤0.01	1,16; 25.03; ≤0.001
	5 – 10	df; <i>F</i> -value; <i>P</i> -value	1,16; 0.20; 0.66	1,16; 5.81; 0.02	1,16; 0.80; 0.38	1,16; 0.86; 0.36	1,16; 3.60; 0.08	1,16; 0.05; 0.83	1,16; 5.01; 0.04	1,16; 10.06; ≤0.01	1,16; 2.95; 0.11
	10 – 60	df; <i>F</i> -value; <i>P</i> -value	1,16; 8.87; ≤0.01	1,16; 3.68; 0.07	1,16; 5.14; 0.03	1,16; 0.39; 0.54	1,16; 4.46; 0.05	1,16; 0.08; 0.78	1,16; 4.58; 0.05	1,16; 2.41; 0.14	1,16; 0.89; 0.36
1500	0 – 5	df; <i>F</i> -value; <i>P</i> -value	1,16; 3.81; 0.07	1,16; 22.30; ≤0.001	1,16; 10.14; ≤0.01	1,16; 6.39; 0.02	1,16; 24.21; ≤0.001	1,16; 0.33; 0.57	1,16; 9.48; ≤0.01	1,16; 2.07; 0.17	1,16; 12.85; ≤0.01
	5 – 10	df; <i>F</i> -value; <i>P</i> -value	1,16; 0.00; 0.99	1,16; 3.40; 0.08	1,16; 0.07; 0.78	1,16; 2.54; 0.13	1,16; 7.06; 0.01	1,16; 0.12; 0.73	1,16; 5.90; 0.02	1,16; 1.18; 0.29	1,16; 0.49; 0.49
	10 – 60	df; <i>F</i> -value; <i>P</i> -value	1,16; 5.09; 0.03	1,16; 1.06; 0.31	1,16; 0.33; 0.57	1,16; 2.16; 0.16	1,16; 12.59; ≤0.01	1,16; 1.01; 0.33	1,16; 12.96; ≤0.01	1,16; 0.03; 0.86	1,16; 0.00; 0.95

Photosynthetic pathway, PP. O₂ concentration, [O₂]. Interaction effect, PP:[O₂]. Table shows degrees of freedom, *F*-value, and *P*-value. Significant ($\alpha < 0.05$) *P*-values are shown in bold.

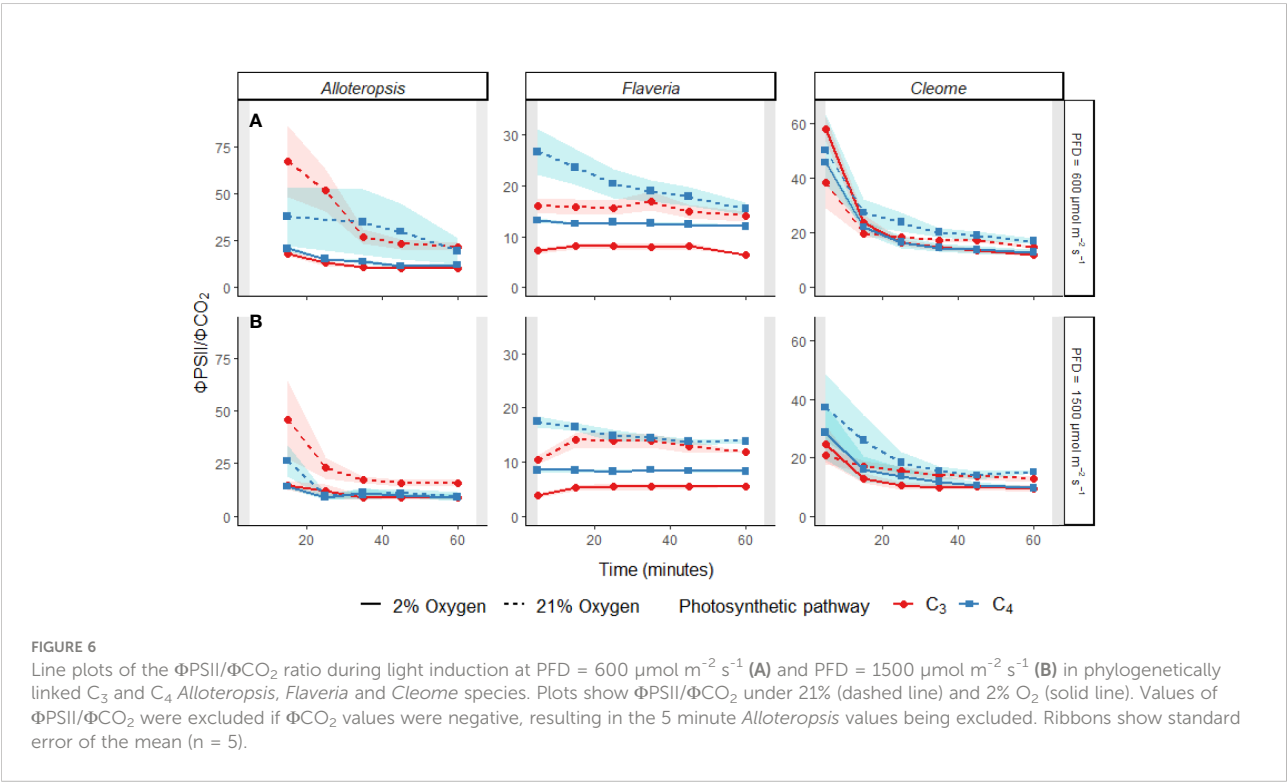


TABLE 4 ANOVA table of the $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio during light induction for phylogenetically linked C_3 and C_4 *Alloteropsis*, *Flaveria* and *Cleome* species.

Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Genus		PP	t	[O ₂]	PP:[O ₂]	PP:t	O ₂ :t	PP:[O ₂]: t
600	<i>Alloteropsis</i>	df; F-value; P-value	1,63; 0.11; 0.74	1,63; 28.69; ≤ 0.001	1,63; 13.07; ≤ 0.001	1,63; 0.71; 0.40	1,63; 0.62; 0.43	1,63; 5.98; 0.02	1,63; 0.93; 0.34
	<i>Flaveria</i>	df; F-value; P-value	1,100; 52.64; ≤ 0.001	1,100; 146.83; ≤ 0.001	1,100; 6.96; ≤ 0.01	1,100; 0.13; 0.73	1,100; 4.23; 0.04	1,100; 6.60; 0.01	1,100; 4.43; 0.04
	<i>Cleome</i>	df; F-value; P-value	1,98; 0.69; 0.41	1,98; 7.71; ≤ 0.01	1,98; 53.53; ≤ 0.001	1,98; 1.57; 0.21	1,98; 0.00; 0.95	1,98; 0.04; 0.83	1,98; 0.30; 0.58
1500	<i>Alloteropsis</i>	df; F-value; P-value	1,70; 3.30; 0.07	1,70; 11.82; ≤ 0.001	1,70; 12.50; ≤ 0.001	1,70; 3.90; 0.05	1,70; 0.60; 0.44	1,70; 4.20; 0.04	1,70; 0.46; 0.49
	<i>Flaveria</i>	df; F-value; P-value	1,101; 67.884; ≤ 0.001	1,101; 478.57; ≤ 0.001	1,101; 0.60; 0.44	1,101; 2.06; 0.15	1,101; 9.33; ≤ 0.01	1,101; 4.51; 0.03	1,101; 2.91; 0.09
	<i>Cleome</i>	df; F-value; P-value	1,101; 4.14; 0.04	1,101; 6.44; 0.01	1,101; 27.65; ≤ 0.001	1,101; 0.52; 0.47	1,101; 3.06; 0.08	1,101; 00.00; 0.96	1,101; 0.64; 0.42

Photosynthetic pathway, PP. Time, t. O₂ concentration, [O₂]. Interaction effects, PP:[O₂], PP:t, and PP:[O₂]:t. Table shows degrees of freedom, F-value, and P-value. Significant ($\alpha < 0.05$) P-values are shown in bold.

induction period. The temporal decrease in $\Phi\text{PSII}/\Phi\text{CO}_2$ was similar under both O₂ concentrations, suggesting that the transient decoupling between electron transport and CO₂ fixation was relatively insensitive to O₂ in both *Cleome* species. Finally, $\Phi\text{PSII}/\Phi\text{CO}_2$ ratios in *Alloteropsis* were not significantly affected by a main effect of photosynthetic pathway ($P = 0.74$ at PFD = $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P = 0.07$ at PFD = $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$), again in contrast to steady state where C_4 *A. semialata* MDG had lower ratios than C_3 *A. semialata* GMT. However, the interaction between photosynthetic pathway and O₂ was significant ($P \leq 0.001$ at both light intensities), due to the fact that the O₂ effect on $\Phi\text{PSII}/\Phi\text{CO}_2$ was much less pronounced in C_4 *A. semialata* MDG than in C_3 *A. semialata* GMT. Similar to the *Flaveria* and *Cleome* results, a significant interaction between O₂ concentration and time ($P \leq 0.001$ at both light intensities) was also observed in *Alloteropsis*, with 2% O₂ more significantly reducing $\Phi\text{PSII}/\Phi\text{CO}_2$ during the start of induction than towards the end.

Discussion

The presented experiments investigated the efficiency of photosynthesis during light induction in phylogenetically linked

Alloteropsis, *Flaveria*, and *Cleome* C_3 and C_4 species. Steady state and photosynthetic induction responses to light were measured to evaluate relative differences between paired species – controlling for evolutionary distance allowed for better differentiation between the effects of photosynthetic pathway and species-specific variation. At the start of light induction C_4 species had greater lag in CO₂ assimilation than C_3 species in all three comparisons (Figure 5A), confirming that the activation of CO₂ assimilation is generally slower in C_4 photosynthesis within the studied genera. However, the underlying reasons for this difference appeared to be genus specific. In C_4 *Flaveria*, slower induction appeared to be explained at least in part by less efficient suppression of photorespiration, since 2% O₂ resulted in increased CO₂ assimilation and fewer transferred electrons per fixed CO₂ (Figure 6). Although decreased photorespiratory electron sinks were also observable in C_4 *Alloteropsis* and *Cleome* induction under 2% O₂, there were no concurrent increases in CO₂ assimilation (Figure 5A), implying alternative limiting factors were at play, such as C_3 cycle activation. In C_3 *Cleome* and both *Alloteropsis* species, 2% O₂ actually suppressed activation of CO₂ assimilation, suggesting that photorespiration may support the induction of photosynthesis in these species.

Slower activation of CO₂ assimilation during light induction in C₄ versus C₃ photosynthesis

In line with previously observed photosynthetic induction responses (Pearcy and Seemann, 1990; Pearcy, 1990; Sassenrath-Cole and Pearcy, 1992; Mott and Woodrow, 2000) a transient reduction in CO₂ assimilation relative to steady state was observed in all species during light induction, with a more pronounced effect found in C₄ species (Figure 5). Greater losses of photosynthetic efficiency have previously been observed in C₄ grown under dynamic light in comparison to C₃ species and linked to mechanisms involving photosynthetic induction (Kubásek et al., 2013). Further fluctuating light work on plants grown under constant light (including C₄ *F. bidentis*) has also shown an increased lag in CO₂ assimilation in C₄ compared to C₃ species following step-increases in light intensity (Li et al., 2021). Similarly, in a study comparing a selection of C₃ and C₄ grasses (Lee et al., 2022), a biphasic increase in assimilation during the low to high light transition was the most significant limitation in maize and big bluestem, again emphasizing the C₄ lag in CO₂ assimilation. However, both Li et al. (2021) and Lee et al. (2022) studies examined the efficiency of fully induced photosynthesis subsequently exposed to stepwise decreases and increases in light intensity, whereas the C₄ lag-time when activation starts from darkness or from prolonged periods of low light may be even more pronounced.

During darkness or low light periods, stomatal closure could subsequently restrict photosynthetic assimilation during light induction due to a lack of coordination between CO₂ influx and assimilation. However, g_{sw} appears to increase in tandem with decreases in C_i , so stomatal opening does not seem to be the major source of limitation (Figures 4C, F, I, L). The lower g_{sw} found in C₄ species during light induction is consistent with studies that show C₄ species to have lower stomatal conductance and greater water use efficiency (Way et al., 2014; McAusland et al., 2016). It is worth noting that C₄ monocots under dynamic light have been reported to have faster stomatal opening and closing than C₃ monocots and dicots (Ozeki et al., 2022) yet stomatal kinetics in C₄ *A. semialata* MDG instead appear to be slower, again emphasizing the importance of accounting for species or genus-specific phenomena. However, although CO₂ availability can affect CO₂ assimilation during induction, other biochemical limitations appeared to dominate the responses observed here, as discussed in more detail below. The different C₄ decarboxylase pathways found across the three C₄ species studied have distinct energetic demands per cell type. The NADP-ME subtype found in C₄ *F. bidentis* and NAD-ME subtype in C₄ *G. gynandra* require substantial transfer of reductant between M and BS cells (Ishikawa et al., 2016) and steeper metabolite gradients for CCM operation, whilst mixed NADP-ME-PEPCK CCM found in C₄ *A. semialata* MDG can

meet ATP and NADPH requirements more cell autonomously (Yin and Struik, 2021). Modelling simulations indicate that reduced metabolite concentrations may be required to sustain this C₄ pathway (Wang et al., 2014a). The greater cell autonomy regarding energetic supply and demand found in C₄ *A. semialata* MDG could suggest a capacity for faster activation of photosynthetic assimilation, yet in the light induction experiments CO₂ assimilation in C₄ *A. semialata* MDG lagged behind the other C₄ species during the first ten minutes after starting light exposure (Figure 5A, B). In the following paragraphs we explore the different mechanisms underlying the slow activation of C₄ photosynthesis across the three genera.

Photorespiration during C₄ photosynthetic induction, disadvantageous or beneficial?

Reduced CO₂ assimilation during induction in C₄ species has been hypothesised to derive from the need to build up C₄ cycle intermediates leading to a lag in the efficient suppression of photorespiration (Sage and Mckown, 2006). If so, induction in C₄ species when the photorespiratory pathway is suppressed by low O₂ should result in an increase in photosynthetic carbon assimilation. This appeared to be confirmed in C₄ *F. bidentis*, where CO₂ assimilation during induction was higher under 2% O₂ than under 21% O₂ (Figure 5), in contrast to CO₂ assimilation in C₄ *F. bidentis* under steady state which showed no difference in A_{CO2} between O₂ concentrations (Figures 2A, D and Table 1). Comparatively, the lack of an equivalent improvement in CO₂ assimilation under 2% O₂ in C₄ *A. semialata* GMT and C₄ *G. gynandra* suggests that in these species the activation of the C₃ cycle (Sassenrath-Cole and Pearcy, 1992; Mott and Woodrow, 2000) could instead be the limiting factor.

Surprisingly, despite the stimulating effect of 2% O₂ on steady state CO₂ assimilation in C₃ *A. semialata* GMT and C₃ *T. hassleriana*, and lack of O₂ sensitivity in C₄ *A. semialata* GMT (Figures 2A, D and Table 1), CO₂ assimilation in all three species during the first 10 min of light induction was lower in 2% O₂ than in 21% O₂ (Figures 5A, B). Reverse sensitivity to O₂ in C₃ species has been linked to limitation by the rate of triose phosphate utilisation (TPU) (Sharkey, 1985). Low O₂ suppresses the net export of photorespiratory intermediates serine or glycine and limits endogenous pools of inorganic phosphate (P_i), as the amino acids come from phosphorylated plastidic metabolites that when used up in the cytosol liberate P_i otherwise used in the glycerate-PGA conversion (McClain and Sharkey, 2019). Additionally, reduced rates of starch biosynthesis from triose phosphates, and phosphoglucose isomerase inhibition have been found in low O₂ conditions (Dietz, 1985). The lower CO₂ assimilation observed in 2% O₂

compared to 21% O₂ during induction in C₃ *A. semialata* GMT and C₃ *T. hassleriana* could thus be due to 2% O₂ causing suboptimal stromal phosphate levels, thereby transiently exacerbating TPU limitation. It remains unclear whether C₄ species suffer from TPU limitation (Zhou et al., 2019), or whether alternative mechanisms may be involved.

The photorespiratory pathway has previously been suggested to help prime the C₄ cycle by providing a carbon source from which to build C₃ and C₄ metabolite pools (Kromdijk et al., 2014; Stitt and Zhu, 2014; Schlüter and Weber, 2020; Fu and Walker, 2022; Medeiros et al., 2022). In C₄ species, photorespiration could help establish CCM metabolic gradients through interconversion of 3-phosphoglyceric acid (3-PGA) and PEP (Arrivault et al., 2016), and in plants with NADP-ME decarboxylase, such as mixed subtype NADP-ME PEPCK C₄ *A. semialata* GMT, models suggest photorespiration could support the activation of redox-regulated C₃ enzymes and contribute to the formation of C₃ cycle intermediates in the BS through the triose phosphate transporter (TPT) (Weber and Von Caemmerer, 2010; Wang et al., 2014b). In C₃ photosynthesis, beyond its photoprotective role (Kozaki and Takeba, 1996), photorespiration has been shown to enhance CO₂ fixation through the assimilation of nitrogen (Busch et al., 2018). A recent metabolomic analysis in maize suggested that photorespiratory intermediates may also provide this supporting role in C₄ species (Medeiros et al., 2022).

Decoupling between electron transport and photosynthesis: Alternative electron sinks and BS leakiness

Particularly at the start of light induction, C₃ and C₄ species had significantly higher $\Phi\text{PSII}/\Phi\text{CO}_2$ ratios (Figure 6) than during steady state measurements (Table 1), indicating less of the reducing power of the electron transport chain was going towards photosynthetic carbon fixation. This was particularly prominent in the C₄ species, both in absolute values and relative to steady state, where C₄ plants had either lower (C₄ *Alloteropsis* and *Flaveria*) or equal (C₄ *Cleome*) $\Phi\text{PSII}/\Phi\text{CO}_2$ values compared to their C₃ counterparts. The build-up of metabolite pools to establish sufficient concentration gradients between M and BS cells required for the efficient operation of C₄ photosynthesis seems a likely contributing factor increasing $\Phi\text{PSII}/\Phi\text{CO}_2$ ratios in C₄ photosynthesis. However, the change in ratio could also be due to a variety of alternative electron sinks having greater presence during induction and drawing electrons away from the C₃ cycle.

Not surprisingly, reductions in $\Phi\text{PSII}/\Phi\text{CO}_2$ ratio under 2% O₂ were observed in all C₃ species as well as in C₄ *F. bidentis*, showing the importance of photorespiration as an electron sink. Although a gradual decrease in $\Phi\text{PSII}/\Phi\text{CO}_2$ across time was

observed during induction in both *Alloteropsis* and *Cleome* C₄ species (Figure 5), in contrast to C₄ *F. bidentis* the temporal changes were not O₂ sensitive. An alternative electron sink to photorespiration could be the Mehler reaction, which reduces O₂ in the chloroplast to hydrogen peroxide and has been suggested to play a role in C₃ and C₄ photosynthesis (Sagun et al., 2021). Suppression of the Mehler reaction under 2% O₂ could be consistent with the small reductions in CO₂ assimilation observed in both *Alloteropsis* species and C₃ *T. hassleriana*, as the Mehler reaction supports ATP formation and the activity of related enzymes has been found to increase when photosynthesis is impaired (Fryer et al., 1998). However, evidence to support a significant contribution of the Mehler reaction to high rates of photosynthesis in both C₃ and C₄ species is generally lacking (Driever and Baker, 2011).

A transient increase in BS leakiness could be an alternative contributing factor to the elevated energetic cost of CO₂ assimilation during induction in C₄ *A. semialata* and C₄ *G. gynandra* that accounts for the lack of O₂ sensitivity. A lag in activation of the C₃ cycle following light exposure would result in an imbalance between the C₃ and C₄ cycles and greater leakage of CO₂ from the BS due to the CCM over-pumping, reducing quantum efficiency by requiring more ATP per CO₂ fixed (Kromdijk et al., 2014; Sage and McKown, 2006). Transient isotope discrimination measurements on sorghum and maize during the first 10 min following a step-increase in light intensity suggested that bundle sheath leakiness could be 60% higher than steady state (Kubásek et al., 2013; Wang et al., 2022) and remain elevated for up to 30 min. This seems consistent with the timing of the decrease in $\Phi\text{PSII}/\Phi\text{CO}_2$ during induction in the *Alloteropsis* and *Cleome* C₄ species. Thus, activation of CO₂ assimilation in these species may be limited by activation of the C₃ cycle, whereas the stable $\Phi\text{PSII}/\Phi\text{CO}_2$ values in C₄ *F. bidentis* under 2% O₂ suggest C₃ cycle activation is faster than the CCM in this species.

Conclusion

This study confirms C₄ photosynthesis experiences greater lag than C₃ photosynthesis during light induction – the greater depression of CO₂ assimilation in C₄ species was independently found in three evolutionary divergent comparisons of phylogenetically linked C₃ and C₄ species, providing experimental support for previous hypotheses and observations of less efficient photosynthetic induction in C₄ photosynthesis (Sage and McKown, 2006; Kubásek et al., 2013; Slattery et al., 2018; Li et al., 2021; Sales et al., 2021). Despite the generally slower induction of CO₂ assimilation found in all C₄ species in comparison to their C₃ pairs, the underlying mechanisms to explain these differences were distinctly different – less effective suppression of photorespiration

seemed to underlie the reduction in CO₂ assimilation in C₄ *Flaveria* whereas delayed activation of the C₃ cycle appeared to be the limiting factor in C₄ species in *Alloteropsis* and *Cleome*, where a potential supporting role for photorespiration in photosynthetic induction was also identified. The substantial variation observed between and across phylogenetic pairs during both steady state and light induction measurements underscores the crucial importance of controlling for evolutionary distance when studying differences between photosynthetic pathways.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JK conceived the study. JK, LAC and ACB designed the experiments. LAC carried out all experiments, data analysis and interpretation, and drafted the manuscript. RLV helped with the 2% O₂ experimental setup and provided support with gas exchange experiments. ELB procured the initial plant material. CRGS helped with data interpretation. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.1091115/full#supplementary-material>

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