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# The negative impact of shade on photosynthetic efficiency in sugarcane may reflect a metabolic bottleneck

Cristina R.G. Sales [a,b,*,1], Rafael V. Ribeiro [c,2], Paulo E.R. Marchiori [a,d,3], Johannes Kromdijk [b,4], Eduardo C. Machado [a,**,5]

[a] *Laboratory of Plant Physiology "Coaracy M. Franco", Center for Agriculture and Post-Harvest Biosystems, Agronomic Institute (IAC), 13020–902 Campinas, SP, Brazil*
[b] *Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK*
[c] *Laboratory of Crop Physiology, Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP), 13083–970 Campinas, SP, Brazil*
[d] *Sector of Plant Physiology, Department of Biology, University of Lavras (UFLA), 37200–900 Lavras, MG, Brazil*

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## ABSTRACT

Plants with $C_4$ metabolism normally have higher photosynthetic rates than $C_3$ ones. As a result, several of the most productive species known are NADP-ME $C_4$ grasses, such as sugarcane and maize. However, the advantages of the $C_4$ cycle are most evident under high light as the $CO_2$ concentrating mechanism comes at the expense of additional ATP. Recent works have suggested a negative impact of shading across NADP-ME $C_4$ grasses, causing a downregulation of maximal quantum efficiency of $CO_2$ assimilation ($\phi CO_{2,max}$). The mechanisms behind the loss in photosynthetic efficiency and whether these results apply for other $C_4$ crops species and within germplasm of a species remain unclear. We analysed the photosynthetic acclimation to shade in four sugarcane genotypes with contrasting yield. To find out whether the effects of leaf history, i.e., shading a leaf developed under full sunlight and exposed later on to shade differ from a leaf fully developed under shading, these two types of leaves were evaluated. Shaded sugarcane plants showed decreased $CO_2$ assimilation efficiency compared to plants grown under full sunlight. Based on the fluorescence measurements, it seems that this reduction coincided with a more reduced $Q_A$ redox state, which could point to a metabolic limitation downstream of the light-dependent reactions. The results were similar for all genotypes and were observed regardless of whether leaves developed under shade or under full sunlight conditions and exposed subsequently to shade, suggesting that light is the main factor affecting photosynthetic efficiency. This study reinforces the notion that this negative impact of shade could reflect a common bottleneck across NADP-ME $C_4$ grasses.

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*Abbreviations:* $A_{CO2}$, net $CO_2$ assimilation rate; $A'_{CO2}$, diurnal-integrated $A_{CO2}$; $A_{operating}$, $A_{CO2}$ measured when $c_a$ is 400 μmol mol$^{-1}$ (from the $A_{CO2}$ x $c_i$ response curve); $A_{potential}$, $A_{CO2}$ measured when $c_i$ is 400 μmol mol$^{-1}$ (from the $A_{CO2}$ x $c_i$ response curve); $A_{sat}$, maximum $CO_2$ assimilation under saturating light (from the $A_{CO2}$ x $Q$ response curve); CCM, $CO_2$ concentrating mechanism; $c_i$, intercellular $CO_2$ concentration; $CO_2$_r, air $CO_2$ concentration in the reference infra-red gas analyser; DAP, days after planting; $F_q'/F_m'$, PSII operating efficiency; $F_q'/F_v'$, PSII efficiency factor; $F_v'/F_m'$, PSII maximum efficiency; $g_s$, stomatal conductance; LCP, light compensation point (from the $A_{CO2}$ x $Q$ response curve); $L_s$, stomatal limitation (from the $A_{CO2}$ x $c_i$ response curve); $Q$, photosynthetic active radiation; $R_d$, dark respiration (from the $A_{CO2}$ x $Q$ response curve); $VPD_{leaf}$, leaf-to-air vapour pressure deficit; $V_{max}$, $c_i$-saturated rate of photosynthesis (from the $A_{CO2}$ x $c_i$ response curve); $V_{p,max}$, maximum carboxylation rate of phosphoenolpyruvate carboxylase (from the $A_{CO2}$ x $c_i$ response curve); θ, convexity (from the $A_{CO2}$ x $Q$ response curve); $\phi CO_2$, instantaneous quantum efficiency of $CO_2$ assimilation; $\phi CO_{2,max}$, maximal quantum efficiency of $CO_2$ assimilation (from the $A_{CO2}$ x $Q$ response curve).

* Corresponding author at: Laboratory of Plant Physiology "Coaracy M. Franco", Center for Agriculture and Post-Harvest Biosystems, Agronomic Institute (IAC), 13020–902 Campinas, SP, Brazil.

** Corresponding author.

*E-mail addresses:* crisgabi.sales@gmail.com, cr673@cam.ac.uk (C.R.G. Sales), rvr@unicamp.br (R.V. Ribeiro), paulo.marchiori@ufla.br (P.E.R. Marchiori), jk417@cam.ac.uk (J. Kromdijk), eduardo.machado@sp.gov.br (E.C. Machado).

[1] ORCID: 0000–0002-8748–7370
[2] ORCID: 0000–0002-1148–6777
[3] ORCID: 0000–0001-9767–9336
[4] ORCID: 0000–0003-4423–4100
[5] ORCID: 0000–0002-6512–5408

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## 1. Introduction

Ongoing rapid human population growth and stagnation of the marginal yield gains of several commodity crops (Ray et al., 2013) have put the subject of crop productivity firmly back on the global research agenda. High photosynthetic efficiency is one important characteristic of highly productive plant species and $C_4$ photosynthesis has a particularly strong association with high productivity. Several of the most productive plant species known are $C_4$ species, such as the established commodity crops maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum* spp.), the second-generation bioenergy crops *Miscanthus* x *giganteus* and switchgrass (*Panicum virgatum*), as well as some of the world's worst weeds, such as barnyard grass (*Echinochloa glabrescens*; Covshoff et al., 2015) and Bermuda grass (*Cynodon dactylon*; Sage, 2016). The $C_4$ photosynthetic pathway concentrates $CO_2$ into specialised leaf internal compartments where the central Calvin-Benson-Bassham cycle enzyme Rubisco is exclusively localised. As a result, RuBP oxygenation and photorespiration are largely reduced, and high $CO_2$ assimilation rates can be achieved. The advantages of $C_4$ photosynthesis are most apparent when radiation is high, but under low radiation the benefits are less apparent. The dicarboxylic acid shuttle across mesophyll and bundle sheath cells which forms the basis of the $CO_2$ concentrating mechanism (CCM) in $C_4$ species comes at the expense of additional ATP needed to drive the regeneration of phosphoenolpyruvate (PEP), the substrate for initial inorganic carbon fixation by PEP-carboxylase. This additional energy expense lowers the maximum quantum yield of the $C_4$ pathway, making the pathway less energetically favourable and thus, less beneficial when light is limiting $CO_2$ assimilation. This may explain the decreased prevalence of $C_4$ species in understorey, deep shade environments, although several notable exceptions exist (Sage, 2013).

Leaves in the dense canopies of $C_4$ crops develop in full sunlight and subsequently get shaded by overtopping leaves and adjacent plants. Considering the importance of light-limited $CO_2$ assimilation for overall canopy $CO_2$ fixation and biomass productivity (Day and Chalabi, 1988; Long, 1993; Marchiori et al., 2010; Qu et al., 2017), efficient acclimation to low light conditions and altered spectrum within the canopy is important for photosynthetic performance, and its importance will likely increase further with the increasing trend in plant density (e.g., Assefa et al., 2018). Leaf acclimation to low light commonly involves the reallocation of resources to increase light harvesting capacity at the expense of photosynthetic capacity. In addition, acclimation to shade at the plant level typically triggers redistribution of photosynthetic capacity to leaves receiving most light (Pons et al., 2001; Ribeiro et al., 2017), as well as adjustment of biomass allocation patterns to above-ground plant parts, to enhance the acquisition of light energy to drive carbon assimilation (Bloom et al., 1985; Poorter et al., 2015). The reallocation of nitrogen from soluble protein into pigment-protein complexes in leaves exposed to low light (Boardman, 1977; Evans and Poorter, 2001; Hubbart et al., 2007; Collison et al., 2020) should sustain or increase maximal quantum efficiency of $CO_2$ assimilation ($\phi CO_{2,max}$) under limiting light. However, recent work has suggested that understorey leaves of maize, *Miscanthus* x *giganteus* (Pignon et al., 2017; Collison et al., 2020) and sorghum (Jaikumar et al., 2021) instead show decreased $\phi CO_{2,max}$. Even though differences in leaf age (Pignon et al., 2017) and angle (Jaikumar et al., 2021) were explored in these experiments, the downregulation of $\phi CO_{2,max}$ appeared to derive specifically from acclimation to the altered light conditions, and not due to leaf aging. However, the specific mechanisms behind the decrease in $\phi CO_{2,max}$ remain unclear. In addition, it is not clear if these findings also apply to other $C_4$ crop species and if they vary within germplasm of a species (Jaikumar et al., 2021).

Several possible mechanisms can underpin the decrease in performance of $C_4$ photosynthesis under limiting light conditions. The efficiency of $C_4$ photosynthesis relies on the coordination of $C_4$ acids produced in the mesophyll cells and their subsequent decarboxylation in the bundle sheath cells. An efficient decarboxylation reaction is essential to achieve high $CO_2$ concentration around Rubisco (Osmond and Harris, 1971; Hatch and Osmond, 1976; Stitt and Heldt, 1985). In low irradiance, however, the coordination between the $C_3$ and $C_4$ cycles could be impacted, resulting in two main outcomes: an inefficient CCM causing an increase in photorespiration; or an excessive CCM, resulting in $CO_2$ leaking back to the mesophyll. While the additional energy expenditure due to leakiness has a small impact on photosynthetic rates under high light conditions, such loss of efficiency could cause a decrease in $\phi CO_{2,max}$ in $C_4$ plants grown under shade (von Caemmerer and Furbank, 2003; Kromdijk et al., 2008).

To address these questions, we analysed the process of acclimation to shaded conditions in sugarcane, testing the hypothesis that $\phi CO_{2,max}$ is decreased in sugarcane leaves under low light conditions, and that such decrease is more pronounced in leaves fully developed under shading conditions than in leaves developed under high light and exposed later on to shade. Four sugarcane genotypes, three originating from the same genetic background but with contrasting yield potential (IAC48–65, SP70–1143 and IAC87–3396, Espironelo et al., 1988; Landell et al., 1997, 1999), and one additional high-yielding variety (IACSP95–5000, Landell et al., 2005) were subjected to full sunlight or artificial shade conditions. The specific objectives were three-fold. Firstly, to test the effects of leaf history, i.e., test for differences in the shade effect between leaves developed under full sunlight condition and exposed later on to shade and leaves fully developed under shade conditions; secondly, to characterise the phenotypic effects of acclimation to shade in terms of photosynthetic efficiency and biomass allocation; and thirdly, to find out whether there is any genotypic variation in shade acclimation traits. The results show that photosynthetic efficiency in sugarcane plants decreased due to shading, regardless of the light environment during leaf development, suggesting that light, not leaf history is the main factor affecting the loss in photosynthetic efficiency.

## 2. Material and methods

### 2.1. Plant material, growth conditions and experimental timeline

Four sugarcane varieties were used: IAC48–65, SP70–1143 and IAC87–3396, originated from the same genetic background (Fig. S1) and released in 1965 (Espironelo et al., 1988), 1982 (Landell et al., 1999), and 1997 (Landell et al., 1997), respectively; and IACSP95–5000, which originates from a different genetic background (Fig. S2) and is known for its high yield and responsiveness to agricultural inputs (Landell et al., 2005).

Stalk segments (15 per cultivar) with one bud were planted in plastic tubes of 120 $cm^3$ containing commercial compost mix (Carolina Soil, Vera Cruz, RS, Brazil) and kept in a greenhouse. At 48 days after planting (DAP) the plantlets were transferred to 12 $dm^3$ pots containing a mixture of soil and compost mix (1:1, v/v), and kept outdoor. Pots were kept distant enough to do not cause shading between plants. Plants were always under well-watered conditions. The substrate water potential ranged from −2 to − 5kPa (WaterMark 200SS, Irrometer, Riverside, CA, USA). Pots were maintained under full sunlight and fertilised as described by Sales et al. (2018). At 89 DAP, five plants per genotype were sampled for biomass analysis. At 90 DAP, half of the remaining plants (five per genotype) was kept under full sunlight, referred as sun treatment hereafter, and the other half transferred to artificial shaded conditions (approximately 30% of full sunlight; referred as shade treatment hereafter; Fig. S3) by using a reflective aluminised polypropylene net (Freshnet, Solpack, Rio das Pedras, SP, Brazil). The photosynthetic active radiation ($Q$) was monitored during the whole experiment with a quantum sensor (Li-190, Licor, Lincoln, NE, USA) and the maximum $Q$ in the sun and shade treatments are shown in Fig. S3. Subsequently, plants were subjected to two series of measurements to determine the effect of shade on leaves developed in full sunlight (samplings between 96 and 109 DAP) and the effect of shade on leaves developed in shade (samplings between 124 and 138 DAP).

For the analyses on leaves developed in full sunlight, measurements were performed on the 2nd youngest fully-expanded leaves that had developed in full sunlight prior to the switch to sun and shade treatments. Diurnal courses of net $CO_2$ assimilation rate ($A_{CO2}$) were measured at six days of treatment (96 DAP). Response curves of $A_{CO2}$ to increasing $Q$ and to increasing intercellular $CO_2$ concentration ($c_i$) were performed between 8 and 9 days of treatment (98–99 DAP) and 15 and 16 days of treatment (105–106 DAP), respectively, in the mid-section of the second youngest fully expanded leaf of each plant. At 19 days of treatment (109 DAP) leaves were sampled and specific leaf area (SLA) calculated.

For the analyses on leaves developed in shade, measurements were performed on leaves that had developed in either under sun or shade treatment, but leaf age was similar to the measured leaves in sampling time 2 (2nd youngest fully expanded leaf). Diurnal courses of $A_{CO2}$ were measured at 34 and 35 days of treatment (124 and 125 DAP, respectively); and $A_{CO2}$ x $Q$ and $A_{CO2}$ x $c_i$ responses were evaluated between 39 and 42 days of treatment (129–132 DAP). At 48 days of treatment (138 DAP) plants were used for biometric measurements via destructive harvest. The measurement sequence is summarised in Fig. S4.

### 2.2. Photosynthetic measurements

Two Li-6400 IRGA equipped with a fluorometer 6400–40 LCF (Licor, Lincoln, NE, USA) were used to assess photosynthetic parameters in the sugarcane varieties. Diurnal courses of $A_{CO2}$ were evaluated with in situ survey-style measurements (around 2 min per measurement, randomised between the two Li-6400 and the light treatments) taken approximately every 2 h from 7.00 h to 17.00 h at six days of treatment (96 DAP), and at 34 and 35 days of treatment (124 and 125 DAP, respectively). At 96 DAP and 124 DAP, $Q$ inside the Li-6400 cuvette mimicked the $Q$ incident in the sun treatment environment (pre-determined just before each time-point), while at 125 DAP, light mimicked the $Q$ incident in the shade treatment environment. The air $CO_2$ concentration in the reference infra-red analyser ($CO_{2\_}r$) was maintained at 400 μmol mol$^{-1}$ and flow rate was 500 μmol s$^{-1}$. The diurnal-integrated $A_{CO2}$ ($A'_{CO2}$) was calculated by using a trapezoidal rule (Jawień, 2014) to obtain the area under the curve (Makowski et al., 2019) of the diurnal courses of $A_{CO2}$. During measurements, the conditions inside the Li-6400 cuvette varied between 25 and 34 °C for leaf temperature and 1.4 and 2.7 kPa for leaf-to-air vapour pressure deficit ($VPD_{leaf}$).

Response curves of $A_{CO2}$ and chlorophyll fluorescence parameters to increasing $Q$ were performed between 8 and 9 days of treatment (98–99 DAP) and between 39 and 41 days of treatment (129–131 DAP). For these measurements, plants were transferred to a glasshouse approximately 1 h before the curves were performed. During measurements, air temperature inside the cuvette was 32 ± 3ºC, $VPD_{leaf}$ was 2.7 ± 0.5 kPa, flow rate was 500 μmol s$^{-1}$ and $CO_{2\_}r$ concentration was 400 μmol mol$^{-1}$. Leaves were enclosed in the cuvette and acclimated to steady-state under $Q$ of 2000 μmol m$^{-2}$ s$^{-1}$. Afterward, $Q$ was then stepped down to 0 μmol m$^{-2}$ s$^{-1}$ and gas exchange and fluorescence data were logged when steady state was reached at each step. Before data for each step were logged, the reference and sample IRGA signals were matched. Light response curves were fitted by a non-rectangular hyperbola (Marshall and Biscoe, 1980) to estimate mitochondrial respiration ($R_d$), maximum $CO_2$ assimilation under saturating light ($A_{sat}$), convexity (θ) and light compensation point (LCP). Instantaneous quantum efficiency of $CO_2$ assimilation ($\phi CO_2$) was calculated at each $Q$ level with the leaf light absorption ($\alpha_{leaf}$) considered as 0.85 (Edwards and Baker, 1993; Baker, 2008).

Although the maximum quantum efficiency of $CO_2$ assimilation ($\phi CO_{2,max}$) is also obtained by the non-rectangular fitting described above, this method can lead to underestimation due to declining PSII efficiency with increasing irradiance (Yin et al., 2014). Thus, $\phi CO_{2,max}$ was instead estimated from the linear regression of $A_{CO2}$ against $Q$ for $Q$ between 50 and 200 μmol m$^{-2}$ s$^{-1}$ (Pignon et al., 2017).

Simultaneously to the gas exchange measurements, chlorophyll *a* fluorescence emission in light-adapted leaves was evaluated and some photochemical variables were estimated according to Edwards and Baker (1993) and Baker (2008): PSII maximum efficiency ($F_v'/F_m'$), PSII operating efficiency ($F_q'/F_m'$), and PSII efficiency factor ($F_q'/F_v'$) were calculated at each $Q$ level of the light response curves.

Response curves of $A_{CO2}$ to increasing $c_i$ were performed between 15 and 16 days of treatment (105–106 DAP) and 39 and 42 days of treatment (129–132 DAP). During measurements, air temperature inside the cuvette was 31 ± 2 ºC, $VPD_{leaf}$ was 2.5 ± 0.4 kPa, flow rate was 500 μmol s$^{-1}$ and $Q$ was 2000 μmol m$^{-2}$ s$^{-1}$. Leaves were enclosed in the cuvette and induced to steady-state under $CO_{2\_}r$ concentration of 400 μmol mol$^{-1}$. The $CO_{2\_}r$ was then stepped down through 300, 200, 100, 75, 50, and back up to 400, 600, 900 and 1200 μmol mol$^{-1}$. Before the data for each step was logged, the reference and sample IRGA signals were matched. The $A_{CO2}$ x $c_i$ response curves were leak-corrected using data obtained by performing curves with heat-killed sugarcane leaves (n = 4), as recommended by Flexas et al. (2007). The $A_{CO2}$ x $c_i$ response curves were fit to a nonrectangular hyperbolic function (von Caemmerer, 2000). The initial part of the curve was used to estimate the maximum carboxylation rate of phosphoenolpyruvate carboxylase ($V_{p,max}$). A linear model of $A_{CO2}$ as a function of $c_i$ was fitted and the breaking point detected. The response of $A_{CO2}$ to $c_i <$ breaking point was used to solve $V_{p,max}$, and $K_p$, the apparent Michaelis-Menten constant of PEPC for $CO_2$, assumed to be 196 μbar at 30 °C (Boyd et al., 2015). The $c_i$-saturated rate of photosynthesis ($V_{max}$) was estimated as the predicted value of each function for $c_i > 2000$ μmol mol $^{-1}$.

The stomatal limitation ($L_s$) of photosynthesis was calculated according to Lawlor (2002) as $[(A_{potential}–A_{operating})/A_{potential}]$x100, where $A_{potential}$ denotes $A_{CO2}$ measured when $c_i$ is 400 μmol mol$^{-1}$ (infinite stomatal conductance, $g_s$), and $A_{operating}$ denotes $A_{CO2}$ measured when $c_a$ is 400 μmol mol$^{-1}$ (finite $g_s$).

### 2.3. Specific leaf area and biomass

Plants were destructively harvested at 89 and 138 DAP. Plant leaf area was measured using a Li-3000 C leaf area metre (Licor, Lincoln, NE, USA). Leaf, shoot and root biomass fractions were separated and dried in an oven (MA032, Marconi, Piracicaba, SP, Brazil) at 60 °C until constant weight after which dry weights were determined. Specific leaf area was calculated as the ratio between whole plant leaf area and leaf dry matter.

### 2.4. Statistical analysis

Statistical analyses were performed in RStudio (version 1.4.1103; R Studio Team, 2021). Analysis of variance was used to test the effect of genotype (G, IAC48–65 vs. SP70–1143 vs. IAC87–3396 vs. IACSP95–5000), light treatment (L, sun vs. shade), leaf history (H, leaves developed under full sunlight condition and exposed later on to shade vs. leaves fully developed under shade conditions), and the three-way interaction (G vs. L vs. H). Data for the different traits were tested for homogeneity of variances by Levene's test ($\alpha = 0.05$) and normality of studentized residual distribution using Shapiro-Wilk test ($\alpha = 0.05$). Bar charts and scatterplots were prepared using ggplot2 (Wickham, 2016). For the $A_{CO2}$ x $c_i$ fitting, package segmented was used (Muggeo, 2008). For the area under the curve to obtain the $A'_{CO2}$, bayestestR library was used (Makowski et al., 2019). Regression analysis between pairs of variables were performed using RStudio.

## 3. Results

### 3.1. Shade effects on physiological determinants of photosynthetic efficiency

In this study, four sugarcane genotypes were subjected to full sunlight (sun) or artificial shade (shade). Photosynthetic responses to sun and shade treatments were analysed in similarly aged leaves which had either already developed under full sunlight prior to the switch to light treatments or which completely developed during the treatments.

Diurnal courses of $CO_2$ assimilation were performed with the light incident in the sun environment at 96 DAP and at 124–125 DAP (Supplementary Fig. S5). The integrated $A_{CO2}$ ($A'_{CO2}$) values of the diurnal curves are shown in Fig. 1A-B. Even though all leaves were measured under the same light intensities, plants grown under shade conditions had a significant reduction in the $A'_{CO2}$ throughout the day (~38 %) when compared to sun treated plants. Responses were similar among all four genotypes and there was no significant interaction between the impact of shade and the sampling timepoint when the measurements were performed. Thus, the decrease due to shading was similar in leaves developed under full-sunlight and later transferred to shaded conditions (Fig. 1A) compared to leaves completely developed under shading (Fig. 1B). Remarkably, when leaves fully developed under each condition were exposed to the diurnal course with the light incident in the shade treatment (Supplementary Fig. S5, Fig. 1C), shaded plants also showed ~36% reduction in $A'_{CO2}$ when compared to sun treated plants. This indicates that the diminished performance of the shaded leaves did not reflect a trade-off between low light and high light performance, but rather an impairment under both light environments.

To further characterise the reduction of photosynthesis under shading conditions, light response ($A_{CO2}$ x $Q$) curves were performed (Fig. 2). Parameters derived from these response curves, i.e. light-saturated $CO_2$ assimilation ($A_{sat}$) per area basis, mitochondrial respiration ($R_d$), the light compensation point (LCP) and the maximum quantum yield of $CO_2$ assimilation ($\phi CO_{2,max}$), were all significantly lower ($p < 0.05$) in the shaded plants (Table 1) without genotypic differences. Of these parameters, only $R_d$ and LCP showed a significant effect of sampling time, i.e., between leaves developed under full-sunlight and later transferred to shaded conditions, or leaves completely developed under shading. These differences appeared to be driven by changes in $R_d$, which in turn impacted the light compensation point. For leaves pre-developed in sunlight and subsequently moved to shade, $R_d$ per area strongly decreased to approximately 43% of the rate in the sun treatment, leading to a reduction in LCP to 46% of the sun treatment (Table 1). These differences were less pronounced for leaves fully developed in the shade where $R_d$ and LCP were 62% and 69% of the sun treatment values, respectively. The maximum quantum yield of $CO_2$ assimilation ($\phi CO_{2,max}$), which is considered a key parameter for photosynthetic light-use efficiency, was significantly lower in sugarcane plants grown under shade ($p = 0.015$) independently of the leaf light history and genotype (Table 1). This reduction was ~4% and ~11% for 98–99 DAP and 129–132 DAP sampling times, respectively. The convexity of the light response curves did not differ significantly between treatments or among genotypes.

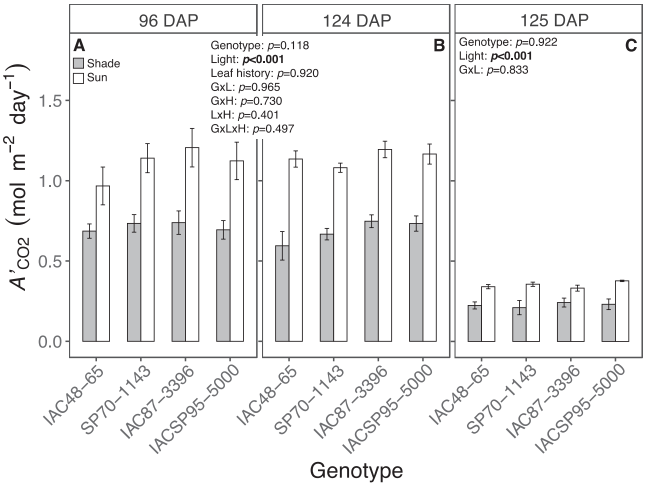


**Fig. 1.** Diurnal-integrated $CO_2$ assimilation ($A'_{CO2}$) calculated from the diurnal change in leaf $CO_2$ assimilation ($A_{CO2}$) in the sugarcane varieties IAC48–65, SP70–1143, IAC87–3396, and IACSP95–5000 grown under full sunlight (sun) or artificial shading conditions (shade) for: (A) 6 days (96 DAP), and (B and C) 34–35 days (124–125 DAP). Data in (A) and (B) were obtained with the incident light in the full sunlight environment while in (C) the incident light was the one incident in the shaded environment. Values are means ± SEM ($n = 5$ biological replicates). $p$-values are from ANOVA testing the effects of genotypes (G), light treatment (L), leaf history (H) and the interactions (GxL, GxH, LxH, GxLxH).

Operating efficiency of PSII ($F_q'/F_m'$) was determined from fluorescence measurements for the different light intensities used in the $A_{CO2}$ x $Q$ curve (Supplementary Fig. S6). Consistent with the $A_{CO2}$ measurements, $F_q'/F_m'$ was lower in plants grown under shade than under sun conditions, independently of the leaf history that the measurement was performed. Consistently, $\phi CO_2$ and $F_q'/F_m'$ were significantly correlated and the slope of the relationship was not different between light treatments and genotypes at both measured points ($p = 0.248$–$0.264$; Fig. 3A and B). The $F_q'/F_m'$ provides an estimation of the efficiency at which light absorbed by PSII is used for $Q_A$ reduction (Baker, 2008) and is given by the product of PSII maximum efficiency ($F_v'/F_m'$, i.e., $F_q'/F_m'$ if all $Q_A$ was oxidised) and PSII efficiency factor ($F_q'/F_v'$, which approximately equates to the proportion of oxidised $Q_A$). Whereas $F_q'/F_v'$ had a similar response to light compared to $F_q'/F_m'$, $F_v'/F_m'$ was not different between sun and shade plants (Supplementary Fig. S7) and indeed, the relationship between shade and sun plants was very close to a linear response (Fig. 3C and D), suggesting that the reduction in photochemical efficiency due to downregulation of light harvesting efficiency was not different between sun and shade treatments. However, shaded leaves showed lower $F_q'/F_v'$ (significant deviation from the 1 by 1 line, Fig. 3E and F), demonstrating that the decrease in $F_q'/F_m'$ in shaded leaves at a common light intensity coincided with an increase in the number of closed PSII reaction centres, hinting at a possible metabolic limitation.

The $CO_2$ response curves were used to further assess the limitations to $A_{CO2}$ (Fig. 4). The $A_{CO2}$ under operating $c_i$ ($A_{op}$) or when $c_i$ equals ambient $c_a$ ($A_{pot}$) were always significant lower ($p < 0.001$) in shade plants, at both sampling timepoints analysed. Interestingly, the oldest variety used in this study, IAC48–65, also displayed the lowest rates of $A_{op}$ and $A_{pot}$ in both light treatments. Stomatal limitation calculated from $A_{op}$ and $A_{pot}$ did not show any clear trend in response to shade.

The maximum carboxylation capacity of PEPC ($V_{p,max}$) did not change significantly between light treatments at either sampling timepoint (Fig. S8). However, the $c_i$-saturated rate of photosynthesis ($V_{max}$) was significantly lower in shade plants ($p < 0.001$), being most pronounced in leaves completely developed in shade (Fig. S8). Again, the oldest genotype, IAC48–65 showed the lowest $V_{max}$ under both light conditions.

### 3.2. Shade effects on leaf morphology and biomass allocation patterns

Shade caused a clear increase in SLA in all varieties in both sampling times analysed ($p < 0.001$; Fig. 5). Plants grown under shaded conditions allocated practically all the new growth to the shoot (Fig. 6B and D), which led to a pronounced increase in the shoot:root DM (Fig. 6F). The biomass yield patterns in the sun grown plants were consistent with the year that the three related genotypes were released, with the oldest one IAC48–65 showing the lowest biomass production, SP70–1143 intermediate, and the newest genotype IAC87–3396 the highest. However, under shade conditions, the differences between genotypes were lost and all plants showed a very pronounced decrease in biomass production, especially in the roots (Fig. 6A-D). These results are consistent with the fact that the decrease in photosynthesis efficiency was not genotype-dependent.

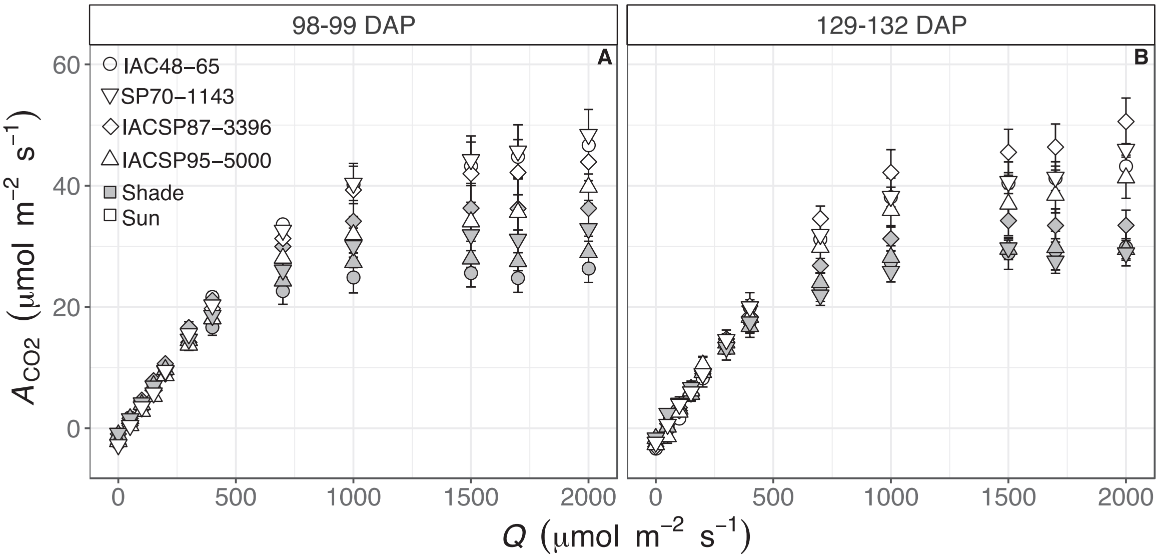


**Fig. 2.** Response curves of leaf $CO_2$ assimilation ($A_{CO2}$) to increasing photosynthetic active radiation ($Q$) in the sugarcane varieties IAC48–65, SP70–1143, IAC87–3396, and IACSP95–5000 grown under full sunlight (sun) or artificial shading conditions (shade) for (A) 8–9 days (98–99 DAP) and (B) 39–42 days (129–132 DAP). Values are means ± SEM ($n = 3$–4 biological replicates).

**Table 1**
Parameters estimated from the response curves of leaf $CO_2$ assimilation ($A_{CO2}$) to increasing photosynthetic active radiation ($Q$) in the sugarcane varieties IAC48–65, SP70–1143, IAC87–3396, and IACSP95–5000 grown under full sunlight (sun) or artificial shading conditions (shade) for 8–9 days (98–99 DAP) and 39–42 days (129–132 DAP).

| Genotype | Light | DAPDAP | $A_{sat}$ (μmol m$^{-2}$ s$^{-1}$) | $A_{sat}$ (μmol g$^{-1}$ s$^{-1}$) | $R_d$ (μmol m$^{-2}$ s$^{-1}$) | $R_d$ (μmol g$^{-1}$ s$^{-1}$) | LCP (μmol m$^{-2}$ s$^{-1}$) | θ (dimensionless) | $\phi CO_{2,max}$ (μmol mol$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| IAC-4865 | sun | 98–99 | 52.0 ± 1.1 | 0.40 ± 0.01 | 2.63 ± 0.21 | 0.020 ± 0.002 | 39.8 ± 2.3 | 0.87 ± 0.02 | 0.060 ± 0.002 |
| SP70–1143 | | | 54.3 ± 5.3 | 0.48 ± 0.05 | 2.93 ± 0.10 | 0.026 ± 0.001 | 45.8 ± 2.3 | 0.88 ± 0.02 | 0.059 ± 0.003 |
| IAC87–3396 | | | 48.5 ± 5.0 | 0.49 ± 0.05 | 2.52 ± 0.41 | 0.025 ± 0.004 | 42.8 ± 6.4 | 0.89 ± 0.08 | 0.057 ± 0.003 |
| IACSP95–5000 | | | 44.6 ± 2.4 | 0.44 ± 0.02 | 2.74 ± 0.36 | 0.027 ± 0.004 | 44.7 ± 4.4 | 0.75 ± 0.07 | 0.054 ± 0.004 |
| IAC-4865 | shade | | 28.2 ± 2.3 | 0.29 ± 0.02 | 1.08 ± 0.25 | 0.011 ± 0.003 | 19.3 ± 4.5 | 0.84 ± 0.08 | 0.053 ± 0.003 |
| SP70–1143 | | | 35.4 ± 4.9 | 0.37 ± 0.05 | 1.08 ± 0.42 | 0.011 ± 0.004 | 18.2 ± 6.5 | 0.90 ± 0.03 | 0.053 ± 0.004 |
| IAC87–3396 | | | 40.1 ± 5.5 | 0.52 ± 0.07 | 1.27 ± 0.25 | 0.016 ± 0.003 | 20.4 ± 3.6 | 0.90 ± 0.04 | 0.061 ± 0.002 |
| IACSP95–5000 | | | 31.3 ± 2.1 | 0.33 ± 0.02 | 1.29 ± 0.13 | 0.001 ± 0.005 | 22.3 ± 1.8 | 0.87 ± 0.05 | 0.055 ± 0.002 |
| IAC-4865 | sun | 129–132 | 50.6 ± 5.6 | 0.38 ± 0.04 | 3.43 ± 0.46 | 0.026 ± 0.003 | 54.2 ± 4.8 | 0.83 ± 0.11 | 0.054 ± 0.004 |
| SP70–1143 | | | 53.1 ± 5.2 | 0.47 ± 0.05 | 2.71 ± 0.60 | 0.024 ± 0.005 | 38.7 ± 1.5 | 0.96 ± 0.02 | 0.054 ± 0.006 |
| IAC87–3396 | | | 55.8 ± 3.9 | 0.47 ± 0.03 | 3.41 ± 0.31 | 0.029 ± 0.003 | 54.2 ± 6.0 | 0.89 ± 0.04 | 0.064 ± 0.005 |
| IACSP95–5000 | | | 47.6 ± 3.2 | 0.39 ± 0.03 | 3.56 ± 0.35 | 0.029 ± 0.003 | 53.3 ± 4.6 | 0.75 ± 0.12 | 0.068 ± 0.008 |
| IAC-4865 | shade | | 34.2 ± 3.5 | 0.36 ± 0.04 | 2.62 ± 0.54 | 0.027 ± 0.006 | 42.0 ± 8.1 | 0.82 ± 0.07 | 0.050 ± 0.002 |
| SP70–1143 | | | 32.8 ± 1.7 | 0.33 ± 0.02 | 1.20 ± 0.22 | 0.012 ± 0.002 | 23.6 ± 8.1 | 0.78 ± 0.06 | 0.044 ± 0.006 |
| IAC87–3396 | | | 38.1 ± 2.6 | 0.42 ± 0.03 | 2.34 ± 0.62 | 0.025 ± 0.007 | 37.4 ± 6.1 | 0.90 ± 0.01 | 0.056 ± 0.004 |
| IACSP95–5000 | | | 35.0 ± 2.6 | 0.42 ± 0.02 | 2.00 ± 0.42 | 0.024 ± 0.005 | 37.0 ± 10.1 | 0.94 ± 0.04 | 0.059 ± 0.003 |
| Genotype | *p*-value | | 0.131 | **< 0.001** | 0.294 | 0.133 | 0.168 | 0.400 | 0.122 |
| Light | | | **< 0.001** | **0.003** | **< 0.001** | **< 0.001** | **< 0.001** | 0.667 | **0.047** |
| Leaf history | | | 0.413 | 0.566 | **< 0.001** | **0.005** | **< 0.001** | 0.705 | 0.656 |
| GxL | | | 0.375 | 0.197 | 0.723 | 0.326 | 0.934 | 0.131 | 0.655 |
| GxH | | | 0.764 | 0.347 | 0.135 | 0.224 | 0.099 | 0.909 | 0.138 |
| LxH | | | 0.872 | 0.512 | 0.457 | 0.086 | 0.153 | 0.721 | 0.276 |
| GxLxH | | | 0.500 | 0.206 | 0.890 | 0.911 | 0.972 | 0.516 | 0.714 |

Abbreviations: $A_{sat}$, $A_{CO2}$ at saturating $Q$ and ambient $CO_2$ (40 Pa); $R_d$, dark respiration, LCP, light compensation point; $\theta$, convexity of the non-rectangular hyperbolic response of $A_{CO2}$ to increasing PAR; $\phi CO_{2,max}$, maximum quantum yield of $CO_2$ assimilation obtained from linear regression of $A_{CO2}$ against $Q$ in the light levels between 50 and 200 μmol m$^{-2}$ s$^{-1}$. Curves were fitted according to Marshall and Biscoe (1980). Values are means ± SEM (n = 3–4 biological replicates). *p*-values are from ANOVA testing the effects of genotypes (G), light treatment (L), leaf history (H) and the interactions (GxL, GxH, LxH, GxLxH).

## 4. Discussion

The acclimation to shaded conditions in four sugarcane varieties with contrasting yield were analysed in this study. We studied leaves developed under full sunlight condition and exposed later on to shade, as well as leaves fully developed under shade conditions to find out whether the leaf developmental history affected photosynthetic acclimation to shade. Even though the present study has been performed under artificial shading, our findings allow interpretation of the effect of lower intensity without the confounding effects of self-shading on both light intensity as well as spectral quality. The results presented here are very robust, considering that the decrease in photosynthetic efficiency under shade was observed in all four genotypes, and both in leaves long-term or short-term acclimated to shade. Moreover, the results are consistent with previous observations on several other NADP-ME species artificially (Yabiku and Ueno, 2019) and naturally shaded (Pignon et al., 2017; Collison et al., 2020; Jaikumar et al., 2021). The fact that Tazoe et al. (2008) have not observed changes in $\phi CO_{2,max}$ in $C_4$ plants grown under artificial shading is probably related to species-specific responses, as they have been performed on the NAD-ME species *Amaranthus*

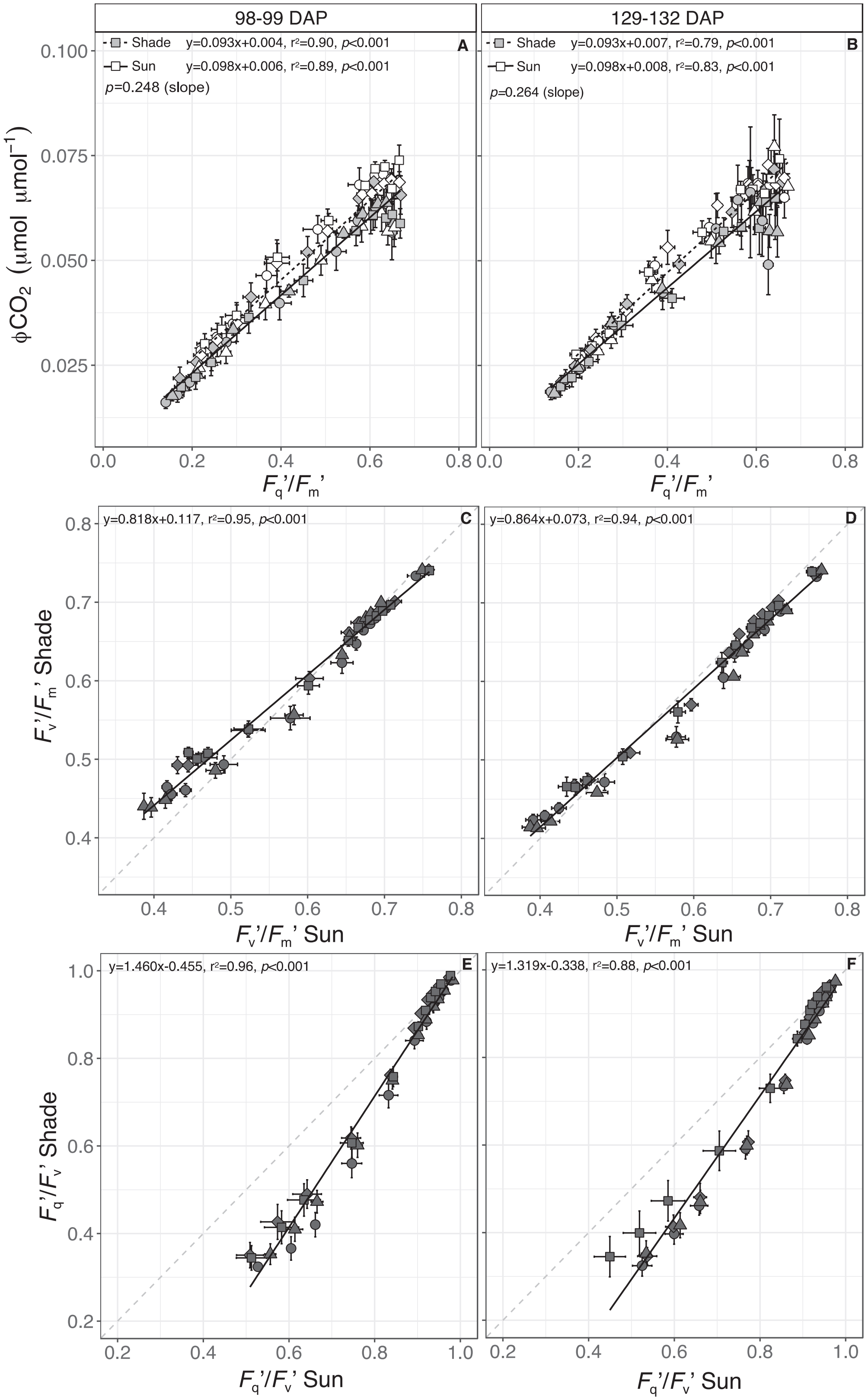
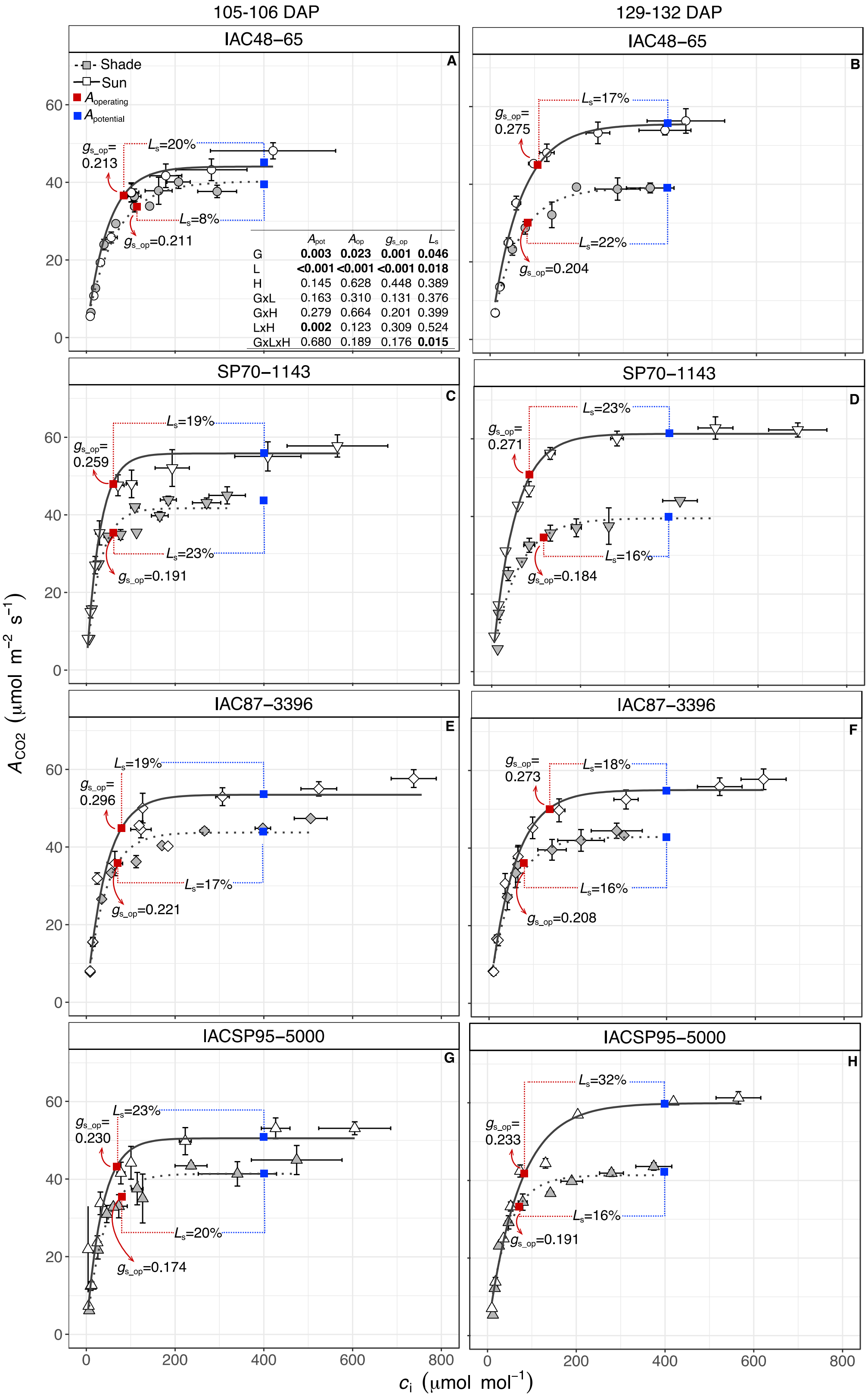


**Fig. 3.** Relationship between (A and B) PSII operating efficiency ($F_q'/F_m'$) and instantaneous quantum yield of $CO_2$ assimilation ($\phi CO_2$), and (C and D) PSII maximum efficiency ($F_v'/F_m'$) and (E and F) PSII efficiency factor ($F_q'/F_v'$) between plants grown under full sunlight (sun) or artificial shading conditions (shade) in the sugarcane varieties IAC48–65, SP70–1143 and IAC87–3396. Measurements were taken at 8–9 days of treatment (98–99 DAP) and 39–42 days of treatment (129–132 DAP). Values are means ± SEM ($n = 3$–4 biological replicates). In A and B the linear regressions between all the light levels in the $A_{CO2}$ x $Q$ curves is shown; in C-F the linear regression between sun and shade plants are shown.



| | $A_{pot}$ | $A_{op}$ | $g_{s\_op}$ | $L_s$ |
|---|---|---|---|---|
| G | **0.003** | **0.023** | **0.001** | **0.046** |
| L | **<0.001** | **<0.001** | **<0.001** | **0.018** |
| H | 0.145 | 0.628 | 0.448 | 0.389 |
| GxL | 0.163 | 0.310 | 0.131 | 0.376 |
| GxH | 0.279 | 0.664 | 0.201 | 0.399 |
| LxH | **0.002** | 0.123 | 0.309 | 0.524 |
| GxLxH | 0.680 | 0.189 | 0.176 | **0.015** |

**Fig. 4.** Response curves of leaf $CO_2$ assimilation ($A_{CO2}$) to increasing intercellular $CO_2$ concentration ($c_i$) in the sugarcane varieties (A and B) IAC48–65, (C and D) SP70–1143, (E and F) IAC87–3396, and (G and H) IACSP95–5000 grown under full sunlight (sun) or artificial shading conditions (shade) for (A, C, E, and G) 15–16 days (105–106 DAP) and (B, D, F and H) 39–42 (129–132 DAP). The stomatal limitation ($L_s$), and the operating stomatal conductance ($g_{s\_op}$, i.e., $g_s$ when $c_a$ = 400 μmol $mol^{-1}$) are shown for each treatment and genotype. Values are means ± SEM ($n$ = 3–4 biological replicates). $p$-values are from ANOVA testing the effects of genotypes (G), light treatment (L), leaf history (H) and the interactions (GxL, GxH, LxH, GxLxH). $A_{operating}$, $A_{CO2}$ when $c_a$ = 400 μmol $mol^{-1}$; $A_{potential}$, $A_{CO2}$ when $c_i$ = 400 μmol $mol^{-1}$.

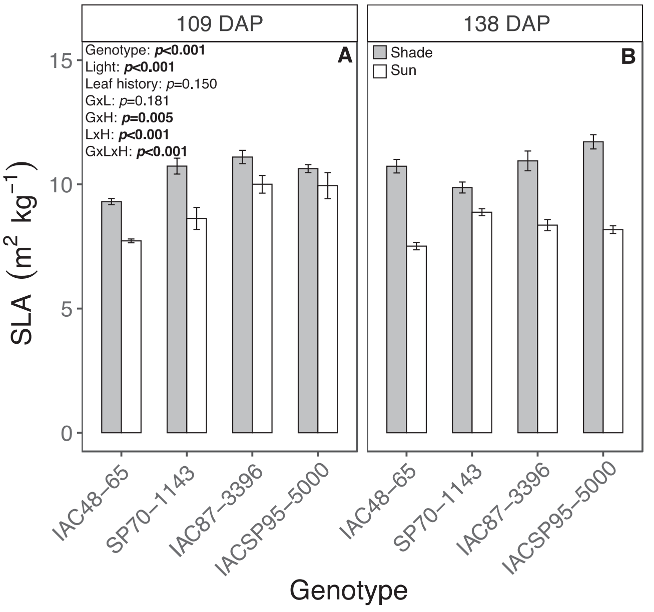


**Fig. 5.** Specific leaf area (SLA) in the sugarcane varieties IAC48–65, SP70–1143, IAC87–3396, and IACSP95–5000 grown under full sunlight (sun) or artificial shading conditions (shade) for (A) 19 days (109 DAP) and (B) 48 days (138 DAP). Values are means ± SEM ($n = 4$–5 biological replicates). *p*-values are from ANOVA testing the effects of genotypes (G), light treatment (L), leaf history (H) and the interactions (GxL, GxH, LxH, GxLxH).

*cruentus*. Further studies will be required to understand whether the downregulation of $\phi CO_{2,max}$ occurs in field-grown sugarcane genotypes, as in our study, although the sugarcane plants were grown outdoors, they were kept in pots, and distant enough to do not cause shading between the plants.

Even though plants grown under full sunlight and shade showed similar convexity of the light response curve, $A_{sat}$ was significantly reduced in shaded leaves, another indication of reduced photosynthetic capacity. Strikingly, when $CO_2$ uptake was measured in response to the diurnal light profile under the shade net, integrated $A_{CO2}$ was also higher in sun than shade plants. This demonstrates that acclimation or development under shade has a significant negative effect on these leaves to perform efficient photosynthesis. These results show clearly that the photosynthetic efficiency decreased as a result of acclimation to low light in all genotypes, independently of the leaf history, as also described by Collison et al. (2020) in maize and *Miscanthus* x *giganteus*, and by Jaikumar et al. (2021) in sorghum, confirming that the trigger for the loss in photosynthetic efficiency in sugarcane is light availability, and not leaf age.

The SLA increased in shaded plants, indicating that shaded plants reduced the construction costs per unit leaf area (Sage and McKown, 2006; Pengelly et al., 2010). Even though leaf thickness was reduced in shaded plants, $A_{sat}$ expressed on a mass basis was still significantly lower than the rates observed in plants grown under full sunlight conditions. Similarly, $R_d$ on mass basis decreased in response to shade, which may be a strategy to reduce the basal metabolism (Bellasio and Griffiths, 2014). Consequently, LCP was reduced in shaded plants. Despite the observed signs of shade acclimation in the sugarcane varieties, $\phi CO_{2,max}$ – the parameter that indicates the intrinsic maximum light use efficiency of photosynthesis (Skillman, 2008) – still significantly decreased in response to shading. Reduction in $\phi CO_{2,max}$ due to shade acclimation observed here in four sugarcane varieties is similar to previous findings in maize, *Miscanthus*, and sorghum (Pignon et al., 2017; Collison et al., 2020; Jaikumar et al., 2021), suggesting that the negative impact of acclimation to shading may be common across these NADP-ME $C_4$ grasses.

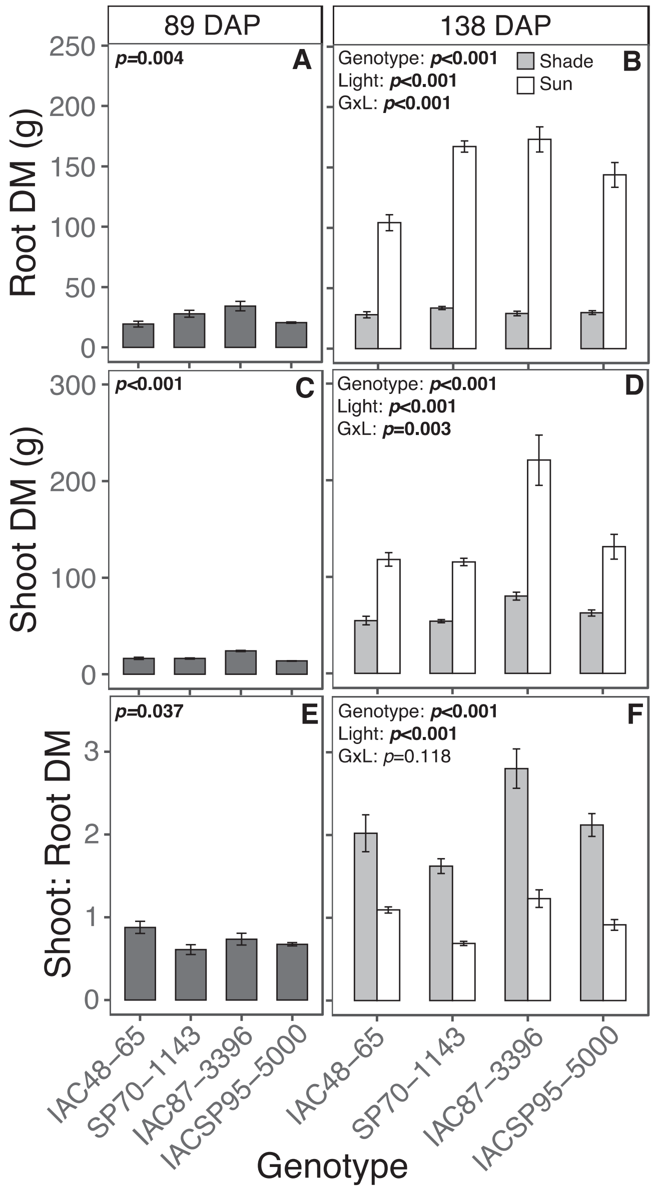


**Fig. 6.** Root (A and B) and shoot (C and D) dry matter, and (E and F) shoot:root ratio in the sugarcane varieties IAC48–65, SP70–1143, IAC87–3396, and IACSP95–5000 before light treatments (A, C and E, 89 DAP) and (B, D and F) after 48 days (138 DAP) grown under full sunlight (sun) or artificial shading conditions (shade). Values are means ± SEM ($n = 4$–5 biological replicates). *p*-values are from ANOVA testing the effects of genotypes (G) at 89 DAP; and the effects of G, and light treatment (L), the interaction between GxL at 138 DAP.

A factor that could potentially affect $\phi CO_{2,max}$ in plants grown under full sunlight or shaded conditions is the effect of temperature, as shaded environments are considerably cooler. However, Björkman and Ehleringer (1977) reported that $\phi CO_{2,max}$ in $C_4$ species appear to be constant in temperatures ranging from 15 °C to 40 °C due to the $C_4$ cycle's suppression of photorespiration. These results suggest that temperature, therefore, is unlikely to have caused the reduction in $\phi CO_{2,max}$ observed in shaded plants in the present study (Björkman and Ehleringer, 1977; Collison et al., 2020).

The possible reasons of low photosynthetic efficiency of sugarcane under shading conditions can be due to diffusional and metabolic limitations. Even though $L_s$ showed variable results between treatments and genotypes, this limitation did not get more pronounced under shaded conditions. Therefore, diffusional limitation linked to stomata seems to not explain the decrease in photosynthetic efficiency observed here. On the metabolic side, however, many factors contributed for a reduced photosynthetic efficiency. The ratio $V_{max}/V_{p,max}$ dropped significantly ($p = 0.011$) in shade (0.347 ± 0.002) compared to sun (0.431 ± 0.002) treated plants without differences between leaf history ($p = 0.902$) or genotype ($p = 0.267$). Decreases in $V_{max}$ were probably due to lower nitrogen investment in the carboxylation enzyme rubisco and/or reallocation of nitrogen from the photosynthetic enzymes into pigment-protein complexes. Such reallocation patterns would explain the decrease in light-saturated $CO_2$ assimilation rates, but not under limiting light.

Increased bundle sheath leakiness at low light, i.e., the retrodiffusion of concentrated $CO_2$ from bundle sheath to mesophyll cells, is often suggested as a mechanism underlying the decrease in performance of $C_4$ under low light (e.g., Kromdijk et al., 2014). Leakiness is energetically costly because PEP regeneration at the cost of ATP still needs to take place, but without $CO_2$ fixation (von Caemmerer and Furbank, 1999; Kromdijk et al., 2014). Likewise, photorespiration could be another alternative electron sink, increasing the costs of CCM, and consequently reducing photosynthetic efficiency in $C_4$ plants under limiting light conditions (von Caemmerer and Furbank, 2003; Sage and McKown, 2006; Kromdijk et al., 2008). If these alternative electron sinks were playing an important role in the decrease of photochemical efficiency in shaded sugarcane plants, the relationship between the efficiencies of the light-dependent and light-independent reactions would be affected. However, the observed relationship between $\phi CO_2$ and $F_q'/F_m'$ was not significantly different between the sun and shade treatments, suggesting that lower photosynthetic efficiency in shade treated plants was probably not due to alternative electron sinks, such as increased leakiness and photorespiration (Krall and Edwards, 1990).

Based on the fluorescence measurements, it seemed that the shade-induced decrease in $CO_2$ assimilation efficiency under low light coincided with a more reduced $Q_A$ redox state, which could point to a metabolic bottleneck downstream of the light-dependent reactions. Due to their location and PSII content, these fluorescence measurements largely reflect mesophyll chloroplasts. Assuming that the main electron sink in the mesophyll cells is the reduction of oxalo-acetate to malate, the more reduced $Q_A$ redox state could reflect an accumulation of malate in the shade-acclimated leaves, relative to the sun-exposed leaves, possibly resulting from a reduction in plasmodesmatal density due to shade acclimation. Plasmodesmata numbers are strongly enhanced in $C_4$ compared to $C_3$ leaves (Danila et al., 2016, 2019) to facilitate the fast metabolite flux between mesophyll and bundle sheath cells (Hatch and Osmond, 1976). However, acclimation to low light significantly reduced the number of plasmodesmata in NADP-ME $C_4$ species *Setaria viridis* and *Zea mays* (Danila et al., 2019), although the functional relationship driving this response was not clear. If plasmodesmata density reduced in the shade treatment, this would have decreased the conductance for diffusive transfer of $C_4$ acids from the mesophyll to the bundle sheath cells (Hatch and Osmond, 1976; Danila et al., 2019; Ermakova et al., 2021), meaning that a larger metabolite gradient would be required to drive the same flux. A larger gradient would imply greater accumulation of $C_4$ acids in the mesophyll cells, decreasing the availability of electron acceptors in the mesophyll chloroplasts, and leading to a more reduced plastoquinone pool and lower PSII efficiency, in line with our observations of lower $F_q'/F_v'$ and $F_q'/F_m'$ in shaded leaves. These results were less apparent at very low light levels, possibly due to a marginal effect of PSII photoinhibition, as it can be observed in the $F_v'/F_m'$ response showed in Fig S7 A and B. Interestingly, Medeiros et al. (2022) recently showed that maize plants subjected to low light had a higher active pool of malate and aspartate than maize plants grown under medium light, suggesting that plants grown under low light were less efficiently using these $C_4$ acid pools to drive the carbon-concentrating shuttle.

Shade-induced decrease in photosynthetic efficiency was observed across all four sugarcane genotypes. The reduction in light availability strongly affected biomass productivity and allocation. Whilst under sun conditions there was a clear difference in biomass production between genotypes, under shade conditions growth was impaired similarly in all genotypes. Biomass root/shoot allocation patterns in the shade also reflected strong light limitation, with almost all new biomass being partitioned to the shoot, which is a well-known strategy to save costs associated to root construction and maintenance in an environment where less energy is available (Givnish, 1988; Poorter and Nagel, 2000; Evans and Poorter, 2001).

In conclusion, this study shows that photosynthetic efficiency decreased due to shading, regardless if the leaf has been developed under full sunlight conditions and exposed later to shade, or if the leaf has been fully developed under shading. These results highlight the fact that the light, and not leaf history is the main factor downregulating photosynthetic efficiency in sugarcane. Decreases in photosynthetic efficiency under shade were seen for all genotypes, and both for long-term and short-term light acclimation, and the consistency with previous observations on several other species suggests that the negative impact of shading on photosynthetic efficiency could reflect a common bottleneck for NADP-ME $C_4$ grasses.

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## CRediT authorship contribution statement

ECM and RVR: funding acquisition; ECM, RVR and CRGS: design of experiments; CRGS: performing experiments with PERM help; CRGS: data analysis; CRGS and JK: writing with input from all authors. All authors read, edited, and approved the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105351.

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