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PHOTOSYNTHESIS AND PHOTOPROTECTION IN C4 SPECIES

FOTOSSÍNTESE E FOTOPROTEÇÃO EM ESPÉCIES C4

CAMPINAS 2024

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Advisor: Prof. Dr. Rafael Vasconcelos Ribeiro

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RESUMO

A eficiência fotossintética e a integridade do aparato fotossintético sob condições ambientais adversas são fatores importantes para o aumento da produtividade das culturas. Uma alternativa para aumentar a produtividade agrícola é o desenvolvimento de cultivares com elevada eficiência de conversão de energia solar em biomassa pelo dossel vegetativo, onde as folhas estão expostas às variações da intensidade luminosa ao longo do dia. Este trabalho visou explorar e caracterizar a variação genotípica na fotossíntese, indução fotossintética, relaxamento do coeficiente de extinção não fotoquímico (NPQ) aclimatação ao auto-sombreamento e distribuição de luz no dossel de Saccharum spp., Sorghum bicolor e Zea mays. No primeiro capítulo, avaliamos o desempenho fotossintético no dossel de dez cultivares de cana-de-açúcar e três espécies de Saccharum em condições de campo. No segundo capítulo, identificamos atributos fotossintéticos que contribuem para a uniformidade da atividade fotossintética em todo o dossel vegetativo de milho e sorgo. No terceiro capítulo, caracterizamos a cinética da indução da fotossíntese e o relaxamento do NPQ no dossel de milho e sorgo. Em geral, nosso estudo revelou que as espécies C₄ exibem aclimatação fotossintética no dossel, com elevada assimilação de CO2 nas folhas do dossel superior e maior responsividade à luz nas folhas localizadas no interior da copa. Isso está associado a uma maior produção de biomassa, destacando o potencial de nossas descobertas na seleção de cultivares modernos mais produtivos pelos programas de melhoramento genético.

Palavras-chave: biomassa, fotossíntese, indução fotossintética, luz.

ABSTRACT

The efficiency of photosynthesis and the integrity of the photosynthetic apparatus under fluctuating environmental conditions are important traits associated with crop yield. One promising strategy to increase crop yield involves the development of cultivars with high sunlight conversion efficiency into biomass throughout the plant canopy, where leaves are exposed to varying light intensities throughout the day. We believe that higher photosynthetic rates, in conjunction with rapid induction of photosynthesis and relaxation of non-photochemical quenching (NPO) along plant canopy can enhance light conversion efficiency into biomass. Our study aimed to explore and characterize the genotypic variation in photosynthesis, photosynthetic induction, NPQ relaxation, the acclimation to self-shading and light distribution across the canopies of Saccharum ssp., Sorghum bicolor and Zea mays. In the first chapter, we evaluated the photosynthetic performance through the canopy of ten sugarcane cultivars and three Saccharum species under field conditions. In the second chapter, we pinpointed key photosynthetic attributes that contribute to the uniformity of high photosynthetic activity throughout the canopy. In the third chapter, we characterized the kinetics of photosynthesis induction and the relaxation of NPQ across the canopy of maize and sorghum species. Overall, our study revealed that C₄ species exhibit photosynthetic acclimation in the canopy, with superior photosynthesis in the upper leaves and heightened responsiveness to increasing light in those leaves located inside the plant canopy. This is associated with higher yield, underscoring the potential of our findings in selecting modern cultivars by breeding programs.

Keywords: biomass, light, photosynthesis, photosynthetic induction.

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GENERAL INTRODUCTION

Growth population has progressively intensified the demand for food and renewable energy sources, pushing the agriculture sector toward higher yields (Ray et al., 2013; Salter et al., 2019). The expansion of agricultural borders to increase productivity is a conceivable possibility, but it lacks long-term sustainability. A more viable alternative lies in harnessing plants that exhibit high efficiency in converting solar energy into biomass, thereby contributing to higher yield (Reynolds et al., 2012; Driever et al., 2014). The ability of these plants to maintain the efficiency of photosynthesis and preserve the integrity of the photosynthetic apparatus, even under fluctuating environmental conditions, is a trait that could enhance crop productivity (Kromdijk et al., 2016; Taylor and Long, 2017; Murchie et al., 2018).

Primary production (PP) is determined by the solar energy that reaches the canopy of plants (Q_{PAR}) and by the efficiencies with which this energy is intercepted (ϵ i) and converted into phytomass (ϵ c) (Zhu et al., 2010):

$$PP = \sum Q_{PAR}. \ \varepsilon i. \ \varepsilon c. \ \varepsilon p \tag{1}$$

being $\sum Q_{PAR}$ the total photosynthetically active radiation (PAR) incident during the growing season; ε i, the interception efficiency of PAR by the plant canopy (nondimensional); ε c, the conversion efficiency of PAR into biomass (g MJ⁻¹); and ε p, the partitioning efficiency (nondimensional) or harvest index – ratio of culms or grains per total plant biomass.

Plant breeding has optimized ε i and ε p to such an extent that there is minimal room for further improvement in crops (Zhu et al., 2010). In modern crops, harvest indices have reached 0.6, leaving little scope for further improvements (Murchie et al., 2009; Zhu et al., 2010). Under optimal conditions the ε p of maize hybrids stands at about 0.52. Interestingly, this figure has remained unchanged for the past two decades. In contrast, sugarcane has a high ε p of 0.8, given that most of the plant is the harvested product (Di Matteo et al., 2016, Waclawovsky et al., 2010).

The balance between photosynthesis and respiration determines ε c. High photosynthesis in plant canopy would imply high crop yield. However, most studies deal with measurements of photosynthesis taken from a small area of the upper leaves of the canopy, which are directly

exposed to light. A significant portion of plant canopy, however, is under low light conditions due to self-shading (Marchiori et al., 2010; 2014). Therefore, it is crucial for studies on photosynthesis and crop yield to consider this reality where leaves are under low light (Marchiori et al., 2014) and can acclimate to such limiting environments (Sales et al., 2018; Almeida et al., 2022). Leaves located inside the canopy presenting high CO₂ assimilation could contribute significantly to the plant's overall carbon gain. Theoretically, only 6% of global solar radiation is converted into biomass by C₄ plants (Zhu et al., 2008), which plays a pivotal role in determining growth and yield (Lawlor, 1995; Long et al., 2006; Marchiori et al., 2010). In practice, crops typically reach εc around 2.5 g MJ⁻¹. However, there have been instances where conversions close to 5.39 g MJ⁻¹ have been observed (Cruz et al., 2021). This untapped potential suggests that plants with greater efficiency in intercepting and converting light could be important targets in breeding programs.

The efficiency of carbon conversion in C₄ plant canopy can be enhanced through improved distribution of PAR. This is because the photosystems are already operating at a high level of efficiency. By increasing the penetration and distribution of PAR within the canopy, photosynthesis rate can be boosted by up to 40% (Long et al., 2006; Marchiori et al., 2014). The architecture of the canopy influences the quantity and quality of light absorbed by leaves. This, in turn, leads to variations in self-shading. Interestingly, high leaf area index (leaf area/soil surface) might result in lower conversion efficiency, since lower PAR availability at the bottom canopy might trigger early senescence (Murchie et al., 1999; Slattery et al., 2018; Tejera et al., 2007).

Leaves within a plant canopy are exposed to changes in light intensity throughout the day, from seconds to minutes. While the upper leaves are typically exposed to direct solar radiation, those located inside the canopy intercept diffuse light or sunflecks - short periods of direct solar radiation inside the canopy profile (Kromdijk et al., 2016; Slattery et al., 2018). These sunflecks account for approximately 90% of the available energy for photosynthesis in this environment. The occurrence of sunflecks through plant canopy is influenced by several factors such as the height of the plant, the overlap of leaves, the flexibility of branches and petioles, and the size of the leaves (Way and Pearcy, 2012). Such light fluctuation requires a sophisticated control mechanism for photosynthetic induction and deactivation of photoprotective mechanisms, which in turn enhances the efficiency of CO₂ assimilation. Physiological factors, such as the diffusion of CO₂ through stomata and mesophyll may pose limitations to the induction of photosynthesis (Lawson and Vialet-Chabrand, 2019; Salter et al., 2019). Furthermore, the slow induction of photosynthesis following the transition from shade/dark to sunlight reduces CO₂ assimilation. The activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is considered a limiting factor for the photosynthetic induction during transitions from low to high light (Soleh et al., 2016, 2017; Taylor and Long, 2017; Morales et al., 2018).

The response of photosynthesis to light is linear under low light intensity. However, for many crops, light saturation (the point at which an increase in light intensity no longer leads to an increment in the rate of photosynthesis) occurs when less than 50% of the maximum available solar energy is utilized (Long et al., 2006). When the irradiance is too high or the energy available for photochemistry rises faster, there is potential damage to photosynthetic proteins and membranes. Photoprotective mechanisms are activated to protect antenna complexes from excessive light energy and this energy is dissipated as heat, increasing the non-photochemical quenching (NPQ) of fluorescence (Kromdijk et al., 2016).

Molecular mechanisms associated with NPQ are not yet fully understood, but it is already known that the pH of the thylakoids lumen and the aggregation state of the antenna complexes in the photosystem II (PSII) are important factors. Three carotenoids - violaxanthin, antheraxanthin, and zeaxanthin, are involved in NPQ and constitute the cycle of xanthophylls. Under high irradiance, violaxanthin is converted into zeaxanthin, via the intermediate antheraxanthin, by the violaxanthin de-epoxidase (VDE). Conversely, when the light level decreases, zeaxanthin is converted back to violaxanthin by zeaxanthin epoxidase (ZEP) (Demming-Adams and Adams, 1996). The induction of NPQ can occur rapidly, within a matter of seconds. However, its relaxation rate (the transition from a quenched to an unquenched state) is slower, taking minutes to hours. This asymmetry between induction and relaxation is exacerbated by repeated exposure to high

light. The slow recovery rate of the PSII antenna complex implies a decrease in the maximum quantum yield of PSII and the photosynthetic quantum yield of CO_2 fixation (Zhu et al., 2004; Kromdijk et al., 2016; Slattery et al., 2018). It is estimated that plants may experience limitations of up to 32% in daily carbon gain when NPQ relaxation is slow (Zhu et al., 2004).

We believe that elevated photosynthetic rates, the fast induction of photosynthesis and rapid relaxation of the NPQ through plant canopy support high biomass production by plants. This in turn leads to a high yield. Variations in the kinetics of photosynthetic induction have been identified in C_3 crops such as soybean and wheat (Soleh et al., 2016, 2017; Taylor and Long, 2017; Salter et al., 2019). However, comprehensive studies evaluating the variability of photosynthesis, photosynthetic induction and photoprotection throughout the canopy structure of C_4 species such as maize, sorghum and sugarcane are currently lacking. Thus, understanding the physiological characteristics of the plant canopy related to biomass could enhance our knowledge about it. It could also reveal genotypic variation in the mechanisms of photosynthetic induction and photoprotection in C_4 plants.

Hypotheses and Objective

This thesis aimed to investigate and characterize the genotypic variation in photosynthesis, photosynthetic induction, NPQ relaxation and light distribution through the canopies of maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) and sugarcane (*Saccharum* spp.).

The study was composed of two experiments, organized in three chapters. In the first chapter, we investigated whether shaded leaves have a photosynthetic capacity similar to that of light-exposed leaves of sugarcane plants. We evaluated the photosynthetic performance across the canopy, including light-exposed and shaded leaves, of ten sugarcane cultivars and three *Saccharum* species. This evaluation was carried out under field conditions, with a particular emphasis on the effects of self-shading and leaf aging. We also addressed the physiological foundations of the observed variability in photosynthetic traits. However, we did not provide an in-depth exploration of photosynthesis, a physiological process that drives the biomass production in plants.

In the second chapter, we identified key photosynthetic related with higher and uniform photosynthesis through the canopy. In the third chapter, we characterized the kinetics of photosynthesis induction and the relaxation of NPQ across the canopy of maize and sorghum species. We tested the hypothesis that rapid induction of photosynthesis and NPQ relaxation throughout the canopy are associated with higher biomass production.

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CHAPTER I: EVIDENCE OF PHOTOSYNTHETIC ACCLIMATION TO SELF-SHADING IN SUGARCANE CANOPIES

Evidence of photosynthetic acclimation to self-shading in sugarcane canopies

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Abstract

Increasing the efficiency of photosynthesis in sugarcane canopy is the key for higher crop yield. Herein, we evaluated the photosynthetic performance along the canopy of ten sugarcane cultivars and three *Saccharum* species. Canopy morphological traits were evaluated and leaf gas exchange was measured in leaves +1 and +4 under low and high light conditions. Similar photosynthetic capacity was found in leaves +1 and +4 under high light in genotypes with high leaf area index and a high fraction of the sky blocked by the foliage (>85%). Interestingly, such canopy characteristics cause low light availability to leaves +4, suggesting the photosynthetic acclimation of these leaves to self-shading in some genotypes. We highlight IACCTC06–8126 and CTC4 as those genotypes with higher canopy photosynthetic capacity, presenting high leaf area, high photosynthetic rates in sun-exposed leaves, and high responsiveness of shaded leaves to increasing light availability.

Keywords: light; photosynthesis; plant canopy; Saccharum spp..

Abreviations

BF₁₀ – Bayes factor; C_i – intercellular CO₂ concentration; DAP – days after planting; Φ CO₂ – instantaneous CO₂ quantum efficiency; g_s – stomatal conductance; k – instantaneous carboxylation efficiency; L_{abs} – leaf light absorbance; LAI – leaf area index; MTA – mean tilt angle; PEPC – phosphoenolpyruvate carboxylase; PEPCK – phosphoenolpyruvate carboxykinase; P_N – photosynthetic rate; Q – photosynthetic photon flux density; R_D – dark respiration.

Highlights

- Responsiveness of shaded leaves to light was evaluated in 13 sugarcane genotypes
- CO₂ uptake varied among genotypes when varying light conditions
- IACCTC06-8126 and CTC4 have high responsiveness of shaded leaves to high light

Introduction

Population growth increases the demand for food and renewable energy sources, challenging global agriculture for higher yield (Ray et al. 2013, Salter et al. 2019). Sugarcane (*Saccharum* spp.), a C₄ crop, is a promising alternative due to its high biomass and sucrose production (Waclawovsky et al. 2010). Increasing the efficiency of photosynthesis in plant canopy is the key for higher crop yield, and studies dealing with canopy photosynthesis or even leaves under different light exposure are limited. In fact, a large proportion of the sugarcane canopy is under low light conditions due to self-shading (Marchiori et al. 2010, 2014). Photosynthetic acclimation to light-limiting conditions has been explored in sugarcane, with plants showing a shift of the main decarboxylation pathway in bundle-sheath cells towards a higher contribution of phosphoenolpyruvate carboxykinase (PEPCK) than NADP-dependent malic enzyme to maximize the quantum efficiency of CO_2 assimilation (Sales et al. 2018).

In theory, about 6% of global solar radiation is converted into biomass by C₄ plants (Zhu et al. 2008), determining crop growth and yield (Lawlor, 1995, Long et al. 2006, Marchiori et al. 2010). Light conversion efficiency in sugarcane may reach ~5.39 g MJ⁻¹ (Cruz et al. 2021, 2022), and such efficiency is driven by canopy photosynthesis and respiration, with high photosynthetic rates and low respiration (mainly maintenance respiration) enhancing biomass production (Zhu et al. 2010). Light interception efficiency is determined by the speed of canopy development, light absorption by leaves, longevity, size and architecture of the canopy (Zhu et al. 2010, Davey et al. 2017), and is dependent on genotype and planting density (Robertson et al. 1996, Tejera et al. 2007). A significant proportion of canopy CO₂ assimilation occurs under light limitation conditions caused by clouds and wind-induced leaf and plant shading (Kromdijk et al. 2016). The selection of plants more efficient in intercepting and converting light into biomass would be a way to increase crop yield in breeding programs (Lawlor 1995, Long et al. 2006, Marchiori et al. 2010).

Marchiori et al. (2010, 2014) highlighted the lack of information about the sugarcane canopy structure in breeding programs and showed that small variations in canopy architecture cause important changes in photosynthesis of three commercial sugarcane cultivars under field conditions. Throughout the decades, sugarcane breeding programs have focused on the development of cultivars with high yield under stressful environments and resistance to pests and diseases. However, the physiological processes underlying the light conversion efficiency and yield

remain poorly understood in field-grown sugarcane plants (Zhu et al. 2010, Lopes et al. 2011, Moore et al. 2014). Recently, Almeida et al. (2021) reported a significant variation of photosynthesis among sugarcane genotypes and identified valuable and heritable photosynthetic traits. However, Almeida et al. (2021) evaluated only one fully expanded and sun-exposed leaf in sugarcane plants, as done by others (Irvine, 1967, 1975, Jackson et al. 2016, Li et al. 2017). Then, an intriguing question arises: would shaded leaves have a photosynthetic capacity similar to the light-exposed leaves in field-grown sugarcane plants?

Herein, our aim was to evaluate the photosynthetic performance of light-exposed and shaded leaves of several sugarcane cultivars originated from crosses between 1948 and 2006 and three *Saccharum* species (*S. officinarum*, *S. spontaneum* and *S. robustum*), emphasizing the effects of self-shading, leaf aging and addressing the physiological bases of such variability in photosynthetic traits.

Material and Methods

Plant material and experimental conditions

A field experiment was conducted between December 2018 and May 2019, with ten sugarcane cultivars and three species (Table 1), grown under rainfed conditions (dystrophic red latosol) in Campinas, SP, Brazil (22°86'S, 47°08'W, 642 m a.s.l). The experimental design was in randomized blocks, with four replications, each composed by six rows (11 m, spaced 1.5 m) of pre-sprouted plants spaced of 0.45 m. Each plant was fertilized with 80 g P₂O₅, 133 g CaCO₃, 28 g KCl, and 34 g (NH₄)₂SO₄, following van Raij et al. (1996). Environmental conditions were continuously monitored by a weather station close to the experimental area. The air temperature was monitored with an HMP–45C probe (Vaisala, Helsinki, Finland) and rainfall with a tipping bucket rain gauge (model CS700, Campbell-Scientific, Logan UT, USA). Data were recorded every 20 min by a data logger (model CR1000, Campbell-Scientific, Logan UT, USA). During the sugarcane growing season, the accumulated rainfall was 653 mm, air temperature ranged from 13.3 to 36.0 °C, with an average air temperature of 25.1 °C (Fig. 1S). Leaf gas exchange and plant canopy were evaluated in four-month-old sugarcane plants 129 days after planting (DAP).

Plant canopy

Leaf area index (LAI), mean tilt angle of the foliage (MTA) and the fraction of the sky that is not blocked by the foliage (visible sky ratio – indicates the absorption of diffuse, short-wave light < 490 nm) were measured with the LI-2000C (LICOR Inc., Lincoln NE, USA), following the manufacturer's instructions. Photosynthetic photon flux density (Q) reaching the first (sunexposed) and the fourth (shaded) fully expanded leaves was measured between 12:00 h and 13:00 h with a linear quantum sensor (model LI-191R-BNC-2, LICOR, Lincoln NE, USA) arranged at 90° with planting line. The first and fourth fully expanded leaves are leaf +1 and leaf +4, respectively, following the Kuijper system of leaf classification (Cheavegatti-Gianotto et al. 2011). The number of tillers per plant was also counted.

Leaf gas exchange

Leaf gas exchange was measured using an infrared gas analyzer (LI-6400XT, LICOR Inc., Lincoln NE, USA) in leaves +1 and +4. Measurements were taken between 8:00 h and 15:00 h, under low (200 µmol m⁻² s⁻¹, at leaf +4) and high (2,000 µmol m⁻² s⁻¹, at leaf +1 and leaf +4) light intensity (Q), air CO₂ partial pressure of 40 Pa, cuvette temperature of 25°C and natural variation of air relative humidity. Data were recorded after temporal stability and when the total coefficient of variation was lower than 2%. Photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular CO₂ concentration (C_i) were measured. Leaf light absorbance (L_{abs}) was estimated by following the LICOR protocol "Estimating Light Absorbance in the 6400-40 Leaf Chamber Fluorometer Using an External Quantum Technical Sensor - Note #128", available in https://licor.app.boxenterprise.net/s/9bgi9ayo5yx7dwjnts8c. We estimated the instantaneous carboxylation efficiency ($k = P_N/C_i$) and the instantaneous CO₂ quantum efficiency [$\Phi CO_2 =$ (P_N+R_D)/(Q× L_{abs})] (Edwards and Baker, 1993). Dark respiration (R_D) was obtained from Almeida et al. (2021), and we assumed that leaves +1 and +4 have similar R_D (unpublished data).

Data analyses

The data were analyzed using Bayesian statistics and mean values were compared using Bayes Factor (BF₁₀): when $1 < BF_{10} < 3$, there is weak support to the alternative hypothesis (H1); $3 < BF_{10} < 20$ indicates positive support to H1; and $BF_{10} > 20$ indicates strong support to the alternative hypothesis, following Miranda et al. (2021). Correlations between traits were evaluated through Spearman's coefficient. All analyses were done using the R software (R Core Team, 2021; version 4.1.1, R-project, packages "Hmisc", "Corrplot" and "Readxl") and the software JASP (JASP Team, 2021; version 0.15, Amsterdam, Netherlands).

Results

Plant canopy and light availability

We found a significant variation for LAI (BF₁₀ = 27.6), tillering (BF₁₀ = 1.3×10^{11}), sky ratio (BF₁₀ = 8.6) and light intensity reaching leaves +4 (BF₁₀ = 956.6). IACCTC06–8126 presented the highest LAI (Fig. 1*A*), while *S. spontaneum* presented the highest tillering (Fig. 1*B*). For the sky ratio, IACCTC06–8126 presented lower values than *S. spontaneum*, *S. officinarum*, SP70–1143, IACSP01–5503 and IAC48–65 (Fig. 1*C*). The light intensity measured at leaves +1 (upper canopy) did not vary among genotypes and was 1,450 µmol (photon) m⁻² s⁻¹ (on average). The light availability for leaves +4 was lowest in IACCTC06–8126 and highest in IACSP01-5503 (Fig. 1*D*). For the mean tilt angle of the foliage, we found the lowest values in IACCTC06–8126 (BF₁₀ = 6.60), with an overall variation between 38 and 63° (Fig. 2S).

Leaf gas exchange

Significant variation among genotypes was found for photosynthetic rates (P_N) measured in leaves +1 (index '+1', BF₁₀ = 7.1×10⁵) and +4 (index '+4', BF₁₀ = 2.2×10⁵) under high light [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹] and in leaves +4 (BF₁₀ = 1.9×10³) under low light [index 'L', Q = 200 µmol (photon) m⁻² s⁻¹]. Differences between P_N^{+1H} and P_N^{+4H} were noticed in

S. spontaneum, S. robustum, S. officinarum, SP80-3280, IACSP01-5503 and RB83-5486 (Fig. 2*A*). We found differences between P_N^{+1H} and P_N^{+4L} for all genotypes (Fig. 3*A*S). In addition, g_s^{+4H} also differed among genotypes (BF₁₀ = 69.9) while g_s^{+1H} and g_s^{+4L} did not (BF₁₀ = 0.9 and 2.38, respectively), as shown in Figs. 2B and 3BS. Differences between g_s^{+1H} and g_s^{+4H} were found for IACSP94–2094 and SP80–3280, while between g_s^{+1H} and g_s^{+4L} for IACSP94-2094 and RB83-5486 Significant variation was also noticed among genotypes (Figs. 2Band 3BS). for ΦCO_2^{+1H} , ΦCO_2^{+4H} and ΦCO_2^{+4L} (BF₁₀ = 9.88×10⁶, 1.54×10⁵ and 156.96, respectively), with differences in ΦCO_2^H between leaves +1 and +4 for S spontaneum, S. officinarum, SP80–3280, IACSP01-5503 and RB83-5486 (Fig. 2C). There was a large variantion in ΦCO_2^{+4L} among genotypes (Fig. 3CS). Regarding the instantaneous carboxylation efficiency (k), differences among genotypes were found only on leaf +4 under high light (BF₁₀ = 289.2) and variations between k^{+1H} and k^{+4H} were found in IACSP94–2094 ($k^{+4H} > k^{+1H}$) and SP80–3280 ($k^{+4H} < k^{+1H}$), with no variation in SP70–1143 for k^{+1H} and k^{+4L} (Figs. 2D and 3DS).

The ratio between photosynthetic rates measured on leaves +4 and +1 under high light $(P_{\rm N}^{+4\rm H:+1\rm H})$, acclimation of the bottom canopy to high light) varied among genotypes (BF₁₀ = 104.40), with *S. officinarum* and CTC4 presenting the lowest and the highest mean values, respectively (Fig. 3). When comparing the same leaf +4 under low and high light conditions (responsiveness of the bottom canopy to light fluctuation), $P_{\rm N}^{+4\rm L:+4\rm H}$, $\Phi \rm CO_2^{+4\rm L:+4\rm H}$ and $k^{+4\rm L:+4\rm H}$ also varied (BF₁₀ = 3.5×10^3 , 156.96 and 63.03, respectively) among genotypes, with the highest values measured in SP80–3280 and the lowest ones found in *S. spontaneum*, IACCTC06–8126 and CTC4 (Fig. 4*A*, *C* and *D*). Regarding $g_{\rm s}^{+4\rm L:+4\rm H}$, only RB83–5486 differed from *S. officinarum* and IACSP94–2094 (BF₁₀=3.31 and 6.84, respectively), as shown in Fig. 4*B*. Under natural conditions, leaves +4 receive less light than leaves +1 and we decided to compare the measurements taken considering such assumption (Figs. 3S and 4S). In fact, $P_{\rm N}^{+4\rm L:+1\rm H}$ and $\Phi \rm CO_2^{+4\rm L:+1\rm H}$ varied (BF₁₀ = 6.7×10^3 and 6.1×10^3 , respectively) among genotypes, with SP80–3280 presenting the highest mean values and *S. spontaneum* the lowest ones (Figs. 4*A* S and *C*). IACSP01–5503 exhibited higher $g_{\rm s}^{+4\rm L:+1\rm H}$ than *S. officinarum* and RB83–5486 (Fig. 4*B*S). IACSP94–2094 showed higher $k^{4\rm L:+1\rm H}$ than *S. spontaneum*, IACCTC06–8126 and CTC4 (Fig. 4*D*S).

Correlations

 $P_{\rm N}^{+1\rm H}$ was correlated with $P_{\rm N}^{+4\rm H}$ (r = 0.62), $g_{\rm s}^{+1\rm H}$ (r = 0.68) and $\Phi \rm CO_2^{+1\rm H}$ (r = 0.96). $P_{\rm N}^{+4\rm H}$ was positively correlated with $g_{\rm s}^{+4\rm H}$ (r = 0.87), $\Phi \rm CO_2^{+4\rm H}$ (r = 0.99), $k^{+4\rm H}$ (r = 0.76) and LAI (r = 0.66), while negatively correlated with sky ratio (r = -0.64) and MTA (r = -0.65) (Fig. 5). Significant correlation was also observed between $k^{+4\rm H}$ and $\Phi \rm CO_2^{+4\rm H}$ (r = 0.72), LAI (r = 0.59) and sky ratio (r = -0.60), as shown in Fig. 5. Positive correlations were noticed for $P_{\rm N}^{+4\rm L:+4\rm H}$ vs. sky ratio (r = 0.60) and MTA (r = 0.60). Tillering correlated negatively (r = -0.65) with the mean tilt angle of the foliage (Fig. 5).

Discussion

We found a high genotypic variation of photosynthesis through the sugarcane canopies, with P_N^{+1H} varying from 32 to 44 µmol (CO₂) m⁻² s⁻¹, and P_N^{+4H} from 22 to 41 µmol (CO₂) m⁻² s⁻¹ (Fig. 2*A*). Such photosynthetic variability was already reported and ascribed to differences in leaf anatomy and morphology, stomatal conductance, leaf nitrogen content and, phosphoenolpyruvate carboxylase (PEPC) and Rubisco abundances and activities (Irvine 1967, 1975, 1983, Marchiori et al. 2010, 2014, Jackson et al. 2016, Li et al. 2017, Almeida et al. 2021). As novelty, our data revealed that some sugarcane canopies have similar P_N in leaves +1 and +4 (IACCTC06–8126, IACSP95–5000, IAC87–3396, SP70–1143, IACSP94–2094, CTC4 and IAC48–65) while others have leaf +1 with higher P_N than leaf +4 (*S. spontaneum*, *S. robustum*, *S. officinarum*, SP80–3280, IACSP01–5503 and RB83–5486) under high light (Fig. 2*A*). P_N was similar in leaves +1 and +4 in sugarcane canopies with high leaf area index and high fraction of the sky blocked by the foliage, with planophile-like leaves (Figs. 1*A*, *C*; 2*A*; 5, and 2S). Such similar photosynthetic performance between shaded (leaf +4) and light-exposed leaves (+1) would be a consequence of increasing photosynthetic nitrogen use-efficiency, instantaneous CO₂ quantum efficiency (as shown herein, Fig. 2*C*) and the maintenance of Rubisco activity (Marchiori et al. 2014) in leaves +4.

While upper leaves are usually exposed to direct solar radiation, those located inside the canopy intercept diffuse light or sunflecks (short periods of direct solar radiation inside the canopy), being progressively shaded by new leaves (Bellasio and Griffiths, 2014). Acclimation

processes to low light would increase light use efficiency and then photosynthesis of shaded leaves or those at the bottom canopy positions, enhancing the amount of CO₂ fixed by the entire canopy. Ideally, a canopy would have (i) top leaves with high photosynthetic rates, (ii) small photosynthetic differences between top and bottom leaves under the same light intensity, and (iii) bottom leaves photosynthesizing close to the maximum even under low light intensity. While such ideotype looks most hypothetical, we were able to find genotypes with interesting characteristics. Under high light, S. spontaneum, S. robustum, S. officinarum, SP80-3280, IACSP01-5503 and RB83-5486 presented the highest $P_{\rm N}$ and $\Phi \rm CO_2$ in leaves +1, *i.e.* at the upper canopy position, as compared to leaves +4 (Fig. 2A, C). On the other hand, IACCTC06-8126, IACSP95-5000, IAC87-3396, SP70–1143, IACSP94–2094, CTC 4, and IAC48–65 had similar P_N and ΦCO_2 in leaves +1 and +4 under high light, with IACCTC06-8126 presenting higher values for sky ratio, low light availability at leaf +4, and the lowest mean tilt angle (Figs. 1*C*, *D* and 2S). Among the biochemical changes underlying shade acclimation of photosynthetic apparatus in leaves +4, we would suggest increases in activity and abundance of PEPC and Rubisco, pyruvate orthophosphate dikinase, NADP-dependent malate dehydrogenase and phosphoenolpyruvate carboxykinase (Sales et al. 2018, Almeida et al. 2021, Jaikumar et al. 2021) – a subject to be further explored in future research. Such acclimation to low light availability leading to the maintenance of the photosynthetic capacity of shaded leaves under high light would improve photosynthesis during sunflecks and then canopy photosynthesis (Waldron et al. 1967, Machado et al. 1982).

Photosynthetic rates in leaves +4 were correlated to ΦCO_2 and k under high light (Fig. 5). In fact, previous studies identified a relationship between photosynthetic acclimation and instantaneous CO₂ quantum efficiency in C₄ grass canopies under shading (Marchiori et al. 2010, 2014, Pignon et al. 2017, Collison et al. 2020). While *S. officinarum* showed a substantial decrease (-43%) in P_N when comparing leaves +4 to +1 under high light, such decrease was around 11% in *S. spontaneum* (Fig. 3). This latter presented higher tillering and likely higher canopy photosynthesis (Figs. 2*A* and 3). Therefore, our data revealed that photosynthetic acclimation due self-shading is not necessarily related to higher photosynthetic rates per leaf area. Instead, such acclimation aims to increase canopy CO₂ assimilation due to high g_s, k and ΦCO_2 (Fig. 2*B*, *C* and *D*). In addition, photosynthesis of leaves +4 under high light was correlated positively with LAI and negatively with visible sky ratio and MTA (Fig. 5), canopy traits that compromise light availability at the bottom canopy layer.

While *S. officinarum* and SP80–3280 – genotypes showing the lowest photosynthesis in leaves +4 (Fig. 2*A*) – presented less responsiveness of P_N , ΦCO_2 and *k* to light, the most light responsive genotypes were *S. spontaneum*, CTC4 and IACCTC06–8126 (Fig. 4*A*, *C* and *D*). This reinforces that the light acclimation of photosynthesis – which means that shaded leaves are able to carry on photosynthesis like sun-exposed ones if light is available – and photosynthetic responsiveness to light are linked.

The commercial cultivars developed by breeding programs are inter or intraspecific hybrids (crosses of *S. officinarum, S. spontaneum, S. robustum* and *S. barberi*) derived mainly from the crossing of the noble species *S. officinarum* (\sim 70–80%) and the wild species *S. spontaneum* (\sim 10–20%) (Moore et al. 2014). As high heritability for photosynthesis and stomatal conductance in sugarcane has been reported by Jackson *et al.* (2016), Li et al. (2017) and Almeida et al. (2021) and there is significant variation in photosynthesis of both light-exposed and shaded leaves reported herein, our data indicate that there is room for improving canopy photosynthesis through breeding. Sugarcane plants with erectophile-like leaves at the upper canopy layer that allows light penetration (Marchiori et al. 2014), and planophile-like leaves at bottom canopy positions (Slattery et al. 2016, Walker et al. 2018), with high photosynthetic capacity due to shading acclimation, would have a phenotype favoring higher conversion of sunlight energy into biomass.

S. officinarum stands out for the high sucrose (up to 25% on stalk fresh mass) and low fiber content in the culms. On the other hand, S. spontaneum presents a higher photosynthetic rate, lower accumulation of sucrose (~2% of stalk fresh mass), vigorous growth, tillering and a higher resistance to abiotic and biotic stresses, compared to S. officinarum (Irvine, 1975, Moore et al. 2014). Herein, our data highlighted the superiority of S. spontaneum for tillering and photosynthesis, fixing about 13% (leaf +1) to 43% (leaf +4) more CO₂ than S. officinarum under high light (Fig. 2A). Such high photosynthetic activity would boost biomass production through breeding for the development of improved sugarcane or energy cane varieties (Cruz et al. 2021), as the herdability of photosynthetic traits is high (Jackson et al. 2016, Li et al. 2017, Almeida et al. 2021).

Conclusion

This study revealed a significant photosynthetic variation in light-exposed and shaded leaves in canopies of *Saccharum* complex, indicating acclimation of shaded leaves towards the maintenance of photosynthetic performance in some sugarcane cultivars and species. This way, shaded leaves are responsive to high light and present photosynthetic rates similar to light-exposed leaves, even with light intensity commonly being less than 25% of reaching light-exposed ones. Our data highlight IACCTC06–8126 and CTC4 as those genotypes with higher canopy photosynthetic capacity due to high leaf area, high photosynthetic rate of light-exposed leaves, and high responsiveness of photosynthesis to high light in shaded leaves, contrasting with *S. officinarum* and SP80–3280.

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Tables

Table 1. Sugarcane hybrids and species, with their year of hybridization, progenitors and institution responsible for breeding.

Genotypes	Year	Progenitors	Institution
IAC48–65	1948	CP27108 ×?	IAC
SP70–1143	1970	IAC48–65 ×?	Copersucar
SP80-3280	1980	SP7-088 × H57-5028	Copersucar
RB83–5486	1983	L60–14 ×?	RIDESA
IAC87-3396	1987	SP70–1143 × Co 740	IAC
CTC4 (92–4221)	1992	SP83–5073 ×?	CTC
IACSP94-2094	1994	SP84–7017 ×?	IAC/Copersucar
IACSP95-5000	1995	SP84–2066 × SP80–85	IAC
IACSP01-5503	2001	IACSP95–2312 × CTC9	IAC
IACCTC06-8126	2006	IACSP95-5000 \times IACSP96-6114	IAC
S. officinarum (Don Cico)		Noble species	
S. spontaneum (19–95)		Wild species	
S. robustum (NG 57–055)		Wild species	



Figure 1



Fig. 1. Leaf area index (LAI, in *A*), tillering (*B*), visible sky ratio – fraction of the sky that is not blocked by the foliage (*C*) and photosynthetic photon flux density on leaf +4 (Q, in *D*) of thirteen sugarcane genotypes. Different letters indicate statistical differences among genotypes (BF₁₀>3, n = 4).
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Fig. 2. Photosynthetic rate (P_N , in A), stomatal conductance (g_s , in B), instantaneous CO₂ quantum efficiency (Φ CO₂, in C) and instantaneous carboxylation efficiency (k, in D) in leaves +1 and +4 of thirteen sugarcane genotypes under high light [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹]. * indicates a difference between leaves +1 and +4 (BF₁₀>3, n = 4).

Figure 3



Fig. 3. Ratio of photosynthesis between leaves +4 and +1 ($P_N^{+4H:+1H}$) of thirteen sugarcane genotypes under high light [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹]. Different letters indicate statistical differences among genotypes (BF₁₀>3, *n* = 4).





Fig. 4. Ratios of photosynthesis ($P_N^{+4L:+4H}$), stomatal conductance ($g_s^{+4L:+4H}$), instantaneous CO₂ quantum efficiency ($\Phi CO_2^{+4L:+4H}$) and instantaneous carboxylation efficiency ($k^{+4L:+4H}$) in leaf +4 of thirteen sugarcane genotypes under low [index 'L', Q = 200 µmol (photon) m⁻² s⁻¹] and high [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹] light. Different letters indicate statistical differences among genotypes (BF₁₀>3, n = 4).





Fig. 5. Correlation of thirteen sugarcane genotypes, based on Spearman's coefficient (P < 0.05). Photosynthetic rate (P_N), stomatal conductance (g_s), instantaneous CO₂ quantum efficiency (Φ CO₂) and instantaneous carboxylation efficiency (k) and ratios considering leaves +1 and +4 and light level [low (L) or high (H)]; photosynthetic photon flux density (Q), leaf area index (LAI), proportion of the sky that is not blocked by the foliage (Sky ratio), mean tilt angle of the foliage (MTA), and tillering.

Supplementary material



Figure 1S

Fig. 1S. Daily rainfall, average air temperature and maximum and minimum range of air temperature. The arrow indicates the date of evaluation.

Figure 2S



Fig. 2S. Mean tilt angle of the foliage of thirteen sugarcane genotypes. Different letters indicate statistical differences among genotypes ($BF_{10}>3$, n = 4).



Fig. 3S. Photosynthetic rate (P_N , in A), stomatal conductance (g_s , in B), instantaneous CO₂ quantum efficiency (Φ CO₂, in C) and instantaneous carboxylation efficiency (k, in D) in leaves +1 under high light [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹] and +4 under low light [index 'L', Q = 200 µmol (photon) m⁻² s⁻¹] of thirteen sugarcane genotypes. * indicates a difference between leaf +1 and +4 (BF₁₀>3, n = 4).





Fig. 4S. Ratios of photosynthesis ($P_N^{+4L:+1H}$), stomatal conductance ($g_s^{+4L:+1H}$), instantaneous CO₂ quantum efficiency ($\Phi CO_2^{+4L:+1H}$) and instantaneous carboxylation efficiency ($k^{+4L:+1H}$) in leaf +4 under low light [index 'L', Q = 200 µmol (photon) m⁻² s⁻¹] and leaf +1 under high light [index 'H', Q = 2,000 µmol (photon) m⁻² s⁻¹] of thirteen sugarcane genotypes. Different letters indicate statistical differences among genotypes (BF₁₀>3, *n* = 4).

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CHAPTER II: INTRA-SPECIFIC VARIATION IN MAIZE AND SORGHUM PHOTOSYNTHESIS

Intra-specific variation in maize and sorghum photosynthesis

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Abstract

Enhancing crop yield through improved photosynthesis is a key for feeding the global population and providing feedstock for a sustainable green economy. However, an integrative perspective considering the canopy profile is needed for effectively linking photosynthetic performance and biomass production in C₄ species. This study aimed for breeding purposes characterize photosynthesis along the canopy profile of five maize and sorghum cultivars, focusing on leaf gas exchange and chlorophyll fluorescence evaluations in leaves from three canopy strata: top; middle and bottom. The responses of photosynthesis to increasing intercellular CO₂ concentration and light (*A*–*C*_i and *A*–PAR curves, respectively) were performed and key photosynthetic traits estimated. The *A*–*C*_i and *A*–PAR curves revealed significant variability in maximum photosynthetic rates across the canopies. Modern maize cultivars exhibited high CO₂ assimilation, stomatal conductance, and the carboxylation rates of PEPC and Rubisco along the canopy profile. In contrast, sorghum cultivars showed significant variation among canopy strata, with DKB560 and Brandelisa standing out as cultivars with enhanced CO₂ uptake and maximum quantum efficiency of CO₂ assimilation through the canopy.

Keywords: light, photosynthesis, plant canopy, Sorghum bicolor, Zea mays.

Introduction

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Higher crop yield is key to feeding global population (Ray et al., 2013; Salter et al., 2019) and provide feedstock for a green economy. As we need highly efficient plants in converting sunlight energy into biomass, C_4 plants play an important role in food and bioenergy supplying due to its high photosynthetic efficiency based on the CO₂ concentration mechanism (CCM). In the mesophyll cells, the phosphoenolpyruvate (PEP) carboxylase (PEPC) fixes the CO₂ into four-carbon molecule, which is transported to the bundle sheath cells and decarboxylated by a malic enzyme dependent on NADP or NAD (NADP-ME or NAD-ME) and by phosphoenolpyruvate carboxykinase (PEPCK). Such decarboxylation increases the [CO₂] in bundle sheath cells, where the ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) refixes the CO₂ into carbohydrates through the C₃ pathway. The C₄ photosynthesis is regulated by the CO₂ diffusion from the atmosphere to the carboxylation sites, by the carboxylation reactions – PEPC and Rubisco activities – and by the regeneration of RuBP and PEP driven by ATP produced through photochemical reactions (von Caemmerer and Furbank, 2003; Yin et al., 2011; Sales et al., 2018).

Light distribution within plant canopy is an important factor limiting the photosynthetic performance in C₄ crops (Marchiori et al., 2010, 2014; Jaikumar et al., 2021; Cruz et al., 2022; Almeida et al., 2022). Architecture, size, composition and longevity of the plant canopy affect both quality and quantity of light reaching leaves, with aging and light acclimation along the canopy also changing CO₂ assimilation (Davey et al., 2017; Almeida et al., 2022). At the canopy scale, plant biomass can be enhanced by changes in plant architecture and higher efficiency of photosynthesis (Marchiori et al., 2010, 2014; Zhu et al. 2010; Slattery et al., 2018). According to Almeida et al. (2022), plant canopy should have three important features for high photosynthesis: (1) top leaves with high photosynthetic rates (high photosynthetic capacity); (2) similar photosynthesis); and (3) bottom leaves photosynthesizing close to the maximum even under low light intensity (light acclimation). These features might boost photosynthesis and then increase yield through genetic breeding, with the selection of top genotypes. However, our knowledge integrating canopy and photosynthesis is limited, mainly in C₄ species.

 C_4 species present a high range of maximum photosynthetic rates, from 14 to 61 μ mol m⁻² s⁻¹, being a possible key trait in breeding programs due its high heritability (Jackson et al., 2016;

Li et al., 2017; Almeida et al., 2021). For instance, most of the literature characterizes the C₄ photosynthetic responses in few genotypes or only in sun-exposed leaves, as done in energy cane (Cruz et al., 2021, 2022), maize and giant *Miscanthus* (Lee et al., 2021), sorghum (Jaikumar et al., 2021) and sugarcane (Marchiori et al., 2010, 2014; Almeida et al., 2021). In addition, no detailed information about how photosynthesis varies along the vertical canopy profile has been provided for modern cultivars of maize and sorghum. An integrative perspective considering the canopy profile is needed for linking photosynthesis along the canopy profile of two 'NADP-ME type' C₄ crop species: maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.), revealing the variability within canopy (three leaf ages) and among genotypes (five cultivars) of each species.

Material and Methods

Plant growth conditions

Five cultivars of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) were studied (Table 1). Seeds were sowed in plastic pots (12 L) filled with commercial substrate composed of *Sphagnum* spp., rice straw and perlite in 7:2:1 ratio (Carolina Soil of Brazil, Vera Cruz RS, Brazil). The plants were fertilized daily with nutrient solution modified from Sarruge (1975): 15 mmol N L⁻¹ (7 % as NH₄⁺); 4.8 mmol K L⁻¹; 5 mmol Ca L⁻¹; 2 mmol Mg L⁻¹; 1 mmol P L⁻¹; 1.2 mmol S L⁻¹; 28 µmol B L⁻¹; 54 µmol Fe L⁻¹; 5.5 µmol Mn L⁻¹; 2.1 µmol Zn L⁻¹; 1.1 µmol Cu L⁻¹; and 0.01 µmol Mo L⁻¹. Four plants per genotype of each species were maintained under greenhouse conditions throughout the experimental period, where air temperature averaged 25.5±5.4°C and air relative humidity 79.6±13.1%. To mitigate the potential impact of environmental variations within the greenhouse, the position of the plants was randomized weekly.

Photosynthesis

Leaf gas exchange and chlorophyll fluorescence were evaluated when plants were approximately two months old, corresponding to the R1 stage (Silking) in maize and the stage 6 (Blooming/Flowering) in sorghum. The measurements were taken in leaves from three canopy strata: top; middle and bottom. For the top canopy, we focused on the flag leaf in maize plants. However, we observed some variations in growth habit among sorghum cultivars. The top canopy was defined by the flag leaf for four sorghum cultivars, with the exception of Santa Elisa. For this cultivar, the top canopy was represented by leaf +1, which is defined as the first fully expanded leaf with visible ligule. Moving to the middle canopy, our attention was on the ear leaf in maize and the third leaf below the flag leaf (or leaf +1) in sorghum. For the bottom canopy, we evaluated the fourth leaf below the ear leaf in maize and the sixth leaf below the flag leaf (or leaf +1) in sorghum, as shown in Fig. 1.

Gas exchange measurements were taken using an infrared gas analyzer IRGA (Li-6400XT, LICOR Inc., Lincoln NE, USA) and a modulated fluorometer (6400-40LCF, LICOR Inc., Lincoln NE, USA) and recorded when the coefficient of variation was less than 2% and there was temporal stability. Measurements were taken under air temperature of 28°C, with a water vapor pressure deficit lower than 1.5 kPa and the flow rate of 500 μ mol s⁻¹. Leaf CO₂ assimilation (*A*), stomatal conductance (g_s) and the intercellular CO₂ concentration (*C*_i) were measured.

Chlorophyll fluorescence was evaluated using signals emitted before and after a saturation pulse (λ <710 nm; photosynthetically active radiation (PAR) ~ 8,000 µmol m⁻² s⁻¹; 0.8 s) and after the excitation of the photosystem I (PSI) by far-red light (λ =735 nm; PAR ~ 5 µmol m⁻² s⁻¹; 3.0 s). At the beginning, the leaves were acclimated in the dark for 30 min. Dark respiration (R_d), the maximum (F_m) and minimum (F_o) fluorescence were measured and the variable fluorescence in dark (F_v = F_m - F_o) and the maximum quantum efficiency of PSII (F_v/F_m) estimated. During leaf gas exchange, we monitored the steady-state (F'), maximum (F_m ') and minimum (F_o ', after excitation of the PSI) fluorescence (Baker, 2008). From these data, the effective quantum efficiency of PSII [Φ_{PSII} =(F_m '-F')/ F_m '] and the non-photochemical quenching [NPQ=(F_m - F_m ')/ F_m '] were calculated. The relative excess of energy (EXC) was estimated as: EXC=[(F_v/F_m - Φ_{PSII})/(F_v/F_m)], according to Bilger et al. (1995).

The response of photosynthesis to increasing intercellular CO₂ concentration (A– C_i curve) was evaluated after leaf acclimation (15 min) to air CO₂ concentration (C_a) of 400 µmol mol⁻¹ and PAR of 2000 µmol m⁻² s⁻¹. After this time, C_a inside the cuvette was changed as follows: 400, 300, 200, 120, 85, 70, 55, 400, 400, 500, 600, 800, 1200, and 1500 µmol mol⁻¹, with each step taking 3 min, or until the coefficient of variation was less than 2%. The A– C_i curves were fitted (r^2 >0.95) using the equation 1, according to Almeida et al. (2021):

$$A = A_{max} \times \left[1 - e^{-k(C_1 - \Gamma CO_2)} \right] \tag{1}$$

where A_{max} is the maximum leaf CO₂ assimilation under high light and CO₂ saturation, Γ CO₂ is the CO₂ compensation point, and *k* is the fitting coefficient.

The apparent maximum carboxylation rates of phosphoenolpyruvate carboxylase (PEPC, V_{pmax}) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, V_{cmax}) were estimated from the $A-C_i$ curves, following Yin et al. (2011):

$$V_{pmax} = s \times \frac{(C_{\rm i} + K_{\rm p})^2}{K_{\rm p}}$$
(2)

$$V_{cmax} = A_{max} + |\mathbf{R}_{\rm d}| \tag{3}$$

where *s* is the angular coefficient obtained by the initial linear part of $A-C_i$ curves ($C_i < 35 \mu mol mol^{-1}$) and K_p is the Michaelis-Menten constant of PEPC for CO₂ (Yin et al., 2016):

$$K_{p} = K_{p25} \times e^{\left(\frac{1}{298} - \frac{1}{T + 273}\right) \times \frac{E_{kp}}{R}}$$
(4)

where K_{p25} is Michaelis–Menten constant of PEPC for CO₂ at 25°C, T is the leaf temperature (°C) during measurements, E_{kp} is the K_p activation energy (68.058 kJ mol⁻¹), and R is the universal gas constant (0.008314 kJ K⁻¹ mol⁻¹).

The stomatal limitation of photosynthesis (L_S) was estimated from $A-C_i$ curve, following Long and Bernacchi (2003):

$$L_{\rm S} = \frac{A^{\prime\prime} - A^{\prime}}{A^{\prime\prime}} \times 100 \tag{5}$$

where A'' and A' are leaf CO₂ assimilation at C_i and C_a of 400 µmol mol⁻¹, respectively.

The metabolic limitation (L_M) of photosynthesis in plant canopy was also calculated, adapted from Lawlor (2002):

$$L_{\rm M} = \frac{A_{\rm H} - A}{A_{\rm H}} \times 100 \tag{6}$$

where $A_{\rm H}$ is the maximum and A is the minimum leaf CO₂ assimilation at $C_{\rm a}$ of 400 µmol mol⁻¹ along the canopy profile, considering only rates measured under high light and CO₂ saturation (curve ceiling).

The response of photosynthesis to light (*A*–PAR curve) was evaluated after leaf acclimation to light and CO₂, as done for the *A*–*C*_i curves. After leaf gas exchange reached the steady state, the light irradiance inside the Li-6400XT cuvette was changed in the following sequence: 2000, 1500, 1000, 500, 300, 200, 100, 50 and 0 μ mol m⁻² s⁻¹, with each step taking 3 min and total coefficient of variation lower than 2%. The *A*–PAR curves were fitted using the equation 7, adapted from Marshall and Biscoe (1980):

$$A = \frac{(\phi \text{ PAR}+A_{\text{sat}}) - [(\phi \text{ PAR}+A_{\text{sat}})^2 - (4\phi \text{ PAR}\theta A_{\text{sat}})]^{0.5}}{2\theta} - R_d$$
(7)

where A and A_{sat} are the area-based net and light saturated CO₂ assimilation, respectively; ϕ is the maximum quantum efficiency of CO₂ assimilation; R_d is the dark respiration (when PAR = 0 µmol m⁻² s⁻¹); and θ is the curvature factor of the response curve. From A–PAR curves, we also estimated the light compensation point (LCPT, PAR when A = 0 µmol m⁻² s⁻¹).

The chlorophyllmeter model CFL1030 (Falker, Porto Alegre RS, Brazil) was used to evaluate the chlorophyll a, b, a + b and a/b indexes.

Biometric measurements

The leaf area (LA) was measured with a planimeter model Li-3000C (LICOR Inc., Lincoln NE, USA) and the dry matter of leaves (DML) evaluated after drying in an oven (60°C) with

forced-air circulation until reaching constant weight. From these data, the specific leaf mass (SLM = DML/LA, in kg m⁻²) was estimated.

Experimental design and data analyses

The experimental design was a randomized 3×5 factorial arrangement, with four biological replicates: three canopy levels (top, middle and bottom) and five genotypes for each of the two species studied (maize and sorghum). Normal distribution and homogeneity of variances were tested using the Shapiro-Wilk and Hartley tests, respectively. Following these preliminary tests, all variables were subjected to the analysis of variance (ANOVA). Mean values were compared by the Tukey test (p<0.05) when statistical significance was detected. Furthermore, the correlations were examined through the Pearson's coefficient. All analyses were conducted using the R software (R Core Team 2024; version 4.4.0, R-project, packages 'corrplot', 'ExpDes', 'Hmisc' and 'Readxl').

Results

Leaf gas exchange

 $A-C_i$ and A-PAR curves revealed significant variation in the maximum photosynthetic rates across the canopies of maize (from 22.5 to 50.4 µmol m⁻² s⁻¹) and sorghum (from 11.2 to 46.0 µmol m⁻² s⁻¹, Figs. 2 and 3). To further analyze these findings, we estimated the photosynthetic canopy homogeneity index (CHI) – the ratio of photosynthesis between the top and bottom leaves of each species under high light and high air CO₂ concentration. For maize, the CHI varied between 1.04 (BM2401) and 1.44 (AG8701). In sorghum, the CHI ranged from 1.19 (DKB560) and 2.16 (Santa Elisa). In terms of metabolic limitation (L_M), we observed differences among the genotypes of both species. L_M varied from 12.9% (B2401) to 30.9% (AG8701) in maize, whereas it ranged from 16.1% (DKB560) to 53.8% (Santa Elisa) in sorghum, as shown in Fig. 2.

Our study revealed no significant interaction (p>0.05) between the canopy position and the maize cultivars for photosynthesis (A_{400} , measured at $C_a = 400 \ \mu mol \ mol^{-1}$) stomatal conductance (g_{s400} , measured at $C_a = 400 \ \mu mol \ mol^{-1}$) and stomatal limitation (L_s). This is also applied to the

saturated CO₂ assimilation (A_{sat}), the maximum quantum efficiency of CO₂ assimilation (ϕ) and light compensation point (LCPT). When comparing cultivars, K7500 exhibited the lowest A_{400} , A_{sat} and ϕ (Figs. 4A and 5A, C). Generally, the bottom leaves displayed lower A_{400} and A_{sat} (30.1±1.1 and 39.9±1.8 µmol m⁻² s⁻¹) compared to the top (38.5±1.4 and 56.1±1.9 µmol m⁻² s⁻¹) and middle leaves (37.3±1.0 and 53.7±1.6 µmol m⁻² s⁻¹, Figs. 4B and 5B). Interestingly, this pattern was reversed for ϕ . The middle and bottom leaves had higher ϕ (0.065±0.003 and 0.067±0.003 µmol µmol⁻¹) than the top leaf (0.056±0.003 µmol µmol⁻¹, Fig. 5D). For stomatal conductance, DKB355 had the highest g_{s400} (0.484±0.028 mol m⁻² s⁻¹) among the maize cultivars (Fig. 4C). In general, bottom leaves presented the lowest g_{s400} when comparing to the middle and top leaves (Fig. 4D). We found no interactions and differences among the genotypes and canopy layers for L_s , which averaged 3.0±0.3%. Regarding the LCPT, K7500 had the highest value (62±5 µmol m⁻² s⁻¹) among maize cultivars (Fig. 5E) and it was lower in bottom leaves (44±2 µmol m⁻² s⁻¹) in relation to the middle and top leaves (Fig. 5F).

Contrary to our findings in maize, there was significant interaction (p < 0.01) between the canopy layers and the sorghum cultivars for photosynthetic parameters such as A_{400} , A_{sat} , ϕ , g_{s400} and $L_{\rm S}$. Enforcer and Santa Elisa presented the largest variation in A_{400} and $A_{\rm sat}$ along the canopy profile (Figs. 6A and 7A). Enforcer, DKB560 and IAC7021 emerged as the superior genotypes for photosynthesis in top leaves, achieving A_{400} close to 46 µmol m⁻² s⁻¹ and A_{sat} about 55 µmol m⁻² s⁻¹ ¹ (Figs. 2, 6A and 7A). Enforcer stood out as the best genotype for ϕ in bottom leaves, with values about 0.077 ± 0.004 umol umol⁻¹ (Fig. 7B). For stomatal conductance, DKB560 showed no differences along the canopy profile (averaging 0.335 ± 0.014 mol m⁻² s⁻¹), while Enforcer, IAC7021, Brandelisa and Santa Elisa exhibited lower g_{s400} in the bottom leaves (Fig. 6B). The stomatal limitation (L_s) was higher in the top (19.2 \pm 3.2%) and middle (16.0 \pm 1.6%) than in the bottom (11.5 \pm 1.9%) leaves of DKB560. Conversely, Santa Elisa presented higher L_s in the bottom (12.4±2.6%) compared to the top and middle leaves (4.8±0.6 and 3.7±1.0%). Enforcer, IAC7021 and Brandelisa showed no differences throughout the canopy, with $L_{\rm S}$ averaging 5.3±0.5%, $12.0\pm1.3\%$ and $8.1\pm0.8\%$, respectively (Fig. 6D). No interaction (p>0.05) between cultivars and canopy layers was found for light compensation point, with DKB560 and IAC7021 presenting the highest LCPT values (about 54 μ mol m⁻² s⁻¹, Fig. 8A). In general, the bottom leaves had a LCPT

of $39\pm2 \mu$ mol m⁻² s⁻¹, which was about 19.0% and 31.3% lower than those of the middle and top leaves, respectively (Fig. 8B).

Mitochondrial dark respiration (R_d) did not change among the maize cultivars (p>0.05), with an average of 3.3±0.1 µmol m⁻² s⁻¹. However, differences were noticed among canopy strata. The top and middle maize leaves (3.6±0.2 and 3.5±0.1 µmol m⁻² s⁻¹, respectively) presented higher R_d than bottom leaves (2.9±0.1 µmol m⁻² s⁻¹). In sorghum, DKB560 and IAC7021 presented the highest R_d (about 2.7 µmol m⁻² s⁻¹), and top leaves had higher R_d (2.9±0.1 µmol m⁻² s⁻¹) when compared to the middle and bottom leaves (2.2±0.1 to 2.4±0.1 µmol m⁻² s⁻¹, respectively).

Biochemistry and photochemistry

No interaction between the canopy layers and maize cultivars was found for V_{pmax} , V_{cmax} , V_{pmax} : V_{cmax} ratio, EXC, Φ_{PSII} and F_v/F_m (p>0.05). However, such interaction was significant for NPQ (p=0.02). AG8701 presented higher V_{pmax} than BM3069 and B2401 (Fig. 4E), whereas K7500 presented the lowest V_{cmax} among maize cultivars (Fig. 4G). The V_{pmax} : V_{cmax} ratio ranged from 0.80 to 2.20, with AG8701 presented higher values than BM3069 and B2401 (Fig. 4I). When comparing leaves, both V_{pmax} and V_{cmax} were lower in the bottom ones (Fig. 4F, H), while the lowest V_{pmax} : V_{cmax} ratio was found in the top leaves (Fig. 4J). Φ_{PSII} and F_v/F_m also varied among the genotypes, with DKB355 and B2401 exhibiting the highest Φ_{PSII} and F_v/F_m values, respectively (Fig. 9A, C). In general, the bottom leaves showed the lowest Φ_{PSII} (0.148±0.004) and F_v/F_m (0.757±0.004) when compared to middle and top leaves (Fig. 9B, D).

In sorghum species, we found significant interaction (p<0.05) between the canopy layers and cultivars for V_{cmax} and F_v/F_m . Nonetheless, no interaction (p>0.05) was observed for V_{pmax} , $V_{pmax}:V_{cmax}$ ratio, NPQ, EXC and Φ_{PSII} . Enforcer presented higher V_{cmax} and V_{pmax} than Santa Elisa (Figs. 6C and 10A), while DKB560 showed the lowest $V_{pmax}:V_{cmax}$ ratio (0.81 ± 0.05) among sorghum cultivars (Fig. 10C). We also observed higher V_{pmax} in top leaves as compared to bottom ones (Fig. 10B). No variation was found for $V_{pmax}:V_{cmax}$ ratio along canopy profile, which averaged 1.06 ± 0.04 (Fig. 10D). Regarding Φ_{PSII} , the top and middle leaves were more efficient than the bottom ones (Fig. 10F). The lowest F_v/F_m was found in the bottom leaves, with Santa Elisa showing the most significant reduction between the top and bottom (Fig. 6E). NPQ was found to be well correlated (p<0.05) with EXC in both maize (r=0.67) and sorghum (r=0.82) canopies (Fig. S1). Among maize cultivars, K7500 presented the largest leaf area $(1.01\pm0.03 \text{ m}^2)$ and the lowest specific leaf mass (SLM, ~44 g m⁻²) when plants were about 2-month-old. When considering sorghum cultivars, Santa Elisa had the largest leaf area $(1.74\pm0.05 \text{ m}^2)$, while Enforcer had the lowest SLM (~26 g m⁻², Fig. 11). No interaction (p<0.05) between the canopy layers and maize cultivars was found for chlorophyll a, b, total (a+b), and a/b ratio. There was no difference in chlorophyll a among maize genotypes, but DKB355 presented higher chlorophyll b (20.5±1.0 vs. 15.2±0.7) and total chlorophyll, and lower a/b ratio when contrasted to B2401 (65.5±1.2 vs. 59.0±1.2; and 2.25±0.11 vs 2.93±0.12), as shown in Fig. S2A, C, E and G. Chlorophyll a remained uniform across the top, middle and bottom maize leaves. Contrarily, chlorophyll b and the total chlorophyll were higher in middle as compared to top leaves, while a/b ratio increased in the top ones (Fig. S2B, D, F and H).

In sorghum, chlorophyll *a* did not show any significant variation (p<0.05) across canopy layers, averaging 40.3±0.2 (Fig. S3B). However, DKB560 showed higher chlorophyll *a* than IAC7021 (Fig. S3A). There were significant interactions (p<0.01) between the canopy layers and sorghum cultivars when considering chlorophyll *b*, *a/b* ratio and total chlorophyll. The top leaves of Enforcer had higher chlorophyll *b* and total than the bottom leaves, while the opposite was true for the *a/b* ratio. Furthermore, IAC7021 differed from Enforcer for chlorophyll *b*, *a/b* ratio and total in both top and bottom leaves (Fig. S4). Unfortunately, chlorophyll data of Brandelisa and Santa Elisa cultivars are missing.

Discussion

Variation of photosynthesis along the maize canopy profile: physiological adjustments for increasing homogeneity

Our data revealed physiological adjustments and no variation in photosynthetic traits when comparing the top and middle canopy leaves. The decline in A_{sat} , A_{400} and V_{cmax} in the bottom leaves could potentially be linked to the aging and nitrogen dynamics. In this context, we believe that the primary source of N being remobilized in the lower canopy might be from Rubisco. This process would redistribute nitrogen to the leaves in the upper canopy, sustaining their ability to perform high photosynthetic rates (Yin et al., 2011; Niinemets et al., 2015; Jaikumar et al., 2021). Interestingly, we found an increase in V_{pmax} : V_{cmax} ratio and ϕ in the middle and bottom leaves (Fig. 4J). According to Hikosaka and Terashima, (1995), N remobilization did not decrease ϕ when light is not a limiting factor, as found in our study and also in giant *Miscanthus* (Pignon et al., 2017). This observation suggests photosynthetic acclimation along the canopy to optimize the photosynthetic capacity in the top and middle leaves as found by Almeida et al., 2022. Simultaneously, it enhances the quantum efficiency of CO₂ assimilation in the bottom leaves, which are several weeks older than those found in the top and middle layers. A reduction in LCPT was also observed in the bottom layer (Fig. 5F), suggesting again a physiological strategy to optimize light-use efficiency.

 V_{pmax} is a physiological index associated with the capacity of PEPC and subsequently with the ability to increase [CO₂] in bundle-sheath cells (Bailey et al., 2000). High activities and abundance of PEPC, Rubisco, pyruvate orthophosphate dikinase (PPDK), phosphoenolpyruvate carboxykinase (PEPCK) and NADP-dependent malate dehydrogenase are crucial for maintaining photosynthesis within the deeper canopy, where leaves are shaded (Vu et al., 2006; Sales et al., 2018, Jaikumar et al., 2021). Here, the efficiency of the CO₂ concentrating mechanism (CCM) plays a significant role in this context. If CCM is effective, photorespiration is inhibited, leakiness is low and the quantum efficiency of CO₂ assimilation is high (Farquhar, 1983; Kromdijk et al., 2008; Sales et al., 2018). Particularly, high $V_{\text{pmax}}:V_{\text{cmax}}$ ratio and a small response of photosynthesis when changing C_a from 400 to 1500 µmol mol⁻¹ (Figs. 2A-E and 4J) provide compelling evidence of a high CO₂:O₂ ratio around the active sites of Rubisco in the middle and bottom leaves.

Overall, maize photosynthesis has a similar pattern among genotypes and canopy layers, which is likely a consequence of rigorous selection pressure in breeding programs. Such programs might have inadvertently selected genotypes with high canopy photosynthesis while aiming high maize yield (Lee and Tollernaar, 2007; Andorf et al., 2019; Muntean et al., 2022).

Underlying factors changing the photosynthetic activity along the sorghum canopy

In contrast to maize, sorghum genotypes exhibited significant variation of photosynthetic traits across the canopy. The source of this variability in sorghum can be traced back to breeding

purposes, such as grain yield, sugar accumulation in stalks and biomass/forage production (Table 1, Fig. 11B). Under identical light and [CO₂] conditions, we identified three distinct patterns of photosynthesis across the canopy of sorghum genotypes: (1) uniform photosynthetic activity across top, middle and bottom leaves, found in DKB560 and Brandelisa; (2) photosynthetic activity in top and middle leaves that differed from the bottom leaves in IAC7021; and (3) photosynthetic activity varying among the top, middle and bottom leaves, as observed in Enforcer and Santa Elisa.

Regarding the pattern (1), DKB560 exhibited high stomatal limitation (L_S) in the top (19.2±3.2%), middle (16.0±1.6%) and bottom (11.5±1.9%) leaves and low V_{pmax} : V_{cmax} ratio as compared to other cultivars (Figs. 6D and 10C). However, high A_{400} and low metabolic limitation (up 16%) found here for DKB560 indicate an enhanced capacity for CO₂ fixation across the canopy. Brandelisa also exhibited a homogeneous CO₂ assimilation throughout the canopy, but with V_{cmax} (31.9±1.3 µmol m⁻² s⁻¹) and L_S (8.1±0.8%) lower than those found in DKB560 (Fig. 6C, D). Both cultivars presented higher ϕ values in the bottom than the top leaves, a response similar to one found in maize.

When considering the patterns (2) and (3), there was a pronounced reduction in photosynthesis along the canopies of IAC7021, Enforcer and Santa Elisa. This decrease could be primarily attributed to the N remobilization from bottom leaves to upper ones. As most of leaf N is found in photosynthetic enzymes (Sage and Pearcy 1987; Sage 2002), we would speculate that such remobilization could reduce Rubisco abundancy and activity (V_{cmax}). If this is true, this phenomenon seems to occur without any decrease in ϕ , as previously found by Hikosaka and Terashima (1995). Overall, leaf CO₂ assimilation and V_{cmax} has a very similar pattern across the sorghum canopy (Fig. 6C), suggesting an association between these traits.

Breeding for enhancing photosynthesis: Is there room to boost canopy efficiency?

While maize genotypes had low variability for photosynthesis and leaf area, sorghum genotypes presented high variability for photosynthetic traits (Figs. 4A, B and 6A). Such variation in sorghum indicates the existence of a broad genetic diversity that could be exploited to enhance crop yield, as high heritability is found for photosynthesis in C₄ plants (Jackson et al., 2016; Li et al., 2017; Almeida et al., 2021). As several studies have shown a strong link between high photosynthetic activity and yield (Yoon et al., 2020; Ainsworth et al., 2021; De Souza et al., 2022;

Wei et al., 2022), photosynthetic traits might be a tool for selecting and screening sorghum genotypes with high yield potential. In maize, the low variability found here is in accordance with the low genetic variation within the maize germplasm, which poses difficulties for enhancing the photosynthetic capacity through breeding (Richards, 2000; Ahmadzadeh et al., 2004).

The biomass-producing sorghum genotypes Santa Elisa and Brandelisa had a large leaf area (Figs. 11). This phenotype not only enhances the visual appeal of the plant, but also suggests functional longevity of canopy in terms of CO_2 uptake and chlorophyll content (Thomas and Howarth, 2000). Interestingly, Brandelisa exhibited a homogeneous leaf CO_2 uptake throughout the canopy and higher ϕ in the bottom canopy (Figs. 6A; 7B), which might be associated with the sustained chlorophyll content as found in DKB560 and IAC7021 genotypes (Figs. S3B; S4). This strategy would maximize the photosynthetic capacity in the middle and bottom layers, an adaptive response aimed at increasing light-use efficiency throughout the canopy with potential to enhance crop yield.

Conclusion

Our study has provided valuable insights into the variation of photosynthetic traits across maize and sorghum canopies. We highlighted maize as homogeneous and efficient C₄ crop for high photosynthesis throughout the canopy. This is attributed to lower stomatal limitation and higher values of ϕ , stomatal conductance, and the carboxylation rates of PEPC and Rubisco. In contrast, sorghum presented significant variability, with DKB560 and Brandelisa standing out as cultivars with enhanced CO₂ uptake and ϕ through the canopy. The CO₂ uptake, ϕ and leaf area variability provides a rich pool for crop improvement in sorghum and could be a key factor, especially considering the current trend towards higher plant density, increased photosynthesis, and large leaf area index. These findings open avenues for future research and breeding programs aimed at enhancing crop productivity in an era of climate change and growing food demand.

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Tables

	Species	Cultivars	Туре	Institution
Maize Sorghum		Santa Elisa	biomass	IAC
	В	Brandelisa	sweet/biomass	IAC
	nyg.	IAC7021	grain	IAC
	Soi	ENFORCER	grain	Nuseed
		DKB560	grain	Dekalb
		BM3069 PRO2	grain/biomass	Biomatrix
		AG8701 PRO4	grain/biomass	Agroceres
	laize	K7500 VIP3	grain	KWS
	Z	DKB355 PRO3	grain	Dekalb
		B2401 PWU	grain	Brevant

Table 1. List of maize and sorghum cultivars, type and the institution responsible for breeding.

Figures



Fig. 1. Scheme of gas exchange evaluations in leaves on top, middle and bottom positions along the canopy of maize (in A) and sorghum (in B). Created in BioRender.com.





Fig. 2. Response curves of leaf CO₂ assimilation rate (*A*) to intercellular CO₂ concentration (C_i) in top (green), middle (yellow) and bottom (red) leaves of maize (A-E) and sorghum (F-J) cultivars, with their respective canopy homogeneity index (CHI) and metabolic limitation (L_M). Each symbol represents the mean±standard error (n=4).





Fig. 3. Response curves of leaf CO₂ assimilation rate (*A*) to increasing light intensity (PAR) in top (green), middle (yellow) and bottom (red) leaves of maize (A-E) and sorghum (F-J) cultivars. Each symbol represents the mean \pm standard error (*n*=4).



Fig. 4. Leaf CO₂ assimilation (A_{400} , A and B) and stomatal conductance (g_{s400} , C and D) at partial air CO₂ pressure (C_a) of 400 µmol mol⁻¹, *in vivo* maximum carboxylation rates of PEPC (V_{pmax} , E and F) and Rubisco (V_{cmax} , G and H) and V_{pmax} : V_{cmax} ratio (I and J) in five maize cultivars. Different letters indicate statistical differences among cultivars (in A, C, E and G, Tukey *p*<0.05, *n*=12), and canopy layers (in B, D, F and H, Tukey *p*<0.05, *n*=20).



Fig. 5. Light saturated leaf CO₂ assimilation (A_{sat} , A and B), maximum quantum efficiency of CO₂ assimilation (ϕ , C and D) and light compensation point (LCPT, E and F) in five maize cultivars. Different letters indicate statistical differences among cultivars (in A, C and E Tukey *p*<0.05, *n*=12), and canopy layers (in B, D and F Tukey *p*<0.05, *n*=20).



Fig. 6. Leaf CO₂ assimilation (A_{400} , in A) and stomatal conductance (g_{s400} , in B) at partial air CO₂ pressure (C_a) of 400 µmol mol⁻¹, *in vivo* maximum carboxylation rate of Rubisco (V_{cmax} , in C), stomatal limitation of photosynthesis (L_s , in D) and maximum quantum efficiency of PSII (F_v/F_m , in E) in top (green), middle (yellow) and bottom (red) leaves of five sorghum cultivars. Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey p<0.05, n=4).



Fig. 7. Saturated leaf CO₂ assimilation (A_{sat} , A) and maximum quantum efficiency of CO₂ assimilation (ϕ , B) in five sorghum cultivars. Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey *p*<0.05, *n*=4).




Fig. 8. Light compensation point (LCPT) in five sorghum cultivars. Different letters indicate statistical differences among cultivars (in A, Tukey p < 0.05, n=12) and canopy layers (in B, Tukey p < 0.05, n=20).



Fig. 9. Effective (Φ_{PSII} , A and B) and maximum (F_v/F_m , C and D) quantum efficiency of PSII in five maize cultivars. Different letters indicate statistical differences among cultivars (A and C, Tukey *p*<0.05, *n*=12) and canopy layers (B and D, Tukey *p*<0.05, *n*=20).





Fig. 10. *In vivo* maximum carboxylation rate of PEPC (V_{pmax} , A and B), V_{pmax} : V_{cmax} ratio (C and D) and effective quantum efficiency of PSII (Φ_{PSII} , E and F) in five sorghum cultivars. Different letters indicate statistical differences among cultivars (A and C, Tukey *p*<0.05, *n*=12) and canopy layers (B and D, Tukey *p*<0.05, *n*=20).





Fig. 11. Leaf area (A and B) and the specific leaf mass (SLM, in C and D) in five maize (A and C) and sorghum (B and D) cultivars. Different letters indicate significant differences among cultivars of each species (Tukey, p < 0.05, n=4).

Supplementary material



Figure S1

Fig. S1. Correlation between non-photochemical quenching (NPQ) and the relative excess of energy at PSII (EXC) of five maize (in A) and sorghum (in B) cultivars, based on Pearson's correlation coefficient (p<0.05, n=60).

Figure S2



Fig. S2. Chlorophyll *a* (in A and B), *b* (in C and D), *a/b* ratio (in E and F) and total (*a+b*) index (in G and H) in five maize cultivars. Different letters indicate statistical differences among cultivars (in A, C and E, Tukey p < 0.05, n=12) and canopy layers (in B, D and F, Tukey p < 0.05, n=20).





Fig. S3. Chlorophyll *a* index in five sorghum cultivars. Different letters indicate statistical differences among cultivars (in A, Tukey p < 0.05, n=12) and canopy layers (in B, Tukey p < 0.05, n=20).

Figure S4



Fig. S4. Chlorophyll *b* (in A), a/b ratio (in B) and total (a+b) index (in C) in top (green), middle (yellow) and bottom (red) leaves of five sorghum cultivars. Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey *p*<0.05, n=4).

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CHAPTER III: FAST PHOTOSYNTHESIS INDUCTION AND PHOTOPROTECTION THROUGH THE CANOPIES OF MAIZE AND SORGHUM CORRELATE WITH BIOMASS PRODUCTION

Fast photosynthesis induction and photoprotection through the canopies of maize and sorghum correlate with biomass production

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Abstract

Light variability along the canopy profile requires a sophisticated control for the induction of photosynthesis and the deactivation of photoprotective mechanisms, which could optimize the CO₂ assimilation and crop yield. This study aimed to investigate the genotypic variation in photosynthesis induction and the relaxation of non-photochemical quenching (NPQ) across the canopy of five maize (BM3069 PRO2, AG8701 PRO4, K7500 VIP3, DKB355 PRO3 and B2401 PWU) and sorghum (DKB560, Enforcer, IAC 7021, Brandelisa and Santa Elisa) cultivars. Measurements of CO₂ uptake, stomatal conductance and NPQ dynamics were taken during the induction of photosynthesis (changing light from 0 to 2,000 μ mol m⁻² s⁻¹) in three canopy strata: top, middle and the bottom canopy positions. The maize cultivars DKB355 PRO3 and AG8701 PRO4 exhibited higher dry matter and faster light responses, which were attributed to the synchrony among CO₂ assimilation, stomatal conductance, quantum yield efficiency of photosystem II, and fast deactivation of NPQ in the middle and bottom canopy layers. In contrast, sorghum cultivars exhibited negative or weak correlations between dry matter and photosynthetic traits. DKB560 was the slowest cultivar in terms of light response, showing no NPQ relaxation throughout the canopy. Interestingly, sorghum dry matter correlated positively with leaf area in the Brandelisa and Santa Elisa cultivars, indicating a compensation mechanism for slower photosynthetic induction and NPQ relaxation compared to maize. These findings provide valuable insights for breeding programs aiming to select new cultivars with improved photosynthesis, light responsiveness and increased leaf area - key traits for enhancing crop productivity under field conditions.

Keywords: biomass, induction, light transition, non-photochemical quenching.

Introduction

The efficiency of photosynthesis and the resilience of the photosynthetic machinery under light transition conditions are critical traits that can be harnessed to boost crop productivity (Kromdijk et al., 2016; Taylor and Long, 2017; Murchie et al., 2018). One promising strategy to increase crop yield involves the development of cultivars with high conversion of light into biomass across the canopy through enhanced photosynthesis (Reynolds et al, 2012; Driever et al., 2014). Leaves distributed along the canopy profile experience a dynamic light environment, with light intensity varying along the day and season. The upper leaves, typically exposed to direct solar radiation, contrast with those inside the canopy, which capture diffuse light or 'sunflecks' - brief periods of direct solar radiation that penetrate the canopy (Kromdijk et al., 2016; Slattery et al., 2018). Interestingly, these sunflecks contribute to approximately 90% of the energy available for photosynthesis inside the canopy and plants must be able to use such resource (Way and Pearcy, 2012).

A sophisticated regulatory apparatus for the induction of photosynthesis and the deactivation of photoprotective mechanisms is needed under light transition, which in turn optimizes CO₂ assimilation. However, slower response of photosynthesis to light reduces carbon gains across the canopy. For many crops, light saturation (the threshold beyond which additional light does not increase the rate of photosynthesis) is reached when less than half of the maximum light energy is available (Long et al., 2006). When the light irradiance is too high or the energy available for photochemistry rises faster, the photosynthetic proteins and membranes risk damage. In response, photoprotective mechanisms spring into action, shielding the antenna complexes from excessive light energy. This energy is then dissipated as heat by increasing the non-photochemical quenching, also known as NPQ (Kromdijk et al., 2016).

The molecular intricacies of NPQ are not yet fully understood, but it is known that the pH of the thylakoid lumen and the aggregation state of the antenna complexes in the photosystem II (PSII) play crucial roles. Three carotenoids - violaxanthin, antheraxanthin, and zeaxanthin - are involved in NPQ and constitute the xanthophylls cycle. Under high light conditions, violaxanthin is converted into zeaxanthin, via the intermediate antheraxanthin by the enzyme violaxanthin de-epoxidase (VDE). When the light irradiance decreases, zeaxanthin is converted back to violaxanthin epoxidase (ZEP) (Demming-Adams and Adams, 1996). The induction

of NPQ can be fast, in a matter of seconds, while its relaxation rate is slower, from minutes to hours. This sluggish recovery of the PSII antenna complex results in a decrease in photochemical efficiency and in quantum yield of CO_2 fixation, reducing the daily carbon gain up to 32% (Zhu et al., 2004; Kromdijk et al., 2016; Slattery et al., 2018).

We believe that the fast induction of photosynthesis and the fast relaxation of NPQ along the canopy profile might support higher biomass production in C₄ species. Variations in the kinetics of photosynthetic induction have been identified in C₃ species (Soleh et al., 2016, 2017; Taylor and Long, 2017; Salter et al., 2019). However, comprehensive studies evaluating the variability of photosynthetic induction and its photoprotection across the canopy of C₄ species are currently lacking. Herein, we investigated the genotypic variation in photosynthesis induction and NPQ relaxation across the canopy profile of two prominent C₄ crop species known for their biomass production: maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.), revealing the variability within canopy (three positions) and among genotypes (five cultivars) of each species.

Material and Methods

Plant growth and experimental conditions

Five cultivars of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) were studied (Table 1). Seeds were sowed in plastic pots (12 L) filled with commercial substrate composed of *Sphagnum* spp., rice straw and perlite in 7:2:1 ratio (Carolina Soil of Brazil, Vera Cruz RS, Brazil). The plants were fertilized daily with nutrient solution modified from Sarruge (1975): 15 mmol N L⁻¹ (7 % as NH₄⁺); 4.8 mmol K L⁻¹; 5 mmol Ca L⁻¹; 2 mmol Mg L⁻¹; 1 mmol P L⁻¹; 1.2 mmol S L⁻¹; 28 µmol B L⁻¹; 54 µmol Fe L⁻¹; 5.5 µmol Mn L⁻¹; 2.1 µmol Zn L⁻¹; 1.1 µmol Cu L⁻¹; and 0.01 µmol Mo L⁻¹. Four plants per genotype of each species were maintained under greenhouse conditions throughout the experimental period, where air temperature averaged 25.5±5.4°C and air relative humidity 79.6±13.1%. To mitigate the potential impact of environmental variations within the greenhouse, the position of the plants was randomized weekly.

Gas exchange and fluorescence analyses

Leaf gas exchange and chlorophyll fluorescence were evaluated when plants were approximately two months old, corresponding to the R1 stage (Silking) in maize and the stage 6 (Blooming/Flowering) in sorghum. These evaluations were performed using an infrared gas analyser (Li-6400XT, LICOR Inc., Lincoln NE, USA) and a modulated fluorometer (6400-40LCF, LICOR Inc., Lincoln NE, USA). For steady-state measurements (prior induction of photosynthesis), the leaf cuvette conditions were set at photosynthetic active radiation (PAR) of 2,000 μ mol m⁻² s⁻¹, air temperature of 28°C, flow rate of 500 μ mol s⁻¹, and air CO₂ concentration (*C*_a) of 400 μ mol mol⁻¹. Leaves were acclimated for a sufficient time to reach temporal stability conditions, when the coefficient of variation was less than 2%.

The induction of photosynthesis was analysed on the overnight-dark-adapted plants. Initially, the leaves were clipped into Li-6400XT cuvette in complete darkness, allowing them to acclimate to a 'dark phase'. Subsequently, the leaves were illuminated with a PAR of 2,000 μ mol m⁻² s⁻¹. Gas exchange and fluorescence were then recorded at one-minute intervals for 23 minutes. A comprehensive summary of all physiological traits derived from the evaluations can be found in

Table 2. The evaluations were taken from leaves positioned at three canopy strata (top, middle, and bottom), between 8h00 and 16h00, under leaf temperature of 28° C. For the top canopy, we focused on the flag leaf in maize plants. However, we observed some variations in growth habit among sorghum cultivars. The top canopy was defined by the flag leaf for four sorghum cultivars, with the exception of Santa Elisa. For this cultivar, the top canopy was represented by leaf +1, which is defined as the first fully expanded leaf with visible ligule. Moving to the middle canopy, our attention was on the ear leaf in maize and the third leaf below the flag leaf (or leaf +1) in sorghum. For the bottom canopy, we evaluated the fourth leaf below the ear leaf in maize and the sixth leaf below the flag leaf (or leaf +1) in sorghum.

Simultaneously to leaf gas exchange measurements, the chlorophyll fluorescence was evaluated using fluorescence signals emitted before and after a saturation pulse ($\lambda < 710$ nm; PAR ~ 8,000 µmol m⁻² s⁻¹; 0.8 seconds) and after the excitation of the photosystem I (PSI) by far-red light ($\lambda = 735$ nm; PAR ~ 5 µmol m⁻² s⁻¹; 3.0 seconds). In overnight-dark-adapted leaves, the maximum ($F_{\rm m}$) and minimum ($F_{\rm o}$) fluorescence signals were recorded. During the induction of photosynthesis, the steady-state (F'), the maximum ($F_{\rm m}'$) and the minimum ($F_{\rm o}'$, observed after the excitation of the PSI) fluorescence signals under light were monitored. Subsequently, the effective quantum efficiency of PSII [$\Phi_{\rm PSII} = (F_{\rm m}' - F')/F_{\rm m}'$] and the non-photochemical quenching [NPQ = ($F_{\rm m} - F_{\rm m}'$) / $F_{\rm m}'$] were calculated following Baker (2008).

The CO₂ uptake loss during the induction phase (C_{loss}), was calculated as:

$$C_{loss} = (A_{steady} \times t) - Ai \tag{1}$$

where A_{steady} represents the steady state CO₂ uptake under high light (PAR= 2,000 µmol m⁻²s⁻¹); and *Ai* is the integrated CO₂ assimilation during induction time (*t*) of 1,400 seconds.

 CO_2 assimilation rate, stomatal conductance, the effective quantum efficiency of PSII and the non-photochemical quenching were normalized (*N*) to values between 0 and 1 at the beginning and the end of the 23-minute illumination, to facilitate comparisons of the kinetics:

$$N = \frac{x - \min}{\max - \min} \tag{2}$$

where *x* is the value recorded at a specific time during the induction phase, *min* and *max* denote the minimum and maximum values recorded during the 23-minute illumination period.

Biometric measurements

The leaf area was measured with a planimeter model Li-3000C (LICOR Inc., Lincoln NE, USA) and the dry matter (DM) of leaves, culms, kernels, panicles and roots evaluated after drying in an oven (60°C) with forced-air circulation until reaching constant weight.

Experimental design and statistical analysis

For each crop species, the experimental design was a randomized 3×5 factorial arrangement, with four biological replicates: three canopy positions (top, middle and bottom) and five genotypes for each species. Normal distribution and homogeneity of variances were tested using the Shapiro-Wilk and Hartley tests, respectively. In cases where the parameters did not follow normal distribution, they were transformed using the function $\log_{10}(x)$ '. Following these preliminary tests, all variables were subjected to the analysis of variance (ANOVA). When statistical significance was detected, mean values were compared using Tukey's test (p < 0.05). In addition, correlations were examined using the Pearson's coefficient. All analyses were conducted using the R software (R Core Team 2024; version 4.4.0, R-project, packages 'corrplot', 'ExpDes.pt', 'Hmisc' and 'Readxl').

Results

Induction of photosynthesis

We found a significant variation in the induction of photosynthesis, stomatal conductance, effective quantum efficiency of PSII, and non-photochemical quenching through the canopies of maize and sorghum species. Herein, these parameters exhibited a biphasic response (especially in

maize) when changing PAR from 0 to 2,000 μ mol m⁻² s⁻¹. During the initial 700 seconds of induction, *A* and *g_s* increased rapidly, followed by a more gradual increase. However, it is important to note that the 1,400-second exposure to high light may not have been sufficient to achieve complete steady-state CO₂ assimilation and stomatal conductance for some maize and sorghum cultivars, as observed in the ratio between *A*_{ind}:*A*_{steady} and *g_s* ind:*g_s* steady (Figs S1 and S2).

When the PAR was increased from 0 to 2,000 μ mol m⁻² s⁻¹, there was an immediate increase in A and g_s of maize plants (Figs. 1 and 2). Following this initial phase, A decelerated, aligning with the rise in g_s by a synchronous temporal response. Here, the middle leaves presented higher mean values for the integrated CO₂ assimilation during the induction (Ai) in BM3069, DKB355 and B2401, while K7500 was less than 30 mmol m⁻² with no differences among top, middle and bottom positions (Fig. 1A-E). When considering the normalized CO₂ assimilation 700 seconds after induction (A_{700}) , the middle and bottom leaves displayed a higher responsiveness to high light than the top leaves. Notably, the genotype AG8701 was the only one that showed no variability of A_{700} across the canopy (Fig. 1I). Regarding g_s , high variability was also found during the induction phase. Stomatal conductance at the end of induction $(g_{s \text{ ind}})$ varied among the genotypes. While K7500 and AG8701 showed higher values in the top $(0.271\pm0.006 \text{ and } 0.352\pm0.007 \text{ mol m}^{-2} \text{ s}^{-1})$ and middle leaves (0.256±0.012 and 0.325±0.006 mol m⁻² s⁻¹), B2401 had higher $g_{s \text{ ind}}$ in the middle position (Fig. 2B, D, E). No differences across the canopy were found for BM3069 and DKB355 and $g_{s \text{ ind}}$ averaged 0.252±0.014 and 0.347±0.012 mol m⁻² s⁻¹, respectively (Fig. 2A, C). Regarding the normalized stomatal conductance 700 seconds after induction (g_s 700), the middle and bottom leaves were the most responsive to illumination during induction and DKB355 and AG8701 showed no differences throughout the canopy. There was no significant interaction (p>0.05) between canopy positions and maize cultivars to the CO₂ uptake loss during induction (Closs) and AG8701 exhibited the lowest mean values (Fig. 3A). Interestingly, Closs varied among top, middle and bottom leaves, being 21.24 ± 1.22 , 15.33 ± 0.91 and 12.16 ± 0.91 mmol m⁻², respectively (Fig. 3B).

Sorghum cultivars exhibited a lack of biphasic response and stability at the end of induction for *A* and g_s , particularly for the cultivar BKB560. When PAR increased from 0 to 2,000 µmol m⁻² s⁻¹, we noticed a 'lag phase' characterized by a delay before the increase in CO₂ uptake and stomatal conductance (Figs. 4 and 5). We found significant interaction between canopy positions and sorghum genotypes for *Ai*. DKB560 and Brandelisa did not show differences in *Ai* along the canopy and averaged 9.50±1.02 and 12.64±1.35 mmol m⁻², respectively. On the other hand, the cultivars Enforcer, IAC7021 and Santa Elisa had higher *Ai* in top leaves (24.63±3.59, 28.03±3.34 and 21.31±3.84 mmol m⁻²) with no differences among them (Fig. 4A-E). Considering A_{700} , DKB560 and Brandelisa showed no differences across the canopy. However, Brandelisa was faster than DKB560 in the bottom leaves, when considering A_{700} . Enforcer and Santa Elisa presented A_{700} in the bottom leaves up to 0.80, while IAC7021 was higher (up to 0.70) in the top ones (Fig. 4F-J). Regarding $g_{s_{ind}}$ Enforcer, DKB560 and Brandelisa showed no differences across the canopy and averaged 0.223±0.010, 0.146±0.011 and 0.148±0.009 mol m⁻² s⁻¹, respectively. In contrast, IAC7021 and Santa Elisa presented higher $g_{s_{ind}}$ in top leaves (Fig. 5A-E). For $g_{s_{-700}}$, Enforcer and Santa Elisa differed along the canopy, with the bottom leaves being more responsive to light than the top and middle leaves (Fig. 5F, J). C_{loss} varied (p<0.05) among canopy positions, averaging 33.93±1.85 mmol m⁻². Enforcer, IAC7021, Brandelisa and Santa Elisa exhibited lower C_{loss} in the bottom leaves (Fig. 6A).

Leaf gas exchange under steady-state

No significant interaction (p>0.05) was found between canopy position and maize cultivars for photosynthesis under high light at steady-state (A_{steady}). The cultivar K7500 (31.61±1.19 µmol m⁻² s⁻¹) presented the lowest A_{steady} values (Fig. 3C). The bottom leaves had lower A_{steady} (30.1±1.1 µmol m⁻² s⁻¹) when compared to the top (39.85±1.1 µmol m⁻² s⁻¹) and middle (39±1.0 µmol m⁻² s⁻¹) leaves (Fig. 3D). For stomatal conductance under high light at steady-state (g_{s_steady}), significative interaction (p<0.05) was found between canopy position and cultivars. Here, DKB355 showed no variation of g_{s_steady} across the canopy and averaged 0.346±0.017 mol m⁻² s⁻¹, while BM3069, K7500 and AG8701 showed higher g_{s_steady} in the top leaves. Regarding the cultivar B2401, the middle leaves (0.345±0.022 mol m⁻² s⁻¹) presented the highest values (Fig. 7A).

In sorghum, significant interaction (p < 0.05) between canopy positions and cultivars were found for both A_{steady} and $g_{\text{s_steady}}$. While A_{steady} of DKB560 was uniform across the canopy, A_{steady} between top to bottom leaves the cultivars varied for Enforcer, IAC7021, Brandelisa and Santa Elisa (Fig. 6B). Regarding g_{s_steady} , no variation along the canopy was found for Enforcer and Brandelisa, averaging 0.290±0.009 and 0.247±0.011 mol m⁻² s⁻¹, respectively. g_{s_steady} in DKB560 was lower at top position as compared to bottom one (0.215±0.026 vs. 0.291±0.006 mol m⁻² s⁻¹). The opposite was found for the genotypes IAC7021 and Santa Elisa (Fig. 7B), with high g_{s_steady} at top positions.

NPQ and Φ_{PSII} *dynamics*

In maize, we observed a rapid increase in NPQ during the induction, which reached a peak before decreasing gradually. Leaves at the bottom of the canopy were slower in relaxing NPQ (Fig. S3). Φ_{PSII} increased rapidly, followed by a more gradual ascent (Fig. S4). No significant interaction (p>0.05) was found along the canopy for the integrated non-photochemical quenching (NPQi), Φ_{PSII} and the normalized effective quantum efficiency of PSII 700 seconds after induction (Φ_{PSII_700}) (Fig. 8). DKB355 and AG8701 exhibited the lowest NPQi (Fig. 8A). Overall, we found no differences in NPQi across the canopy positions of maize cultivars (Fig. 8B). The cultivar DKB355 (0.198±0.008) presented the highest Φ_{PSII} (Fig. 8C), and top and middle leaves had higher Φ_{PSII} than bottom ones (Fig. 8D). Regarding Φ_{PSII_700} , DKB355 and AG8701 emerged as the fastest genotypes (Fig. 8E). Overall, the middle and bottom leaves were faster than the top leaves when considering Φ_{PSII_700} (Fig. 8F).

In sorghum, NPQ increased rapidly and eventually reaching a 'plateau phase', without any relaxation across the canopy (Fig. S5). Φ_{PSII} raised and did not exhibit any 'plateau phase' as found for NPQ in some cases (Fig. S6). No significant interactions between canopy positions and sorghum cultivars were found for NPQ*i*, Φ_{PSII} and Φ_{PSII_700} . DKB560 presented the highest NPQ*i* and the lowest Φ_{PSII} and Φ_{PSII_700} (Fig. 9A, C, E). Overall, no differences were found along the canopy profile for NPQ*i* and Φ_{PSII_700} (Fig. 9B, F), whereas Φ_{PSII} differed between the top and bottom leaves (Fig. 9D).

Leaf area and biomass

The maize cultivar AG8701 (336±7 g plant⁻¹) presented the highest biomass, while B2401 showed lower biomass of leaves compared to AG8701 and K7500. However, DKB355 had the lowest biomass in kernels and AG8701 presented the highest biomass in roots (Fig. 10A). Among

sorghum cultivars, the biomass of leaves, culms and roots ranged from 10 to 72; 23 to 167; and 9 to 66 g plant⁻¹, respectively. Brandelisa and Santa Elisa presented the highest biomass of leaves, culms, roots and total, and IAC7021 had biomass of panicles 2.5 times lower than those found in Enforcer and DKB560 (Fig. 10B). Regarding leaf area, the maize cultivar K7500 and sorghum cultivar Santa Elisa exhibited the largest values $(1.01\pm0.03 \text{ and } 1.74\pm0.05 \text{ m}^{-2}, \text{ data no showed})$

Correlations

Ai was correlated with A_{ind} (r = 0.77 and 0.89), g_{s_ind} (r = 0.72 and 0.85), Φ_{PSII_ind} (r = 0.57 and 0.72), A_{steady} (r = 0.71 and 0.55) and A_{700} (r = 0.55 and 0.56) for maize and sorghum, respectively (Figs. S7 and S8). All these traits were correlated with maize biomass production. In sorghum, we found either a negative or no correlation with the biomass production. Sorghum biomass was correlated with LA (r = 0.90, Fig. S8). For both species, C_{loss} correlated positively with NPQ*i* (r = 0.54 and 0.34) and negatively with A_{700} (r = -0.92 and -0.82), g_{s_700} (r = -0.85 and -0.81) and Φ_{PSII_700} (r = -0.73 and -0.52). Interestingly, we noticed a negative correlation between NPQ*i* and *Ai* (r = -0.61), and between NPQ*i* and biomass (r = -0.60) in maize (Fig. S7).

Discussion

In this study, we have made a pioneering discovery of high variability in photosynthesis during the transition from dark to light conditions along the canopies of *Zea mays* and *Sorghum bicolor*. As found previously under steady-state conditions (chapter II), maize showed no interaction between canopy position and cultivars for leaf CO₂ assimilation. In contrast, sorghum presented a significant interaction and high variability for A_{steady} and stomatal conductance ($g_{s_{\text{steady}}}$; Figs. 3, 6 and 7). In general, such variability in sorghum can be addressed to differences in photosynthetic nitrogen use efficiency (Marchiori et al., 2014; Almeida et al., 2021), stomatal limitation, apparent quantum efficiency of CO₂ assimilation (ϕ), quantum efficiency of PSII and in carboxylation rates of PEPC (V_{pmax}) and Rubisco (V_{cmax}).

During the transition from dark to light, maize presented A_{ind} and $g_{s_{ind}}$ aligned with those observed under steady-state conditions, contrasting to the behavior observed in sorghum cultivars. The A_{ind} : A_{steady} and $g_{s_{ind}}$: $g_{s_{s_{ind}}}$: $g_{s_{ind}}$:g

(Figs. S1 and S2). This indicates high responsiveness to high light, characterized by a rapid induction and elevated carbon gain by the higher Ai and $\Phi_{PSII_{ind}}$ across the canopy profile (Figs. 1 and 8). A strong relationship between A and Φ_{PSII} was confirmed for both species (Figs. S7 and S8). In fact, ATP and NADPH are the products of light reactions of photosynthesis, encompassing electron transport in the thylakoid membranes of chloroplasts. For higher and faster carboxylation reactions, a quick supply of these components is essential (Baker, 2008; Malone et al., 2021; Ermakova et al., 2023). The sorghum cultivars DKB560, IAC7021 and Brandelisa exhibited a 'lag phase' as given by lower A_{700} and $g_{s,700}$ (ranging from 0.30 to 0.60) compared to those observed in maize during induction (Figs. 2 and 4). An increase in the leakiness of Z. mays, S. bicolor and S. officinarum species during dark to high light transition has been predicted by modelling, which suggests that the activation of the pyruvate phosphate dikinase (PPDK) in the mesophyll by the PPDK regulatory protein (PDRP) is faster than activation of Rubisco in the bundle sheath by Rubisco activase (Wang et al., 2021). Our findings suggest that higher responsiveness to light in Zea mays is linked to a better synchronism between C_3 and C_4 cycles. We would argue that high activity of both PPDK and Rubisco, when associated with higher stomatal response, might be the primary targets to enhance the coordination between the C₃ and C₄ cycles and thereby improve the responsiveness to high light. In fact, Rubisco activity is a key factor controlling photosynthesis (Taylor and Long, 2017). Carboxylation reactions might be also limited by the build-up of Calvin cycle intermediates under light transitions, such as the ribulose-1,5-bisphoshate (RuBP). When increasing light, RuBP requires almost 60 seconds for regeneration and supply, while Rubisco activation takes more than 10 minutes in cowpea and wheat plants (Taylor and Long, 2017; Deans et al., 2019; Acevedo-Siaca et al., 2020; Taylor et al., 2022).

Regarding A_{700} , we observed two patterns of light responsiveness in maize and sorghum species during the induction of photosynthesis: (1) A_{700} in the middle and/or bottom leaves being faster than the top ones; and (2) no variation in A_{700} among top, middle and bottom leaves. In (1), A_{700} in middle and/or bottom leaves reached up to 80% of the final induction values (Figs. 1 and 4) and exhibited low C_{loss} (Figs. 3B and 6A). This responsiveness to high light may indicate a canopy acclimation to light fluctuation, which is supported by our previous findings (Almeida et al., 2022), and a significant decrease in V_{cmax} while the ϕ and Φ CO₂ increased in the bottom leaves (chapter II). This phenomenon has also been described in maize, *Miscanthus*, sorghum, and sugarcane (Marchiori et al., 2010, 2014; Pignon et al., 2017; Collison et al., 2020). Our data also suggest that middle and bottom leaves might have fast and enhanced coordination between C₄ and C₃ cycles during transitions from dark to high light, despite the top leaves presenting high A_{ind} and $g_{s_{ind}}$ (Figs.1, 2, 4 and 5).

In general, bottom leaves experience severe light transitions throughout the day. This could potentially affect their responsiveness from dark to high light due to fast activation of key enzymes, metabolic reactions between mesophyll and bundle sheath cells, the accumulation of intermediates or/and the balance of ionic fluxes for fast stomatal opening (Vialet-Chabrand et al., 2017; Papanatsiou et al., 2019; Wang et al., 2021, 2022). In (2), A₇₀₀ was similar along the canopy profile of C₄ species studied. Specifically, the maize cultivar AG8701 was responsive to light, showing normalized A_{700} greater than 80% (Fig. 11). In contrast, normalized A_{700} in sorghum cultivars varied from 30% (DKB560) to 65% (IAC7021), as shown in Fig. 4G, H. Interestingly, A700 was strongly correlated with $g_{s,700}$ (Figs. S7 and S8), and this indicates that the responsiveness of stomatal conductance to changing light was similar to photosynthesis. In fact, photosynthesis could potentially be constrained by the slow response of stomata to increasing light intensity as stomata can take several minutes to fully open (McAusland et al., 2016; Vialet-Chabrand et al., 2017). Herein, A_{ind} and A_{steady} correlated positively well with g_s ind and g_s steady during induction (Figs. S7) and S8). Indeed, stomatal conductance is determined by the stomatal density and size of the pore (Drake et al., 2013; Tanaka et al., 2013), which affects stomatal dynamics and then diffusional limitation of photosynthesis during the induction (McAusland et al., 2016; Yoshiyama et al., 2024).

Regarding NPQ dynamics, we found no interaction among canopy positions and maize and sorghum species (Figs. 8 and 9). Maize genotypes experienced a rise to a peak in NPQ, followed by a decline, while sorghum cultivars displayed a peak without subsequent relaxation during the induction of photosynthesis (Figs. S3 and S5). This trend was also documented in the first fully expanded leaf with apparent ligule (leaves +1) of maize, sorghum and sugarcane (Wang et al., 2021; Ermakova et al., 2023). Lower NPQ*i* and accelerated relaxation of NPQ during induction found herein, could potentially improve $\Phi_{PSII_{ind}}$ and boost CO₂ assimilation in top and middle leaves during the latter part of the induction phase – as shown by enhanced A_{700} and $g_{s_{2}700}$ (Figs. S7 and S8). The decline in NPQ*i* along the canopy profile coupled with rapid photosynthesis and stomatal opening up to 75% of the maximum observed in the middle and bottom leaves (Figs. 1, 2 and 9) likely facilitated a faster establishment of the proton gradient-dependent quenching (qE) during the induction of photosynthesis in maize cultivars DKB355 and AG8701. NPQ occurs

through the rapid response of qE in the PSII antenna pigments bound to the light-harvesting proteins (Demming-Adams and Adams, 1992). Recently, Ermakova et al. (2023) highlighted the importance of the cytochrome b_6f complex (Cytb₆f) and the overexpression of its Rieske FeS subunit during the dark to light transition had significant impact on CO₂ uptake, NPQ, biomass and grain yield in *Sorghum bicolor*.

Taken together, our data indicate that biomass production by maize and sorghum species can be attributed to high CO₂ uptake, fast induction of photosynthesis, rapid NPQ relaxation and high leaf area. We found strong correlations between *Ai* and *A*_{ind}, *g*_{s_ind}, Φ_{PSII_ind} , *A*_{steady} and *A*₇₀₀ for maize and sorghum cultivars (Figs. S7 and S8). The maize cultivar AG8701 accumulated more biomass and presented the lower C_{loss} and higher *A*₇₀₀, *g*_{s_700} and Φ_{PSII_700} (Figs. 1-3, 8 and 10). Here, high CO₂ uptake and responsiveness to light during the transition from dark to high light improved light-use efficiency and stimulated biomass production in maize under greenhouse conditions (Fig. 10A). Sorghum biomass presented a negative or weak correlation with photosynthetic parameters on leaf-area basis and correlated positively with total leaf area and leaf biomass (Fig. S8). This is an evidence that high leaf area (as found in Brandelisa and Santa Elisa cultivars) might compensate the limitations found during the photosynthetic induction (Fig. 10B). This finding paves the way for breeding programs to select new cultivars with improved photosynthesis, responsiveness to light fluctuations and leaf area, which could potentially lead to enhanced crop productivity in the face of changing environmental conditions.

Conclusion

We found high variability in photosynthesis during induction along plant canopies, which was correlated with biomass production. The maize cultivar AG8701 showed higher biomass and fast responsiveness of photosynthesis during induction, attributed to a synchronism among stomatal conductance, quantum efficiency of photosystem II and fast deactivation of NPQ in the middle and bottom canopy. The sorghum cultivars Enforcer and IAC7021 presented higher *Ai* in the top leaves. Sorghum biomass correlated positively with total leaf area, as observed in Brandelisa and Santa Elisa cultivars. This suggests a compensatory mechanism for photosynthetic limitations, including the higher C_{loss} and lower CO₂ uptake, stomatal conductance and effective quantum efficiency of PSII after 700 seconds after transitioning from dark to high light. Our

findings provide valuable insights for breeding programs to select new cultivars with improved photosynthesis, light responsiveness and increased leaf area, potentially enhancing crop productivity in changing environmental scenarios.

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Tables

Species	Cultivars	Туре	Institution
Sorghum	Santa Elisa	biomass	IAC
	Brandelisa	sweet/biomass	IAC
	IAC7021	grain	IAC
	ENFORCER	grain	Nuseed
	DKB560	grain	Dekalb
Maize	BM3069 PRO2	grain/biomass	Biomatrix
	AG8701 PRO4	grain/biomass	Agroceres
	K7500 VIP3	grain	KWS
	DKB355 PRO3	grain	Dekalb
	B2401 PWU	grain	Brevant

Table 1. List of maize and sorghum cultivars, type and the institution responsible for breeding.

Trait	Description	Unit
Asteady	CO_2 uptake under high light at steady-state (PAR= 2,000 μ mol m ⁻² s ⁻¹)	μ mol m ⁻² s ⁻¹
g_{s_steady}	Stomatal conductance under high light at steady-state (PAR= 2,000 μ mol m ⁻² s ⁻¹)	mol $m^{-2} s^{-1}$
$A_{ m ind}$	CO ₂ assimilation rate at the end of induction	μ mol m ⁻² s ⁻¹
g_{s_ind}	Stomatal conductance at the end of induction	$mol \ m^{-2} \ s^{-1}$
A_{700}	The normalized c 700 seconds after induction	unitless
g s_700	The normalized stomatal conductance 700 seconds after induction	unitless
Ai	Integrated CO ₂ assimilation during the induction	mmol m ⁻²
C_{loss}	The CO ₂ uptake loss during the induction	μ mol m ⁻² s ⁻¹
A_{ind} : A_{steady}	The ratio of A_{ind} and A_{steady}	unitless
g_{s_ind} , g_{s_steady}	The ratio of $g_{s_{ind}}$ and $g_{s_{isteady}}$	unitless
$\Phi_{\mathrm{PSII_ind}}$	Effective quantum yield efficiency of PSII at the end of induction	unitless
Φ_{PSII_700}	The normalized effective quantum efficiency of PSII 700 seconds after induction	unitless
NPQi	Integrated non-photochemical quenching during the induction	unitless
LA	Leaf area	m^2
LDM	Leaf dry matter	g plant ⁻¹
CDM	Culm dry matter	g plant ⁻¹
RDM	Root dry matter	g plant ⁻¹
DM	Total dry matter	g plant ⁻¹

Table 2. A summary of all biometric and photosynthetic traits measured and mentioned in the text, under steady-state and non-steady-state conditions. Units are also included.

Figures

Figure 1



Fig. 1. Photosynthesis induction in five maize cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻²s⁻¹ for 1,400 s). CO₂ assimilation rate (*A*, A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Insert graph, the integrated CO₂ assimilation of the induction (*Ai*, A-E) and the normalized CO₂ assimilation 700 seconds after induction (*Ai*, A-E). Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey *p*<0.05, *n*=4). Data are means±standard error.





Fig. 2. Photosynthesis induction in five maize cultivars to a step change in light irradiance (from dark to 2,000 µmol m⁻²s⁻¹ for 1,400 s). Stomatal conductance (g_s , A-E) and its normalized values (F-J) in top (green), middle (yellow) and bottom (red) leaves. Insert graph, the stomatal conductance under high light at steady state ($g_{s_{ind}}$, A-E) and the normalized stomatal conductance 700 seconds after induction ($g_{s_{ind}}$, F-J). Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey p<0.05, n=4). Data are means±standard error.





Fig. 3. The CO₂ uptake loss during the induction (C_{loss}, A and B) and the CO₂ uptake under high light at steady-state (A_{steady} , C and D) in five maize cultivars. Different letters indicate statistical differences among cultivars (in A and C Tukey *p*<0.05, *n*=12), and canopy layers (in B and D Tukey *p*<0.05, *n*=20).





Fig. 4. Photosynthesis induction in five sorghum cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻²s⁻¹ for 1,400 s). CO₂ assimilation rate (*A*, A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Insert graph, the integrated CO₂ assimilation of the induction (*Ai*, A-E) and the normalized CO₂ assimilation rate 700 seconds after induction (*Ai*, A-E). Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey *p*<0.05, *n*=4). Data are means±standard error.





Fig. 5. Photosynthesis induction in five sorghum cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻²s⁻¹ for 1,400 s). Stomatal conductance (g_s , A-E) and its normalized values (F-J) in top (green), middle (yellow) and bottom (red) leaves. Insert graph, the stomatal conductance under high light at steady state (g_{s_ind} , A-E) and the normalized stomatal conductance 700 seconds after induction (g_{s_700} , F-J). Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey p<0.05, n=4). Data are means±standard error.

Figure 6



Fig. 6. The CO₂ uptake loss during the induction (C_{loss}, A) and the CO₂ uptake under high light at steady-state (A_{steady} , B) in five sorghum cultivars. Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey *p*<0.05, *n*=4).





Fig. 7. Stomatal conductance under high light at steady-state (g_{s_steady}) in five maize (A) and sorghum (B) cultivars. Different capital and lowercase letters indicate statistical differences among cultivars and canopy layers, respectively (Tukey p < 0.05, n=4).




Fig. 8. Integrated non-photochemical quenching of the induction (NPQ*i*, A and B), effective quantum efficiency of PSII at the end of induction (Φ_{PSII_ind} , C and D) and the normalized effective quantum efficiency of PSII 700 seconds after induction (Φ_{PSII_700} , E and F) in five maize cultivars. Different letters indicate statistical differences among cultivars (in A, C and E, Tukey *p*<0.05, *n*=12), and canopy layers (in B, D and F, Tukey *p*<0.05, *n*=20).





Fig. 9. Integrated non-photochemical quenching of the induction (NPQ*i*, A and B), effective quantum efficiency of PSII at the end of induction (Φ_{PSII_ind} , C and D) and the normalized effective quantum efficiency of PSII 700 seconds after induction (Φ_{PSII_700} , E and F) in five sorghum cultivars. Different letters indicate statistical differences among cultivars (in A, C and E, Tukey *p*<0.05, *n*=12), and canopy layers (in B, D and F, Tukey *p*<0.05, *n*=20).





Fig. 10. Dry matter (DM) partitioning (leaves, culms, kernels, panicles and roots) of five maize (in A) and sorghum (in B) genotypes. Different letters indicate significant differences among genotypes of each species: capital letters compare total dry matter, and lowercase compare dry matter of a given organ (Tukey p < 0.05, n=4).

Supplementary Material



Figure S1

Fig. S1. Ratio of CO₂ assimilation rate (A_{ind} : A_{steady} , in A) and stomatal conductance (g_{s_ind} : g_{s_steady} , in B) in five maize cultivars, at the end of induction (index '__ind') and under high light at steady state (index '__steady').





Fig. S2. Ratio of CO₂ assimilation rate (A_{ind} : A_{steady} , in A) and stomatal conductance ($g_{s_{ind}}:g_{s_{steady}}$, in B) in five sorghum cultivars, at the end of induction (index '__ind') and under high light at steady state (index '__steady').





Fig. S3. Photosynthesis induction in five maize cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻²s⁻¹ for 1,400 s). Non-photochemical quenching (NPQ, A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Data are means±standard error (*n*=4).





Fig. S4. Photosynthesis induction in five maize cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻²s⁻¹ for 1,400 s). Effective quantum efficiency of PSII (Φ_{PSII} , A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Data are means±standard error (*n*=4).





Fig. S5. Photosynthesis induction in five sorghum cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻² s⁻¹ for 1,400 s). Non-photochemical quenching (NPQ, A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Data are means±standard error (*n*=4).





Fig. S6. Photosynthesis induction in five sorghum cultivars to a step change in light irradiance (from dark to 2,000 μ mol m⁻² s⁻¹ for 1,400 s). Effective quantum efficiency of PSII (Φ_{PSII} , A-E) and its normalized values (F-J), in top (green), middle (yellow) and bottom (red) leaves. Data are means±standard error (*n*=4).

Figure S7



Fig. S7. Correlation of five maize genotypes, based on Pearson's coefficient (p<0.05). CO₂ assimilation rate (A), stomatal conductance (g_s), effective quantum efficiency of PSII (Φ_{PSII}), non-photochemical quenching (NPQ) and ratios during induction (index '__ind'), steady state conditions (index '__steady'), integrated (index 'i') or normalized 700 seconds after induction (index '__700'); the CO₂ uptake loss during the induction (C_{loss}); leaf area (LA); leaf (LDM), culm (CDM), root (RDM) and total dry matter (DM).

Figure S8



Fig. S8. Correlation of five sorghum genotypes, based on Pearson's coefficient (p<0.05). CO₂ assimilation rate (A), stomatal conductance (g_s), effective quantum efficiency of PSII (Φ_{PSII}), non-photochemical quenching (NPQ) and ratios during induction (index '__ind'), steady state conditions (index '__steady'), integrated (index 'i') or normalized 700 seconds after induction (index '__700'); the CO₂ uptake loss during the induction (C_{loss}); leaf area (LA); leaf (LDM), culm (CDM), root (RDM) and total dry matter (DM).

GENERAL DISCUSSION

With this study, we were able to understand the photosynthetic traits driving higher CO_2 assimilation across plant canopies, highlighting genotypic variations. Herein, we underscored the photosynthetic dynamics of three major C_4 crops – maize, sorghum and sugarcane, and analyzed their relationship with biomass production, offering valuable insights for breeding selection.

In the first chapter, we observed significant photosynthetic variation in light-exposed and shaded leaves in *Saccharum* complex canopy. Notably, some sugarcane cultivars and species showed an impressive ability to maintain high photosynthetic performance in shaded leaves, which responded well to high light conditions. IACCTC06-8126 and CTC4 stood out with the best canopy photosynthetic capacity, attributed to their larger leaf area and elevated CO₂ uptake in light-exposed leaves and the best responsiveness to high light in the shaded leaves.

In the second chapter, we found high variability of photosynthesis along the canopies of maize and sorghum species. Maize demonstrated stability and efficiency for high photosynthesis due to low stomata limitation and high quantum efficiency of CO₂ assimilation, high stomatal conductance and high V_{pmax} : V_{cmax} ratio in bottom leaves, which increased the photosynthetic efficiency of such canopy layer. In contrast, there was significant interaction between the canopy layers and the sorghum cultivars for CO₂ uptake, stomatal conductance, quantum efficiency of CO₂ assimilation, stomatal limitation and V_{cmax} . Sorghum showed varying leaf areas and enhanced CO₂ assimilation rates across the canopy and among the genotypes. These findings might have implications for breeding goals related to grain, sugar or biomass production.

In chapter 3, we linked the rapid induction of CO_2 assimilation and NPQ relaxation across the canopy to higher photosynthesis and biomass production in maize. In fact, maize exhibited greater dry matter accumulation and quick responsiveness to light transition as a consequence of the synchronism among photosynthesis, stomatal conductance, quantum efficiency of photosystem II and fast deactivation of NPQ. Conversely, sorghum biomass was positively associated with total leaf area. This suggests a compensatory mechanism for photosynthetic limitations, including the higher CO_2 uptake loss during the induction and lower responsiveness of CO_2 assimilation, stomatal conductance and effective quantum efficiency of PSII during dark-to-light transitions.

CONCLUSION

Our findings provide an integrative view on photosynthesis variability, photosynthetic induction and NPQ dynamics across the canopy of C_4 species. These insights are important for breeding programs to select cultivars with improved photosynthesis and light responsiveness with increased leaf area. Future studies are essential to understand the molecular bases of such complex mechanisms by which plants acclimate/adapt to changing environments and how such capacity can be leveraged through breeding for improving yield in modern cultivars.

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Chapter I: Actavia;

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