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Flexibility of C_4 decarboxylation and photosynthetic plasticity in sugarcane plants under shading

Short running title: Sugarcane C_4 decarboxylation under shading

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Highlights

- Sugarcane grown under low light showed increased PEPCK activity.
- Changes in chloroplast arrangements in bundle sheath cells were also observed.
- Such morpho-physiological adjustments maintained C_4 photosynthetic efficiency.
- A model considering carboxylation and decarboxylation pathways is proposed.

Abstract

The flexibility between C_4 photosynthetic sub-types NADP-malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PEPCK), recently identified in some C_4 species, confers high photosynthetic efficiency under varying light conditions. Theoretically, PEPCK decarboxylation uses less quanta *per* CO_2 fixed than NADP-ME, suggesting an increase in PEPCK activity could be advantageous under shading, as CO_2 leakiness increases under low light. Thus, we hypothesize that sugarcane plants have flexibility among the decarboxylation pathways, i.e., more than one decarboxylation route occurs independent of the environmental condition; furthermore, low light availability induces biochemical and anatomical adjustments resulting in increased PEPCK activity, which could contribute to maintaining or even increasing quantum efficiency of CO_2 assimilation under limiting light. Two sugarcane varieties were evaluated and both presented activities of the three decarboxylases, either under full sunlight or shading. *In vitro* PEPCK activity increased in plants grown under low light, suggesting an upregulation of this decarboxylation pathway. Accordingly, changes in chloroplast arrangement of bundle sheath cells from centrifugal to evenly distributed were found. Our data suggest that such biochemical and anatomical adjustments found in sugarcane grown under shading were important to maintain the maximum quantum efficiency of CO_2 assimilation. Finally, we propose a model highlighting the integration between the decarboxylation pathways under shading, considering carboxylation and decarboxylation pathways in sugarcane plants.

Abbreviations: A_n, leaf CO_2 assimilation; *Abx*, abaxial surface epidermis; *Adx*, adaxial surface epidermis; A_{max}, leaf CO_2 assimilation under light saturation; *BS*, bundle sheath;

C_i, CO_2 molar fraction in the leaf intercellular spaces; *DAP*, days after planting; *ETR*, apparent electron transport rate through PSII; F_q'/F_m', PSII operating efficiency; g_s, stomatal conductance; *Lb*, leaf blade; *LA*, leaf area; *LDM*, leaf dry matter; *M*, mesophyll thickness; *NAD-ME*, NAD-malic enzyme; *NADP-ME*, NADP-malic enzyme; *PEP*, phosphoenolpyruvate; *PEPC*, phosphoenolpyruvate carboxylase; *PEPCK*, phosphoenolpyruvate carboxykinase; *PPDK*, pyruvate orthophosphate dikinase; *Q*, photosynthetic active radiation; R_d, dark respiration; *RDM*, root dry matter; *SLA*, specific leaf area; $\phi CO_{2,max}$, maximum quantum efficiency of CO_2 assimilation; $\Delta^{13}C$, carbon isotope discrimination; ϕ, bundle sheath leakiness; ϕCO_2, instantaneous quantum efficiency of CO_2 assimilation

Keywords: C_4 photosynthesis, leakiness, low light, NADP-ME, PEPCK, *Saccharum* spp.

1. Introduction

C_4 photosynthesis is a specialized adaptation of C_3 plants in which CO_2 is firstly fixed via phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells, producing a C_4 acid that is transported to the bundle sheath cells (Sage et al., 2014). The next step is the decarboxylation of C_4 acids, with C_4 plants being classified in accordance to the major decarboxylating enzyme: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) or phosphoenolpyruvate carboxykinase (PEPCK). Chloroplast distribution in the bundle sheath cells and metabolite transport vary among C_4 subtypes (Sage et al., 2014). In addition, Ehleringer and Pearcy (1983) have suggested that NAD-ME plants present lower values of maximum quantum efficiency of CO_2 assimilation ($\phi CO_{2,max}$) than NADP-ME and PEPCK subtypes. Higher $\phi CO_{2,max}$ in PEPCK subtype is caused by ATP synthesis in the mitochondria of bundle sheath cells and direct return of phosphoenolpyruvate to the mesophyll cells without extra expense of ATP through the activity of pyruvate orthophosphate dikinase (PPDK; Furbank, 2011; Bellasio and Griffiths, 2014a).

Regarding the classical grouping of C_4 species according to the decarboxylating enzymes, many studies suggest flexibility within the C_4 subtypes (Pick et al., 2011; Bellasio and Griffiths, 2014a, b; Kromdijk et al., 2014; Sharwood et al., 2014; Wang et al., 2014). Flexibility is the concurrent operation of more than one decarboxylation enzyme, indicating that C_4 subtypes co-exist and contribute to the overall decarboxylation rate (Furbank, 2011). In sugarcane, a C_4 species classified as NADP-ME subtype, analysis of gene expression suggests that decarboxylation through PEPCK would predominate over NADP-ME in mature leaves (Calsa Jr and Figueira, 2007). From an ecological viewpoint, C_4 decarboxylation by more than one enzyme could confer advantages through maintaining high photosynthetic efficiency under a range of environmental conditions (Wang *et al.*, 2014). Besides the flexibility among the decarboxylation enzymes, C_4 species present metabolic plasticity (Pengelly et al., 2010; Bellasio and Griffiths, 2014b), i.e., the ability to acclimate to environmental changes through adjustments in morphological and physiological traits (Pigliucci, 2001; Sage and McKown, 2006).

The coordination between C_3 and C_4 cycles is essential for a high photosynthetic efficiency in C_4 species under shading (Kromdijk et al., 2008; Tazoe et al., 2008; Pengelly et al., 2010; Sharwood et al., 2014). In this sense, an important parameter to evaluate photosynthetic efficiency under limiting light is $\phi CO_{2,max}$, accessed from the initial slope of the light response curve (Zhu et al., 2004; Pignon et al., 2017). In sugarcane plants, photosynthesis occurs under non-saturating light conditions in lower canopy leaves (Dohleman and Long, 2009; Marchiori et al., 2010), where decreases in *in vivo* Rubisco capacity, nitrogen and chlorophyll contents limit photosynthetic capacity (Marchiori et al., 2014). In addition, imbalance in metabolite fluxes between C_3 and C_4 cycles leads to increased leakiness in plants grown under low light (Henderson et al., 1992; Kromdijk et al., 2008; Tazoe et al., 2008; Pengelly et al., 2010; Bellasio and Griffiths, 2014b), an additional factor reducing the efficiency of C_4 photosynthesis under shading conditions. While the additional energy expenditure due to leakiness has a small impact on photosynthetic rates under high light conditions, such loss of efficiency could limit C_4 photosynthesis under shading (von Caemmerer and Furbank, 2003; Kromidijk et al., 2008).

As the PEPCK pathway uses less quanta *per* CO_2 fixed than NADP-ME (Furbank, 2011), an increase in PEPCK activity in plants grown under low light conditions could maintain the efficiency of leaf photosynthesis. Such flexibility of C_4 decarboxylation would confer robustness to plants under fluctuating environment conditions (Wang et al., 2014). Thus, hypothesize that sugarcane plants have flexibility among the decarboxylation pathways, i.e., more than one decarboxylation route occurs independent of the environmental condition; furthermore, low light availability induces biochemical and anatomical adjustments resulting in increased in PEPCK activity, which could contribute to maintaining or even increasing $\phi CO_{2,max}$ under limiting light. Here, two varieties of sugarcane were grown in full sunlight or under shading and photosynthetic characteristics, enzyme activities and leaf anatomy were compared in order to detect acclimation responses related to the flexibility of C_4 photosynthetic metabolism.

2. Material and Methods

2.1. Plant material and experimental conditions

The photosynthetic acclimation to shading was evaluated in two sugarcane (*Saccharum* spp.) varieties SP70-1143 and IAC87-3396, which have high productivity in the field (Landell et al., 1997; 1999). Stalk segments (around 3 cm in length) with one bud were planted in plastic tubes of 120 cm^3 containing soilless substrate (Carolina Soil, Vera Cruz RS, Brazil). At 48 days after planting (DAP), the plantlets were transferred to 12 L plastic pots containing a mixture of soil and substrate (1:1, v/v). Pots were maintained under full sunlight and fertilized with 1.2 L of a solution containing (w/v) 21.4% $Ca(NO_3)_2$, 6.3% monoammonium phosphate, 8.6% g KCl, 2.5% $MgSO_4$, 0.2% H_3BO_3, 0.2% $MnSO_4$, 0.02% $CuSO_4$, 0.02% $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.10% $ZnSO_4$ and 0.02% Fe-EDDHA, supplied in six applications of 0.2 L each at 59, 77, 90, 106, 116 and 123 DAP.

After 90 DAP, one group of plants was transferred to shaded conditions (ca. 20% of full sunlight) by using a reflective aluminized polypropylene net (Freshnet, Solpack, Rio das Pedras SP, Brazil) and another group was maintained under full sunlight. Plants were grown for 48 days in each light condition (until 138 DAP; Supplementary Fig. S1). Regardless light environments, sugarcane genotypes were at the same phenological stages during the experimental period. The soil water availability during all the experimental period was monitored in both light conditions with soil moisture sensors (WaterMark 200SS, Irrometer, Riverside CA, USA). Soil matric water potential was maintained between −2.0 and −5.0 kPa, which represents high water availability for the plants. The photosynthetic active radiation (*Q*) was also monitored in both light conditions with a Li-190 light sensor (Licor Inc., Lincoln NE, USA) and recorded every 15 min with a Li-1400 data logger (Licor Inc., Lincoln NE, USA). The temperature was monitored with a Hobo U12-011 device (Onset, Bourne MA, USA). Environmental conditions during the experimental period are shown in Supplementary Fig. S2. The maximum *Q* in the full sunlight and the shaded environment were 2349 and 932 μmol m^{-2} s^{-1}, respectively.

2.2. Photosynthetic responses to light

Leaf gas exchanges were evaluated with a portable infrared gas analyzer (Li-6400, Licor Inc., Lincoln NE, USA) and chlorophyll fluorescence was measured with a modulated fluorometer (6400-40 LCF, Licor Inc., Lincoln NE, USA) coupled to the Li-6400. The response of leaf CO_2 assimilation (A_n) and stomatal conductance (g_s) to increasing Q was evaluated between 39 and 41 days of treatment (i.e. 129-131 DAP). During the measurements, the air temperature was 32.4 ± 2.7 °C and the leaf-to-air vapor pressure deficit was 2.7 ± 0.5 kPa. Leaf tissue was briefly acclimated for 10 min under Q of 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 40 Pa prior to the measurements. After acclimation, Q inside the leaf chamber was varied as follows: 2000, 1700, 1500, 1100, 700, 400, 300, 200, 150, 100, 50, and 0 μmol m^{-2} s^{-1}. The light response curves were fitted according to Marshall and Biscoe (1980):

$$A = \frac{\left(\phi CO_{2,max} Q + A_{max} + R_d\right) - [(\phi CO_{2,max} Q + A_{max} + R_d)^2 - [4\phi CO_{2,max} Q\theta (A_{max} + R_d)]^{0.5}}{2\theta} - R_d \quad (1)$$

where $\phi CO_{2,max}$ is the maximum quantum efficiency of CO_2 assimilation; A_{max} is the leaf CO_2 assimilation under light saturation, i.e., Q = 2000 μmol m^{-2} s^{-1}; θ describes curve convexity; and R_d is the dark respiration.

PSII operating efficiency (F_q'/F_m') and the apparent electron transport rate through PSII (*ETR*) were calculated for each Q level of the light response curve. For ETR calculation, the fraction of light energy partitioned to PSII (f) was considered 0.4 and the light absorption (α_{leaf}) 0.85 (Baker, 2008). Instantaneous quantum efficiency of CO_2 assimilation (ϕCO_2) was calculated according to Edwards and Baker (1993). The relationship between F_q'/F_m' and ϕCO_2 was used to provide information about alternative electron sinks (Edward and Baker, 1993).

2.3. Leaf sampling

The first and second fully expanded leaves with visible ligules were collected between 10h00 and 11h00, immediately frozen in liquid nitrogen, and stored at −80 °C (Revco PLUS model ULT1786-4-D46, Thermo Fisher Scientific, Waltham MA, USA). These samples were used for pigment contents, leaf nitrogen, enzyme assays and carbon isotope discrimination.

2.4 Leaf nitrogen and pigment contents

Leaf samples were ground and the analysis of nitrogen was analyzed using the Kjedahl method (Bremner, 1965). Pigment extraction was performed in segments of ca. 0.1 g with ethanol solution (96%, v/v). After extraction, the absorbance of samples was read in a spectrophotometer (Genesys 10S UV-Vis, (Thermo Fisher Scientific, Waltham MA, USA) and the equations proposed by Lichtenthaler and Wellburn (1983) used to determine Chl *a* and *b* concentrations. The ratio Chl *a*:*b* was also calculated

2.4. Photosynthetic enzymes

The activities of photosynthetic enzymes were measured spectrophotometrically (Genesys 10S UV-Vis, Thermo Fisher Scientific, Waltham MA, USA) at 340 nm, in a final volume of 1 mL, in four replicates. Protein extraction was performed using approximately 0.3 g of leaves, which were ground in liquid nitrogen using a pre-cooled ceramic mortar and pestle and 2% (w/v) insoluble PVPP, resulting in a fine powder. The extraction buffer and the specific procedures for each enzyme are described below. All reagents were purchased from Sigma-Aldrich (St. Louis MO, USA).

NADP-ME: The leaf powder was homogenized with 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 14,000 *g* for 25 min at 4 °C and the supernatant collected for the analysis (adapted from Ferreira et al., 2002). The standard assay medium for NADP-ME activity containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.5 mM NADP was incubated continuously at 30 °C. The reaction was started by addition of 100 mM L-malate with 100 μL of plant extract. The increase of the absorbance (340 nm) was a consequence of L-malate decarboxylation by NADP-ME, with the reduction of the co-factor NADP+ to NADPH (Maurino et al., 1997).

NAD-ME: The plant material was homogenized with 1.5 mL of 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM DTT and 2.5 mM pyruvate. The homogenate was centrifuged at 14,000 *g* for 25 min at 4 °C and the supernatant collected for the analysis (adapted from Du et al., 1996). NAD-

ME activity was measured using a standard reaction mixture containing 50 mM MES-NaOH (pH 6.5), 4 mM NAD, 5 mM DTT, 10 mM $MnCl_2$ and 10 units of malate dehydrogenase (MDH). The reaction was started by addition of 100 mM L-malate with 100 μL of plant extract (Tronconi et al., 2008). The increase of the absorbance (340 nm) was due to the decarboxylation of L-malate by NAD-ME and consequently, the increase in NADH (Chapman and Hatch, 1977).

PEPCK: The protein extraction was carried out as described for NAD-ME activity. The assay mix for PEPCK activity containing 100 mM HEPES-NaOH (pH 8.0), 2 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ATP, 0.4 mM ADP, 0.35 mM NADH, 5 units of lactate dehydrogenase, 2 units of pyruvate kinase was incubated continuously at 30 °C. The reaction was started by addition of 5 mM oxaloacetate with 100 μL of plant extract in the cuvette. The oxidation of NADH was accompanied by a subsequent reduction in absorbance (340 nm; Ashton et al., 1990). As PEPCK activity was evaluated in crude leaf extract instead using purified protein, we performed previous tests to confirm that PEPC activity was not interfering in PEPCK analysis (Supplementary Fig. S3).

Rubisco: The leaf powder was homogenized for 2 min with 1.5 mL of 100 mM bicine-NaOH (pH 7.8), 1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF and 10 μM leupeptin. The homogenate was centrifuged at 14,000 *g* for 5 min at 4 °C and the supernatant kept and maintained at 4 °C (adapted from Reid et al., 1997; Scales et al., 2014). Total Rubisco activity was measured using an assay mix containing 100 mM Bicine-NaOH (pH 8.0), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase and 80 nkat creatine phosphokinase. Seventy μL of plant extract was incubated at 30 °C for 10 min with the assay mix in the absence of ribulose-1,5-bisphosphate (RuBP), to enable carbamylation of the enzyme. The oxidation of NADP was started by adding 16 mM RuBP in the cuvette (adapted from Reid et al., 1997).

PEPC: The protein extraction was carried out as described in NADP-ME activity. The reaction mixture for PEPC activity containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase and 0.3 mM NADH was incubated continuously at 30 °C. The reaction was started by addition of 40 mM PEP with 100 μL of plant extract. The carboxylation of PEP and

subsequent oxidation of NADH causes reduction in absorbance at 340 nm (Degl'innocenti et al., 2002).

PPDK*:* The protein extraction was carried out as described in NAD-ME activity. PPDK activity was measured using a standard reaction mixture containing 25 mM HEPES-KOH (pH 8.0), 8 mM $MgSO_4$, 2.5 mM K_2HPO_4, 5 mM $(NH_4)_2SO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 1 mM *D*-glucose 6-phosphate, 1 mM ATP, 0.2 mM NADH, 2 units MDH, and 0.5 units mL^{-1} pyruvate carboxylase was incubated continuously at 25 °C. The reaction was started by adding 50 mM pyruvate with 50 μL of plant extract. The production of PEP through NADH oxidation via PEPC and MDH was accompanied by a reduction in absorbance at 340 nm (adapted from Ashton et al., 1990; Du et al., 1996).

2.5. Carbon isotope discrimination

Sugarcane leaves were oven-dried at 60 °C, ground to a fine powder and dried again overnight. Approximately 1.5 mg of leaf material was weighted in tin capsules. Samples were combusted and the relative abundance of ^{13}C and ^{12}C and the CO_2 produced was analyzed by mass spectrometry in the Stable Isotope Facility of University of California (Davis CA, USA). Stable carbon isotope composition was expressed as the ^{13}C:^{12}C ratio relative to Pee Dee Belemnite standard ($\delta^{13}C$, ‰). The carbon isotope discrimination ($\Delta^{13}C$) was calculated according to Equation 2 (Meinzer et al., 1994):

$$\Delta^{13}C = \frac{\delta_a - \delta_p}{1 + \delta_p} \qquad (2)$$

where δ_a is the isotopic composition of the air, standardised as –8.1‰ (Meinzer et al., 1994; Saliendra et al., 1996) and δ_p is the isotopic composition of the plant material.

2.6. CO_2 leakiness

Bundle sheath CO_2 leakiness (ϕ) was calculated according to Equation 3 (Ubierna et al., 2011):

$$\phi = \frac{\frac{1}{C_m}[C_a\Delta^{13}C - a(C_a - C_i)] - b_4}{b_3 - s}, \quad (3)$$

where C_m is CO_2 molar fraction in the mesophyll cell (in this case C_m=C_i was assumed considering infinite mesophyll conductance, g_m, to CO_2); C_a is CO_2 molar fraction in the atmosphere, 380 μmol mol^{-1}; a is the ^{13}C fractionation during diffusion of CO_2 in air (4.4‰); C_i is CO_2 molar fraction in the leaf intercellular spaces, here calculated as an average of measurements taken in light response curve (Supplementary Fig. S4); and s is the fractionation during leakage of CO_2 out of the bundle-sheath cells (1.8‰, according to Henderson et al., 1992).

The terms b_3 (^{13}C fractionation during carboxylation by Rubisco) and b_4 (net fractionation by CO_2 dissolution, hydration, and PEPC, including respiratory fractionation) were defined by Equations 4 and 5, respectively (Farquhar, 1983; Ubierna et al., 2011):

$$b_3 = b_3' - \frac{eR_d}{V_c} - \frac{f'V_0}{V_c} \quad (4)$$

$$b_4 = b_4' - \frac{eR_m}{V_p} \quad (5)$$

where b_3' is the fractionation by Rubisco (30‰, according to Roeske and O'Leary, 1984); e is the ^{13}C fractionation during decarboxylation (0‰, according to Ubierna *et al.*, 2013); f' is the ^{13}C fractionation during photorespiration (11.6‰, according to Ubierna et al., 2013); V_0 is the oxygenation rate (solved from equation 4.1 in von Caemmerer, 2000); V_c is Rubisco carboxylation rate (calculated from equation 4.33 in von Caemmerer, 2000); b_4' is the net fractionation by CO_2 dissolution, hydration and PEPC activity (−5.7‰, according to Farquhar, 1983); R_m is the mesophyll mitochondrial respiration rate (considered 0.5R_d according to von Caemmerer, 2000); and V_p is PEP carboxylation rate (solved from equation 4.31 in von Caemmerer, 2000).

2.7. Leaf anatomy

Samples of 3-4 cm from the middle portion of the second fully expanded leaf with visible ligule were taken after 48 days of treatment, between 10h00 and 11h00. Slices were fixed in a solution containing 1% glutaraldehyde and 4% paraformaldehyde in 0.2 M sodium phosphate buffer, pH 7.2 (Karnovsky, 1965) under vacuum for 24 h and subsequently dehydrated in an ethanol series and stored in 70% ethanol. For the analysis, the leaf pieces were dehydrated in an ethanol series (until 100%), infiltrated and embedded with plastic resin (Leica Microsystems, Wetzlar, Germany). Leaf sections of 2 or 7 μm thickness were prepared using a rotary microtome (Leica RM 2245, Leica Microsystems, Wetzlar, Germany), stained with 0.05% toluidine blue (Sakai, 1973) in phosphate and citrate buffer, pH 4.5 (McIlvaine, 1921). Permanent slides were mounted in synthetic resin Entellan New (Merck, Darmstadt, Germany). Images were captured with an Olympus BX53 microscope equipped with an Olympus Q-Color 5 camera (Olympus, Tokyo, Japan), using Image-Pro Express software, v. 6.3 (Media Cybernetics, Bethesda MD, USA). We evaluated leaf blade thickness (Lb), mesophyll thickness (M), adaxial (Adx) and abaxial (Abx) surface epidermis thickness and chloroplast distribution. Such variables were analyzed in a region with *ca.* 700 μm length, close to the third large vascular bundle (Supplementary Fig. S5).

2.8. Plant leaf area and biomass

After 48 days of treatment, leaf area (LA) was determined using a Li-3100C (Licor Inc., Lincoln NE, USA). Leaf (LDM) and root (RDM) dry matters were measured after drying plant material in an oven (60 °C), until constant weight. Specific leaf area (SLA) was determined as LA/LDM.

2.9. Statistical analysis

The experimental design was completely randomized and the effect of light treatment (full sunlight or shading) was evaluated for each variety. There were four plants per variety and each plant was treated as a single replicate; except for anatomical measurements (three plants × three replicates per plant). Statistical

significance tests were carried out using one-way ANOVA and means were ranked using Student's *t*-test at 95% significance level. Regression analyses between pairs of variables ($P < 0.01$) were performed using Origin 9.1.0 software (OriginLab Corp., Northampton MA, USA).

3. Results

3.1. Light photosynthetic responses

Shaded plants exhibited lower light-saturated CO_2 assimilation rates and stomatal conductance when compared to plants under the full-sunlight condition (Fig. 1A−D). Dark respiration was also lower in plants grown under shading (*ca.* 3.0±0.2 μmol m^{-2} s^{-1} in full sunlight and *ca.* 1.3±0.2 μmol m^{-2} s^{-1} in shaded plants; inset Fig. 1A, B). The maximum quantum efficiency of CO_2 assimilation ($\phi CO_{2,max}$) was similar in both light conditions, varying between 0.066 and 0.062 μmol μmol^{-1} in IAC87-3396 and between 0.060 and 0.059 μmol μmol^{-1} in SP70-1143 (inset Fig. 1A, B). Shading decreased the apparent electron transport rate (*ETR*) measured under high light in both varieties (Fig. 1E, F).

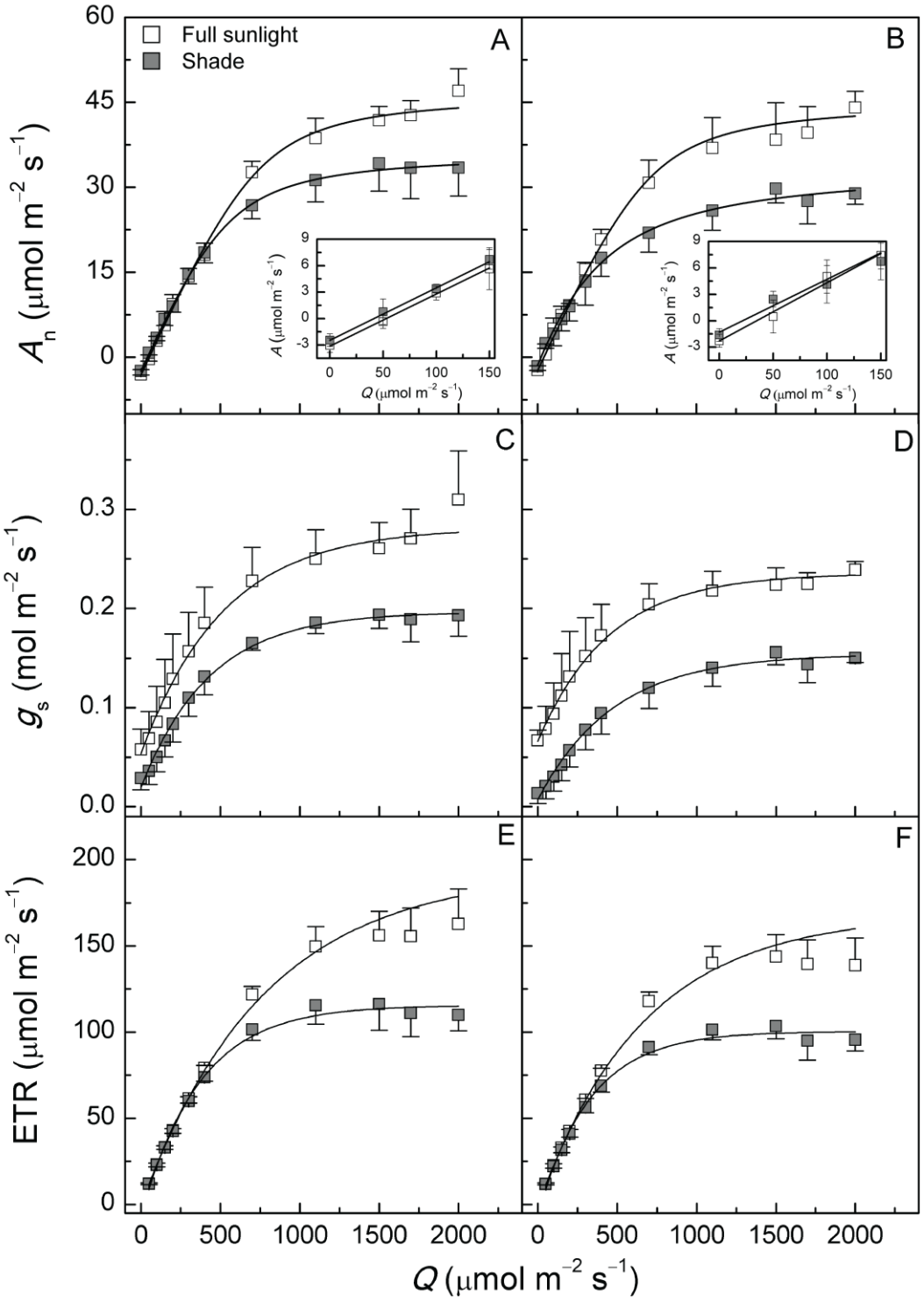

Fig. 1. Response of leaf CO_2 assimilation rate (A_n in A and B), stomatal conductance (g_s, in C and D) and apparent electron transport rate (*ETR*, in E and F) to increasing photosynthetic active radiation (Q) in sugarcane varieties IAC87-3396 (A, C and E) and SP70-1143 (B, D and F) grown under full sunlight (open symbols) or shading (closed symbols) for 39-41 days. Curves in A and B were fitted according to Marshall and Biscoe (1980). Values are mean ± SD (n=4).

The relation between PSII operating efficiency (F_q'/F_m') and the instantaneous quantum efficiency of CO_2 assimilation (ϕCO_2) was not changed by light treatments (Fig. 2).

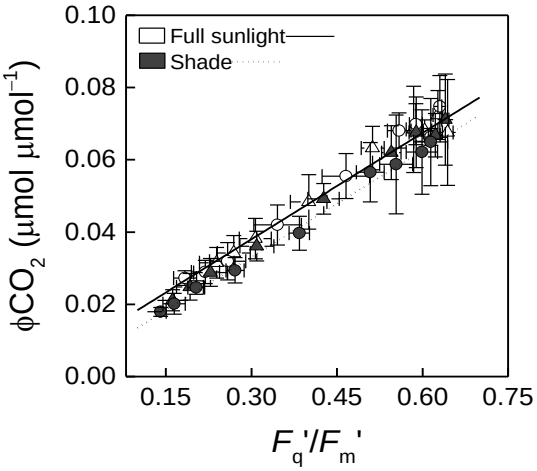

Fig. 2. Relationship between the PSII operating efficiency (F_q'/F_m') and the instantaneous quantum efficiency of CO_2 assimilation (ϕCO_2) in sugarcane varieties IAC87-3396 (triangle) and SP70-1143 (circle) grown under full sunlight or shading for 39-41 days. Values are mean ± SD (n=4).

3.2. Rubisco, PEPC and PPDK

Shade induced significant decreases in *in vitro* activity of both Rubisco and PEPC (Fig. 3A, B). Consequently, PEPC: Rubisco ratios did not change due low light (Fig. 3C). The activity of PPDK, the enzyme responsible for PEP regeneration, was significantly decreased in IAC87-3396 (by 24%) and SP70-1143 (by 54%) under shading (Fig. 3D).

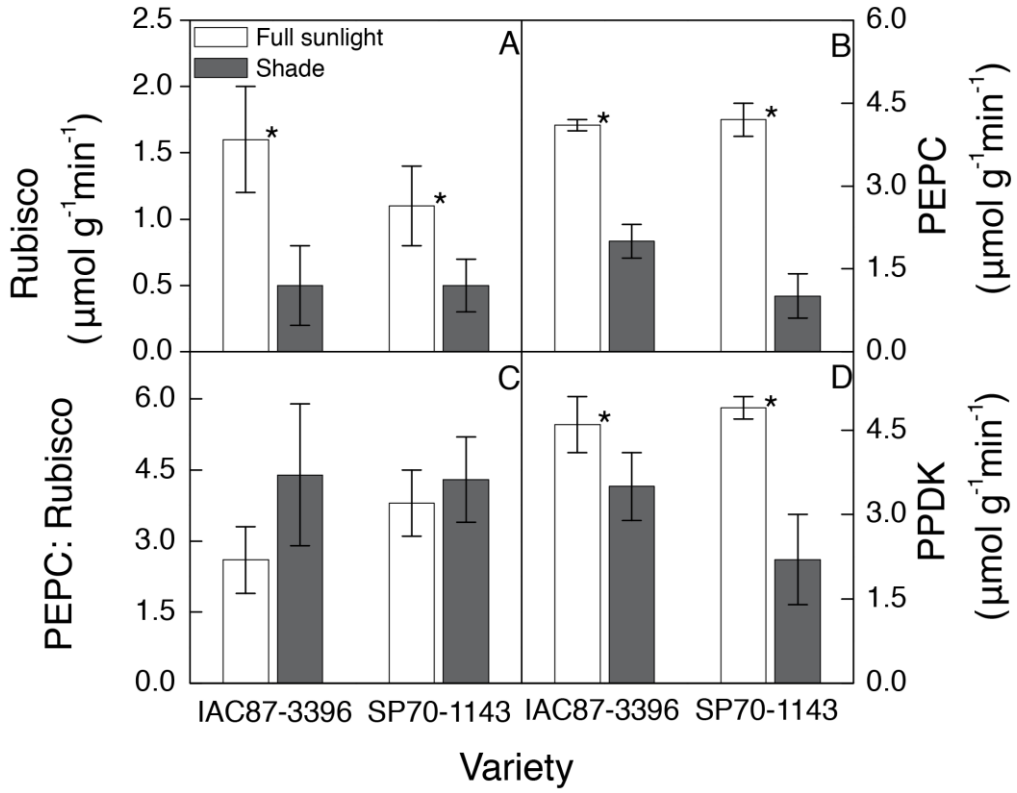

Fig. 3. Activity of Rubisco (A) and PEPC (B), PEPC:Rubisco ratio (C) and PPDK activity (D) in sugarcane varieties IAC87-3396 and SP70-1143 grown under full sunlight or shading for 48 days. Values are mean ± SD (*n*=4). * indicates significant difference ($P < 0.05$) between light treatments in a given variety.

3.3. Decarboxylase activities, chloroplast distribution and leakiness

Activities of NADP-ME, NAD-ME and PEPCK decarboxylases were detected in both varieties (Fig. 4). The activity of the primary decarboxylase NADP-ME was decreased (by 13%) due to shading only in SP70-1143 (Fig. 4A), which also showed a large increase in PEPCK activity in response to shading (Fig. 4C). IAC87-3396 also presented a significant increase in PEPCK activity due to shading (Fig. 4C). Regarding the overall decarboxylation process, the relative contribution of PEPCK increased in both sugarcane varieties under shading (Fig. 4D).

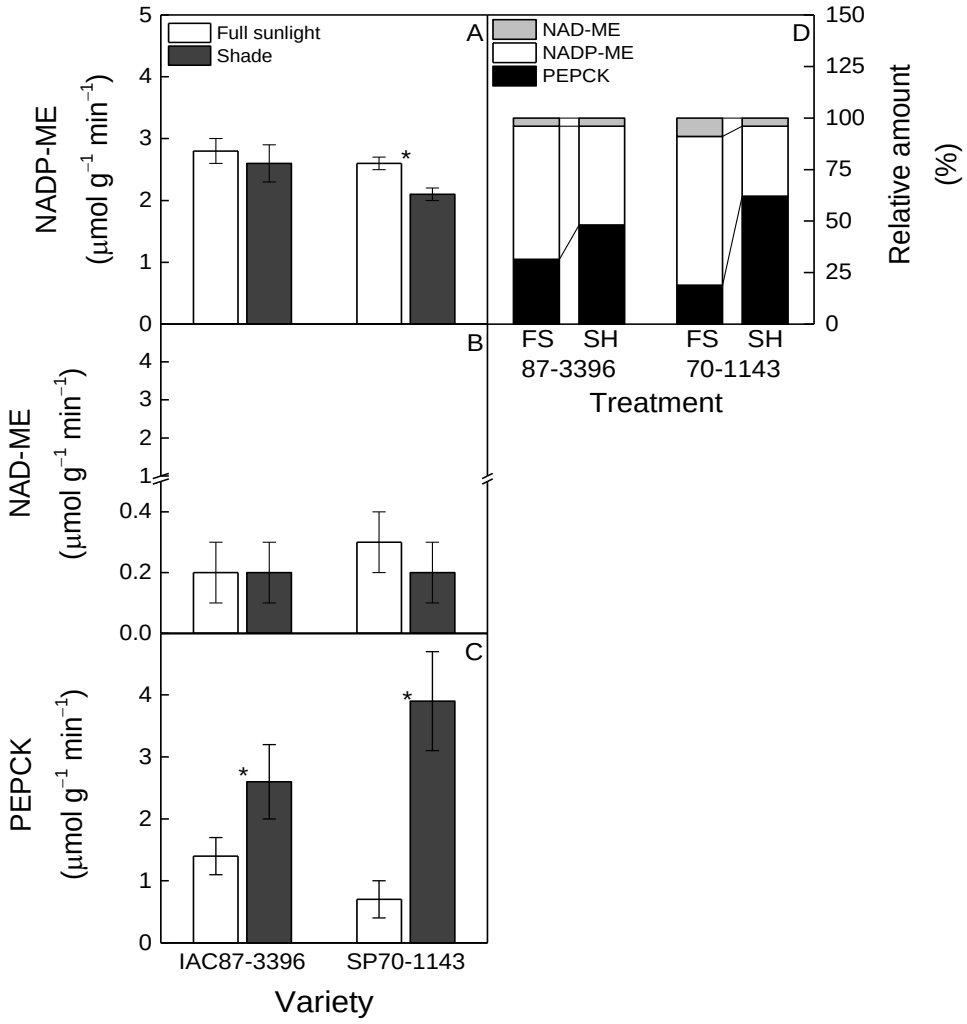

Fig. 4. Activity of NADP-ME (A), NAD-ME (B) and PEPCK (C), and their relative contribution of the overall decarboxylation (D) in sugarcane varieties IAC87-3396 and SP70-1143 grown under full sunlight (FS) or shading (SH) for 48 days. Values are mean ± SD (*n*=4). * indicates significant difference ($P < 0.05$) between light treatments in a given variety.

The chloroplast arrangement in bundle sheath cells was also changed by shading (Fig. 5). In general, the bundle sheath chloroplasts of C_4 grasses with NADP-ME subtype are usually arranged in a centrifugal position (Supplementary Fig. S6A), whereas their arrangement varies between evenly and centrifugal distribution in PEPCK subtypes (Supplementary Fig. S6B). We have noticed that

both varieties grown under full sunlight showed a centrifugal chloroplast distribution in the bundle sheath cells (Fig. 5A−D), whereas shaded plants presented chloroplasts arranged evenly (Fig. 5E−H).

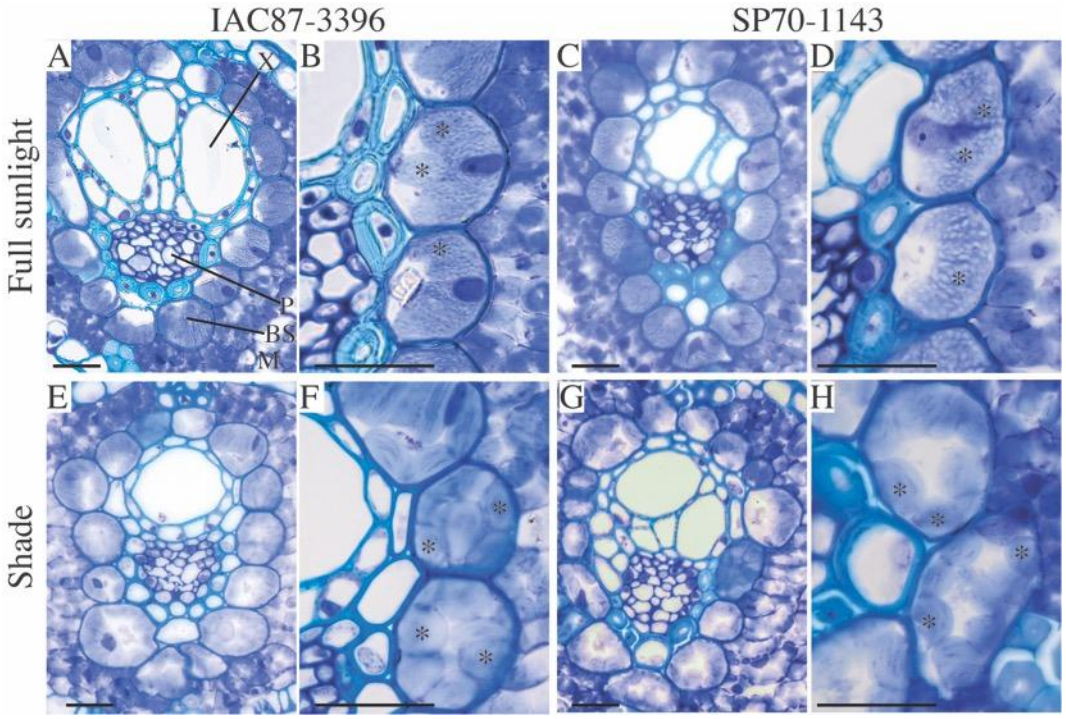

Fig. 5. Leaf cross sections of sugarcane varieties IAC87-3396 (A, B, E and F) and SP70-1143 (C, D, G and H) grown under full sunlight (A-D) or shading (E-H) for 48 days. General view of the vascular bundle (A, C, E, G) and detail of the bundle sheath cells (B, D, F, H) showing the chloroplast distribution: centrifugal (A-D) or evenly (E-H) positions. BS, bundle sheath; M, mesophyll, P, phloem; X, xylem; *, chloroplasts. Scale bar: 25 µm.

For both varieties, $\delta^{13}C$ of shaded plants was lower than the one found in plants grown under full sunlight (12.5±0.1 *vs*. 12.0±0.1 ‰). Compared to full sunlight conditions, $\Delta^{13}C$ increased by 12% for both varieties (Fig. 6A), and leakiness by 14% for IAC87-3396 and 34% for SP70-1143 under shading (Fig. 6B).

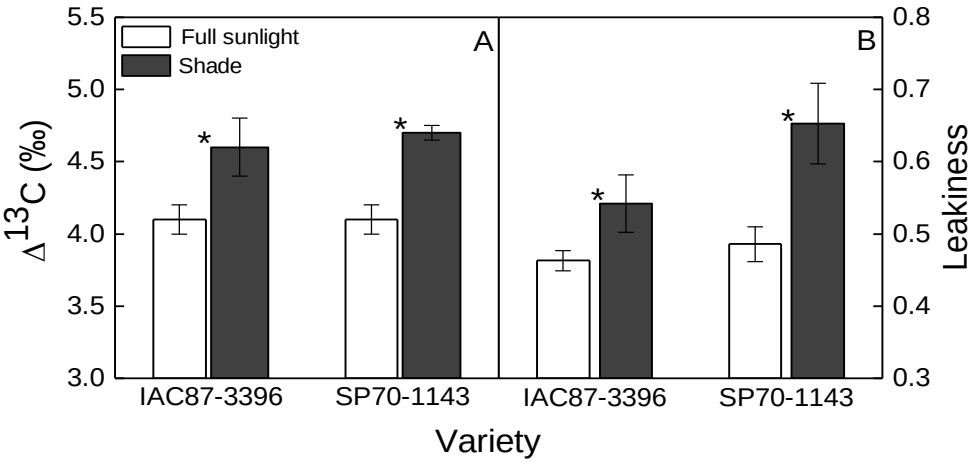

Fig. 6. Carbon isotope discrimination ($\Delta^{13}C$, in A) and bundle sheath leakiness (ϕ, in B) in sugarcane varieties IAC87-3396 and SP70-1143 grown under full sunlight or shading for 48 days. Values are mean ± SD (*n*=4). * indicates significant difference ($P < 0.05$) between light treatments in a given variety.

3.4. Plant growth, chlorophyll, nitrogen and anatomy of leaves

Shading decreased both leaf and root dry matter, with a more pronounced effect being found in the root system (Supplementary Table S1). As compared to plants exposed to full sunlight, specific leaf area was increased in both varieties under shading (Supplementary Table S1). Leaf chlorophyll *a* concentration was not affected by light treatments, whereas chlorophyll *b* concentration was 25% higher under shading in both sugarcane varieties. Leaf nitrogen concentration was also increased in plants grown under shading (Table 1).

Shading caused reductions in thickness of both abaxial and adaxial surface epidermis in both varieties and of leaf blade only in SP70-1143, without changing mesophyll thickness (Supplementary Table S1).

Table 1. Chlorophyll a and b concentrations and their ratio, and total leaf nitrogen content (N) in sugarcane varieties IAC87-3396 and SP70-1143 grown under full sunlight or shading after 48 days of treatment.

	IAC87-3396		SP70-1143	
Variable	Full sunlight	Shading	Full sunlight	Shading
Chl *a* (mg g^{-1})	4.5 ± 0.6a	4.6 ± 0.4a	4.6 ± 0.3a	5.1 ± 1.2a
Chl *b* (mg g^{-1})	1.5 ± 0.2b	1.8 ± 0.4a	1.6 ± 0.1b	2.0 ± 0.5a
Chl *a*:*b*	3.0 ± 0.3a	2.8 ± 0.2a	2.9 ± 0.2a	2.8 ± 0.2a
N (g kg^{-1})	21.3 ± 1.3b	22.5 ± 0.6a	19.2 ± 0.6b	21.7 ± 1.0a

Values are mean ± SD (n = 4). Different letters indicate significant difference ($P < 0.05$) between light treatments in a given variety.

4. Discussion

In this study, we show activity of the three decarboxylases NADP-ME, NAD-ME and PEPCK in sugarcane plants grown either under full sunlight or shading (Fig. 4). Although such flexibility among pathways has already been described in other species (Walker *et al*., 1997; Furbank, 2011; Pick *et al.*, 2011; Bellasio and Griffiths, 2014a, b; Sharwood *et al*., 2014), only indirect evidence was found in sugarcane. In fact, Calsa Jr and Figueira (2007) reported higher relative expression of PEPCK than NADP-ME in bundle sheath transcripts in mature sugarcane leaves. Herein, we found such decarboxylation flexibility in sugarcane varieties taking into account the enzymatic activities. The presence of more than one decarboxylation pathway could be advantageous for the acclimation of C_4 species to varying environmental conditions, such as changes in light availability (Furbank, 2011; Bellasio and Griffiths, 2014a; Sharwood *et al*., 2014).

Shading caused increases in the decarboxylation through PEPCK in both varieties (Fig. 4). Accordingly, leaf cross sections of both sugarcane varieties revealed that chloroplast arrangement in bundle sheath cells changed from centrifugal (full sunlight) to even (shading) distribution (Fig. 5). Such patterns of chloroplast distribution are in agreement with the increased importance of PEPCK

as the main decarboxylase under shading (Fig. 4D). The intracellular orientation of chloroplasts instead of centrifugal position increases the length of the CO_2 diffusion between bundle-sheath and mesophyll cells, reducing CO_2 leakiness (Dengler and Nelson, 1999). Our data also revealed the coupling between leaf biochemistry and anatomy in sugarcane plants, indicating the coordination of C_4 syndrome under varying light conditions.

Before addressing the possible advantages of decarboxylation plasticity, we should first consider the photosynthetic changes induced by low light conditions. There were large decreases in Rubisco and PEPC activities (Fig. 3A, B). As already described for C_4 species (Henderson *et al.*, 1992; Kubásek *et al.*, 2007; Ubierna *et al.*, 2013), sugarcane plants exhibited increases in $\Delta^{13}C$ and leakiness under shading (Fig. 6). High efficiency of CO_2 pumping in C_4 species is closely associated with low leakiness (Farquhar, 1983; Kromdijk *et al.*, 2008). For increasing CO_2 concentration around Rubisco, C_4 plants consume two molecules of ATP by PPDK to regenerate PEP from pyruvate. Then, increases in leakiness lead to additional cost of energy as plants need to refix the CO_2 that leaks to the mesophyll (Henderson *et al.*, 1992; Tazoe *et al.*, 2008).

What would be the advantage of changing the decarboxylation pathway under low light conditions? While plants with NADP-ME subtype have low leakiness due to the release of CO_2 close to the Rubisco carboxylation sites in bundle sheath chloroplasts (Furbank, 2011) and a suberized lamellae acting as a physical barrier (Farquhar, 1983), plants with PEPCK pathway would have an energetic advantage. Most of the oxaloacetate in bundle sheath cells is converted to PEP through PEPCK in the cytoplasm, with NAD-ME acting in the mitochondria and forming pyruvate and NADH. This cofactor is used in oxidative phosphorylation, generating ATP that can be used in PEPCK decarboxylation (Kanai and Edwards, 1999). Another energetic advantage of increasing PEPCK activity under limiting light is the low energetic expenditure in PEP regeneration, as it occurs in bundle sheath cells and uses half of the energy as compared to the PPDK pathway (Bellasio and Griffiths, 2014a). In accordance with this, both varieties presented significant reductions in PPDK activity under shading (Fig. 3C). It is important to point out that more studies are necessary to understand in which extent NAD-ME activity detected in this study is from the photosynthetic metabolism, providing CO_2 for the Calvin cycle, or from the respiratory pathway

(Rao and Dixon, 2016). In this sense future work investigating with gene expression and/or metabolite flux between bundle-sheath and mesophyll compartments are essential.

The increase in PEPCK activity in shaded plants is a likely physiological strategy for reducing the energetic costs of C_4 uptake under low light availability (Fig. 4) and keeping the equilibrium of metabolites and energy fluxes between mesophyll and bundle sheath cells (Bellasio and Griffiths, 2014a). Both anatomical and biochemical adjustments noticed in shaded plants represent ways to cope with low light availability and energetic costs of re-fixing CO_2 induced by leakiness under shading. This advantage can be notice in both varieties as sugarcane plants grown under shading and full sunlight had a similar $\phi CO_{2,max}$ (inset Fig. 1A, B). As recently highlighted by Pignon *et al.* (2017), $\phi CO_{2,max}$ is a key factor of photosynthetic efficiency under light-limiting conditions. Even with the increase in leakiness (Fig. 6B) and the additional energetic cost to re-assimilate CO_2, we may argue that the biochemical and anatomical adjustments were important to maintain $\phi CO_{2,max}$ in plants grown under light-limiting.

A model to integrate the shade-induced changes in sugarcane photosynthetic metabolism is proposed, considering carboxylation/decarboxylation reactions and leakiness (Fig. 7). Firstly, shading may reduce CO_2 availability for photosynthesis by causing low stomatal conductance (Fig. 1C, D). Considering the decarboxylation pathways, there is a pronounced increase in PEPCK activity and a reduction in NADP-ME (Fig. 4); thereby, it is possible to infer that both aspartate and malate are translocated from mesophyll to bundle sheath cells (Fig. 7). In the mitochondria of bundle sheath cells, NAD-ME activity is essential for malate decarboxylation, forming pyruvate and generating NADH, which is used for oxidative phosphorylation and production of ATP consumed by PEPCK (Fig. 7). The aspartate translocated to bundle sheath cells can form oxaloacetate in the cytoplasm or malate into the chloroplasts. The latter will be decarboxylated by NADP-ME, forming pyruvate, CO_2 and NADPH. This malate decarboxylation in bundle sheath cells is important to balance energetic molecules (NADPH) and amino groups (alanine) between both leaf compartments.

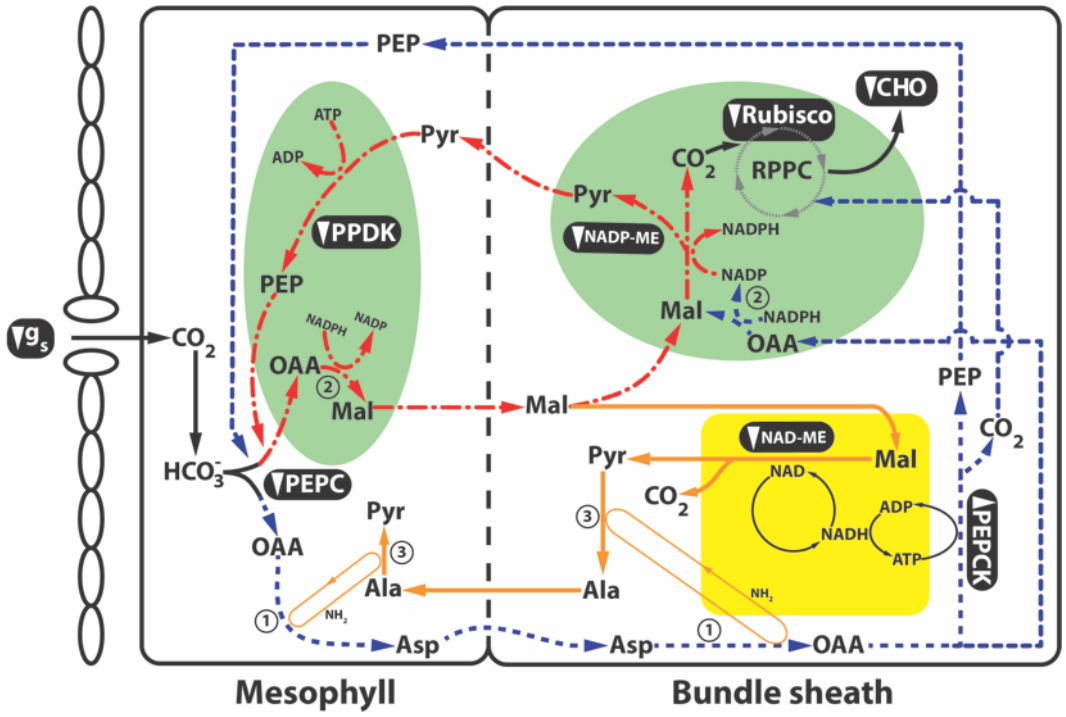

Fig. 7. Proposed model of sugarcane decarboxylation flexibility under shading. Reactions via NADP-ME (dash-point-dash lines), via NAD-ME (continuous lines) and via PEPCK (dashed lines). As it was not possible to discern in which extent *in vitro* NAD-ME activity detected in this study is from the photosynthetic metabolism, providing CO_2 for the Calvin cycle, or from the respiratory pathway, CO_2 released via this decarboxylation pathway are not directed to RPPC. Abbreviations: Ala, alanine; Asp, aspartate; CHO, carbohydrate; RPPC, reductive pentose phosphate cycle; g_s, stomatal conductance; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate. (1), (2) and (3) represent the enzymes aspartate aminotransferase, NADPH-malate dehydrogenase, and alanine aminotransferase, respectively. Chloroplasts are in green (ellipsis) and mitochondria in yellow (square). Variables outlined in black were evaluated herein and arrows represent the response to shading: increase (▲) or decrease (▼). Adapted from Furbank (2011).

As Rubisco activity decreased due to shading, CO_2 leakiness from bundle sheath to mesophyll increased. While decreases in PEP regeneration through PPDK activity were found under shading, increases in PEPCK activity suggest that PEP was translocated from bundle sheath to mesophyll without ATP consumption.

5. Conclusions

Sugarcane plants have flexibility among the decarboxylation pathways, which could balance energetic and metabolic fluxes between mesophyll and bundle sheath cells and improve plant acclimation to low light availability (Furbank, 2011). As leakiness increases in plants under shading and reduces the photosynthetic efficiency, an increase in the contribution of PEPCK pathway in decarboxylation process likely occurs to compensate the energetic costs for refixing CO_2; these biochemical and anatomical adjustments were efficient to maintain $\phi CO_{2,max}$ in plants grown under light-limiting.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at XXXXXXXXXXX

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