EXOGENOUS GLUCOSE SUPPLYING DECREASE PHOTOSYNTHESIS THROUGH *IN VIVO* PEPCASE ACTIVITY RESTRICTION AND METABOLIC LIMITATION IN SUGARCANE PLANTS

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Abstract – Sugarcane photosynthesis and production are deeply regulated by its source-sink relationships, however the mechanisms involved in this process are not fully understood to date. Aiming to verify whether photosynthesis inhibition in sugarcane plants is regulated by glucose or fructose, attached leaves of 4-month-old plants were sprayed with 50 mM exogenous glucose or fructose over five days in growth chamber conditions. Steady-state CO_2 assimilation decreased, whereas intercellular CO_2 partial pressure increased and stomatal conductance did not change after glucose and fructose application, indicating that photosynthesis was most inhibited by biochemical limitation. Photosystem II activity was slightly reduced by exogenous glucose and did not change by exogenous fructose. The initial slope of A-Ci curve (*k*), related to PEP carboxylation activity, strongly decreased by glucose (65%) and fructose (47%), while the metabolic limitation increased by 44% and 28%, while the stomatal limitation did not change after glucose and fructose application, respectively. The sugar profile had slight changes in leaves of glucose- and fructose-supplied plants, with increases of sucrose content of both treatments and increases of sucrose/hexose ratio only in glucose-treated plants. These results were not enough to identify which sugar is directly involved with photosynthesis down-regulation, but it seems clear that glucose is a promising candidate to be involved in this process in sugarcane plants. These data suggest that exogenous glucose is more effective than exogenous fructose to inhibit CO_2 assimilation by reducing *in vivo* PEPCase activity through metabolic limitations.

Keywords: CO_2 assimilation; Fructose; Sugar; Source-sink; *Saccharum* spp.

Introduction

Photosynthetic rate of sugarcane plants show a large variation during its development and is deeply regulated by source-sink relationship (McCormick et al., 2009). It is believed that an increase of sink demand, due to active growth or sugar compartmentalization in sink tissues, is able to stimulate photosynthesis in source leaves and the opposite, when plants are dormant or in case of sucrose export from leaves are reduced, promote a negative feedback in the photosynthetic reactions (Inman-Bamber et al., 2011; Watt et al., 2014). According to theoretical analysis, the content of sucrose in sugarcane stalk is capable to reach 30% of its fresh weight (Grof and Campbell, 2001), suggesting that current yields are still only 65% of the predicted capacity for sugarcane culm tissues (Jackson, 2005). Photosynthesis negative feedback in sugarcane is a phenomenon known over many decades and despite intense studies around the world focusing to understand this process to improve sugarcane yield, the increase of sucrose amount in this species has been little so far (Jackson, 2005; Watt et al., 2014).

It has been discussed if photosynthetic down-regulation is signaled by the sugar level in sink or source tissues (Inman-Bamber et al., 2011; McCormick et al., 2008a). Recently was demonstrated that sugar accumulation (Inman-Bamber et al., 2011) or changes in sucrose/hexose metabolism in leaves, after spraying a sucrose solution in the shoot, inhibits photosynthesis by decreasing *in vitro* Rubisco activity and abundance and *in vivo* PEPCase activity (Lobo

et al., 2015; Ribeiro et al., 2017). Sugar metabolism in plants is very dynamic and complex, which some of them are constantly synthesized and degraded and small fluctuations of these reactions activate a range of cellular signaling processes (Eveland and Jackson, 2012; Figueroa and Lunn, 2016). In the previous studies, it was verified that exogenous sucrose supply in leaves strongly decreased photosynthesis in sugarcane, but it was not clear which sugar was directly involved in this regulation. Therefore, the main goal of this study was to investigate whether sucrose constituents, glucose and fructose, are related to photosynthesis impairment in sugarcane plants. According to our results, exogenous glucose was more effective to inhibit photosynthesis by restricting *in vivo* PEPCase activity and PSII activity through metabolic limitations.

Materials and Methods

Plant material and growth conditions

Sugarcane plants (*Saccharum* spp.), cv. IACSP94-2094 supplied by the Agronomic Institute (IAC), Brazil, were propagated by sowing stalk segments with a single bud. The plants were cultivated according to Lobo et al. (2015) in a greenhouse (3°44´S; 38°34´W; 31 m of altitude). The average air temperature, relative humidity and the maximum photosynthetic photon flux density (PPFD) were 27±3 °C, 58±5% and 1,100±100 µmol photons m^{-2} s^{-1} respectively, with 12 h of photoperiod inside the greenhouse. All secondary tillers were removed during the experimental period to retain only the primary stalk per pot and avoid excessive self-shading and related changes in source-sink relationships. At the beginning of the experiments, the plants exhibited a stalk diameter of 2.5 cm and five internodes per stalk.

For the experiment, 4-month-old plants (at tillering stage) initially grown under natural conditions in a greenhouse were transferred to a growth chamber with the following controlled conditions: 29/24 °C day/night; RH 70%; air CO_2 partial pressure of 38 Pa, PPFD of 800 µmol m^{-2} s^{-1} and a 12 h photoperiod. After an acclimation period of 24 h inside the growth chamber, a 50 mM glucose and fructose solution was sprayed on the shoot until a complete leaf wetting. A 0.01% (v/v) Triton-100 solution was mixed or not (control) with 50 mM of glucose or fructose solutions to allow a more effective infiltration into the leaf tissues. The solutions were sprayed on all expanded leaves twice a day (10:00 a.m. and 4:00 p.m.) for five consecutive days. In the last day gas exchange and chlorophyll *a* fluorescence measurements were performed in the leaf +1 of each plant and samples (leaves +1 and +2) were collected, washed with 1.5 mM $CaCl_2$ to residue elimination and then immediately stored at -80 °C for further analysis.

Gas exchange, chlorophyll a fluorescence and sugar measurements

Leaf CO_2 assimilation (A), stomatal conductance (g_S), intercellular CO_2 partial pressure (C_i) and chlorophyll *a* fluorescence were measured by using a portable infrared gas analyzer system (LI-6400XT, LI-COR, Lincoln, NE, USA), equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA) according to Lobo et al. (2015). For A/*Ci* curve, PPFD and the temperature at the chamber were maintained at 1,000 µmol photons m^{-2} s^{-1} and 28 °C, respectively, the CO_2 concentration was changed from 0 to 800 µmol m^{-2} s^{-1}. The curve fitting was performed according to von Caemmerer and Furbank (1999) and the stomatal and metabolic limitations were estimated. The stomatal limitation of photosynthesis (Ls) was calculated as $L_S = [(A_{pot} - A_c)/A_{pot}] * 100$, where A_{pot} denotes A measured when *Ci* = 38 Pa (infinite g_S) and A_c denotes A measured when C_a = 38 Pa (finite g_S). A_{pot} is the potential leaf CO_2 assimilation or photosynthetic capacity, A_c is the actual leaf CO_2 assimilation and C_a is the air CO_2 partial pressure. The metabolic limitation of photosynthesis between the treatments (L_M) was calculated as $L_M = [(A_1 - A_2)/A_1] * 100$ (Lawlor, 2002), where A_1 is the leaf CO_2 assimilation of the control and A_2 is the leaf CO_2 assimilation of the glucose or fructose treatment. The parameter related to V_{pmax} (PEPC carboxylation activity) – *k*, was calculated

from the initial slope of the A/*Ci* curve until *Ci* of 100 µbar (von Caemmerer and Furbank, 1999). The concentration of total soluble sugars was measured by the phenol-sulfuric acid method (Dubois et al., 1956), whereas the content of sucrose, starch, glucose, and fructose were quantified as described by Lobo et al. (2015). The sucrose/hexose ratio was calculated as follows: Suc/hexose ratio = [Sucorse]/([Glucose] + [Fructose]). The contents of all sugars were expressed in µmol g^{-1} DW.

Results and Discussion

The application of both hexoses decreased CO_2 assimilation around 15%, increased the intercellular CO_2 partial pressure (*Ci*) circa 32% and did not change stomatal conductance when compared to control plants (Figure 1A-C). The actual quantum efficiency and the electron transport rate of PSII were modified only by glucose supply, which decreased around 11%, and did not change in fructose-treated plants. The maximum quantum efficiency of PSII was not altered by glucose or fructose supply regarding control plants (Figure 2A-C). These results indicate that exogenous glucose was more effective than exogenous fructose to decrease photosynthesis mainly by biochemical restrictions in Calvin-Benson cycle. Moreover, our data reveal that exogenous glucose can also affect the instantaneous PSII activity, reinforcing that both photosynthetic reactions, in the thylakoid membranes and in the stroma, are independent and mutually regulated (Takahashi and Murata, 2008).

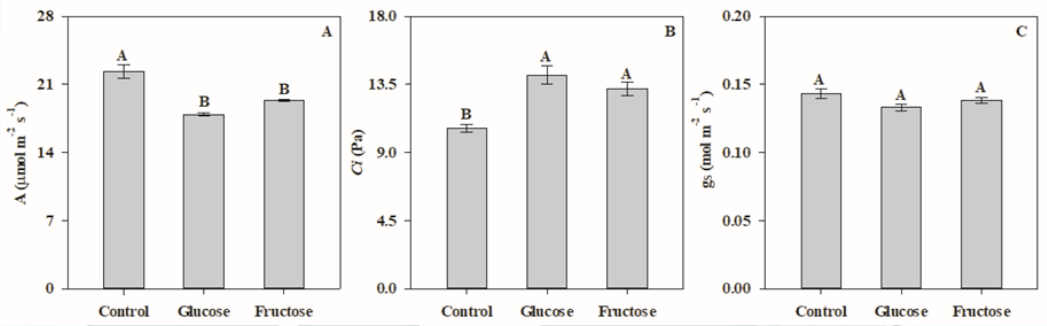

Figure 1: Gas exchange parameters measured in leaves of sugarcane plants sprayed with 50 mM exogenous glucose or fructose for five days. Leaf CO_2 assimilation - A (A), intercellular CO_2 partial pressure - *Ci* (B) and stomatal conductance - g_s (C). Each point represents the average of four independent replicates (± SE), different letters represent significant differences between treatments according to Tukey's test ($P \leq 0.05$).

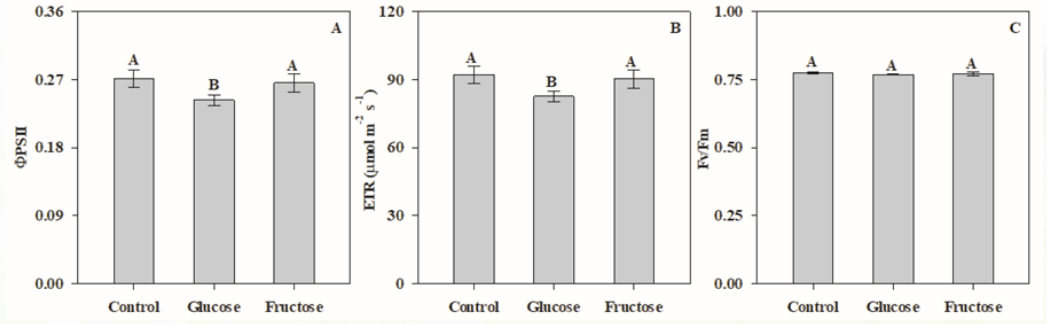

Figure 2: Photochemical parameters measured in leaves of sugarcane plants sprayed with 50 mM exogenous glucose or fructose for five days. Actual quantum efficiency of PSII – ΦPSII (A), electron transport rate – ETR (B) and maximum quantum efficiency of PSII – Fv/Fm (C). Each point represents the average of four independent replicates (± SE), different letters represent significant differences between treatments according to Tukey's test ($P \leq 0.05$).

The restrictions in the CO_2 assimilation by exogenous glucose and fructose were also investigated by estimating some of the A-*Ci* curve parameters. PEP carboxylase (PEPCase) is the first enzyme which incorporates CO_2 in organic molecules in C4 plants, such as sugarcane, therefore the initial slop of sugarcane A-*Ci* curve is attributed to *in vivo* PEPCase activity (*k*). In this study, PEPCase activity (*k*) was strongly decreased by glucose (65%) and fruc-

tose (47%) regarding to control plants. This greater reduction in the *in vivo* PEPCase activity was corroborated with larger increases of metabolic limitation after glucose (44%) and fructose (28%) supply, whereas the stomatal limitation did not change when compared to control plants (Figure 3A-C). These data, in fact, demonstrate that exogenous glucose supply, more than fructose, down-regulates CO_2 assimilation by decreasing *in vivo* PEPCase activity through metabolic restrictions.

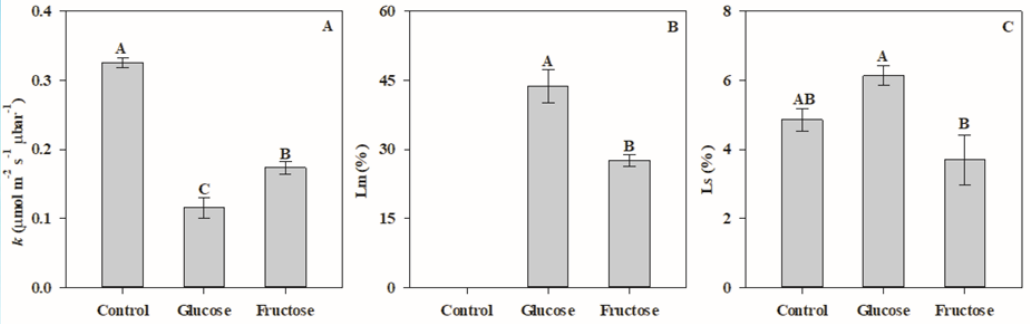

Figure 3: A-*Ci* curve parameters measured in leaves of sugarcane plants sprayed with 50 mM exogenous glucose or fructose for five days. Initial slope of A-*Ci* curve – *k* (A), metabolic limitation – Lm (B) and stomatal limitation – Ls (C). Each point represents the average of four independent replicates (± SE), different letters represent significant differences between treatments according to Tukey's test ($P \leq 0.05$).

The sugar profile in leaves was also analyzed aiming to verify the interaction between source-sink alterations and photosynthesis down-regulation in sugarcane. The concentration of total soluble sugars, glucose, fructose, and starch did not change in leaves of glucose- and fructose-treated plants. Differently, sucrose content increased in leaves of both glucose- and fructose-treated plants. These changes in sucrose and hexoses relations promoted increases of sucrose/hexose ratio in leaves of glucose-treated plants, while in fructose-treated plants this ratio did not change, all compared to control plants (Table 1). It is already known that sugar metabolism involves a range of signaling sugar and enzymes which can regulates many cellular processes in plants, however the mechanisms related to this process are not well understood (Häusler et al., 2014).

Table 1: Concentration of total soluble sugars (TSS), sucrose, glucose, fructose, starch and sucrose/hexose ratio in leaves of sugarcane plants sprayed with 50 mM exogenous glucose or fructose for five days. Each point represents the average of four independent replicates, different letters represent significant differences between treatments according to Tukey's test ($P \leq 0.05$).

Sugars (μmol g^{-1} DW)	**Treatments**		
	Control	**Glucose**	**Fructose**
TSS	205.57 A	248.93 A	235.19 A
Sucrose	28.22 B	34.86 A	33.45 A
Glucose	39.78 A	36.13 A	48.99 A
Fructose	55.27 A	53.57 A	59.48 A
Suc/hexose ratio	0.30 B	0.39 A	0.31 B
Starch	132.08 A	122.07 A	117.90 A

According to the results obtained in this study, it was not possible to classify which sugar is directly involved with photosynthesis down-regulation in sugarcane plants, once sugar metabolism is very dynamic and complex. In fact, exogenous sucrose was much more efficient to down-regulates photosynthesis in sugarcane by decreasing CO_2 assimilation more than 34% and inhibiting *in vivo* PEPcase and Rubisco activity (Lobo et al., 2015; Ribeiro et al., 2017). However, in these previous works, it was not possible to separate the direct role of sucrose signaling from its constituent hexoses (glucose and fructose). Indeed, exogenous glucose changed sugar metabolism in leaves by increasing sucrose content and, consequently, sucrose/hexose ratio. It is possible that these alterations in sugar profile could modify PEPCase posttranslational regulation by signaling process and decrease it's *in vivo* activity (McCormick

et al., 2008b; Lobo et al., 2015). Despite our results are superficial and did not show whether enzymes of sugar metabolism or other signaling sugars, such as trehalose, are involved to photosynthesis downregulation in sugarcane, it is very conclusive that glucose is a target sugar capable to change sugar profile and inhibits photosynthesis by reducing PEPCase activity. Therefore, further studies are needed to better understand these mechanisms and identify the sugar direct involved to photosynthesis restrictions in sugarcane plants.

Conclusion

In summary, our results demonstrate that exogenous glucose is more effective than fructose to negatively modulate photosynthesis in sugarcane plants by changing sugar metabolism, increasing metabolic limitations in the CO_2 assimilation and decreasing *in vivo* PEPCase activity and PSII activity.

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References

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Eveland, A.L., Jackson, D.P., 2012. Sugars, signalling, and plant development. J. Exp. Bot. 63, 3367–77.

Figueroa, C.M., Lunn, J.E., 2016. A Tale of Two Sugars: Trehalose 6-Phosphate and Sucrose. Plant Physiol. 172, 7–27.

Grof CPL, Campbell JA. 2001. Sugarcane sucrose metabolism: scope for molecular manipulation. Australian Journal of Plant Physiology 28, 1–12.

Häusler, R.E., Heinrichs, L., Schmitz, J., Flügge, U.I., 2014. How sugars might coordinate chloroplast and nuclear gene expression during acclimation to high light intensities. Molecular Plant 7, 1121–1137.

Inman-Bamber, N.G., Jackson, P.A.A., Hewitt, M.A., 2011. Sucrose accumulation in sugarcane stalks does not limit photosynthesis and biomass production. Crop Pasture Sci. 62, 848–858.

Jackson PA. 2005. Breeding for improved sugar content in sugarcane. Field Crops Research 92, 277–290.

Lawlor DW. 2002. Limitations to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Annals of Botany 89, 871–885.

Lobo, A.K.M., de Oliveira Martins, M., Lima Neto, M.C., Machado, E.C., Ribeiro, R.V., Silveira, J.A.G., 2015. Exogenous sucrose supply changes sugar metabolism and reduces photosynthesis of sugarcane through the down-regulation of Rubisco abundance and activity. J. Plant Physiol. 179, 113–121.

McCormick, a J., Watt, D. a, Cramer, M.D., 2009. Supply and demand: sink regulation of sugar accumulation in sugarcane. J. Exp. Bot. 60, 357–64.

McCormick, a. J., Cramer, M.D., Watt, D. a., 2008a. Culm sucrose accumulation promotes physiological decline of mature leaves in ripening sugarcane. F. Crop. Res. 108, 250–258.

McCormick, a J., Cramer, M.D., Watt, D. a, 2008b. Changes in photosynthetic rates and gene expression of leaves during a source-sink perturbation in sugarcane. Ann. Bot. 101, 89–102.

Ribeiro, R.V., Machado, E.C., Magalhães Filho, J.R., Lobo, A.K.M., Martins, M.O., Silveira, J.A.G., Yin, X., Struik, P.C., 2017. Increased sink strength offsets the inhibitory effect of sucrose on sugarcane photosynthesis. J. Plant Physiol. 208, 61–69.

Takahashi S, Murata N. 2008. How do environmental stresses accelerate photoinhibition? Trends in Plant Science 13, 178–182.

von Caemmerer, S., Furbank, R.T., 1999. Modeling C4 photosynthesis. In: Sage, R.F., Monson, R.K. (Eds.), C4 Plant Biology. Academic Press, Toronto, pp. 173–211.

Watt, D. a, McCormick, a J., Cramer, M.D., 2014. Source and sink physiology, Sugarcane: Physiology, Biochemistry, and Functional Biology.