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ANA KARLA MOREIRA LOBO

PHOTOSYNTHESIS REGULATION BY SUCROSE METABOLISM UNDER WATER DEFICIT AND SOURCE-SINK ALTERATIONS IN SUGARCANE

FORTALEZA
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Tese apresentada ao Curso de Doutorado em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Bioquímica. Área de Concentração: Bioquímica Vegetal

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira
Coorientador: Prof. Dr. Marcio de Oliveira Martins

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A minha família

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"Nossas virtudes e nossos defeitos são inseparáveis, assim como a força e a matéria.
Quando se separados, o homem deixa de existir."

(Nikola Tesla)

ABSTRACT

Water deficit stress is the major limiting factor for plant growth and development, constraining food production. In order to survive in such dry conditions, many biochemical and physiological changes must be triggered by plants. In general, the responses to drought are loss of water content, reductions of stomatal conductance and photosynthesis and increase of carbohydrates. Soluble sugars play a key role in plant metabolism, acting as substrates and modulators of enzyme activity in carbon-related pathways and controlling the expression of different genes related to carbon, lipid and nitrogen routs. However, the mechanisms involved with photosynthesis down-regulation by drought and sugars in C4 plants are not fully understood. The aim of this study was to investigate how drought and source-sink perturbation regulate photosynthesis in sugarcane plants. Therefore, two studies were conducted with sugarcane plants with four months old cultivated under greenhouse conditions. In the first study sugarcane plants (cv. IACSP94-2094) were subjected to water deficit for 5 days (WD) with concomitant spraying of 50 mM exogenous sucrose (WD + Suc). While in the second study source-sink relationship was perturbed in two sugarcane cultivars (cv. IACSP94-2094 and cv. IACSP95-5000) by imposing partial darkness, spraying 50 mM exogenous sucrose and their combination for 5 days. The negative effects of WD in the gas exchange and photochemical parameters were aggravated by exogenous sucrose. Photosynthesis reductions were related to both stomatal and biochemical limitations, but exogenous sucrose intensified metabolic restrictions mainly through down-regulation of Rubisco initial activity and PSII effective quantum efficiency in drought-stressed plants. In addition, Rubisco activation state was decreased by WD + Suc, indicating perhaps that the activity of this enzyme was reduced by tight-binding inhibitors, such as sugars phosphates. Sucrose metabolism enzymes and sugars amount were also differently altered by WD and WD + Suc in leaves, sheath and stalk in WD and WD +Suc plants. Interestingly, Sucrose/hexose ratio decreased in both leaf and sheath whereas it was increased in stalk, suggesting that sucrose and related sugars were intensely metabolized and transported in drought-stressed plants. In well-watered conditions, photosynthesis was inhibited by sucrose spraying in both genotypes, through decreases in maximum Rubisco carboxylation rate (V_{cmax}), initial slope of A-C_i curve (k), stomatal conductance (g_s) and ATP production driven by electron transport (J_{atp}). The partial darkness and sucrose spraying combination did not change photosynthesis in both genotypes. Significant increases in V_{cmax}, g_s and J_{atp} and marginal increases in k were noticed when combining partial darkness and sucrose spraying compared with sucrose spraying alone. Altogether, these results

suggest that CO_2 assimilation impairment is aggravated by exogenous sucrose in drought-stressed plants. This limitation was mainly related to biochemical restrictions, specially associated with Rubisco initial activity and PSII quantum efficiency. In contrast, *in vitro* PEPCase activity and amount were increased in sucrose-treated plants, suggesting that C4 cycle efficiency was reduced *in vivo* by C3 cycle inhibition under drought conditions. Moreover, sucrose amount was increased in the stalk, suggesting the feedback regulation from stalk to source leaves in drought-stressed plants. Our data also revealed that increases in sink strength due to partial darkness offset the inhibition of sugarcane photosynthesis caused by sucrose spraying, enhancing the knowledge on endogenous regulation of sugarcane photosynthesis through the source-sink relationship.

Keywords: CO_2 assimilation. *Saccharum* spp. Rubisco. PEPCase. Drought.

RESUMO

A deficiência hídrica é o principal fator limitante para o crescimento e desenvolvimento das culturas. Para sobreviver nessas condições adversas, várias modificações bioquímicas e fisiológicas são desencadeadas pelas plantas. Em geral, os efeitos da seca em plantas são diminuição do status hídrico, reduções da condutância estomática, fotossíntese e crescimentos e aumentos nos níveis de carboidratos. Os açúcares solúveis desempenham papéis chave no metabolismo das plantas, atuando como substratos e moduladores da atividade enzimática em vias relacionadas com o carbono. Além disso, os açúcares controlam a expressão de genes associados com as rotas do metabolismo do carbono, lipídios e nitrogênio. Entretanto, os mecanismos envolvidos com a regulação negativa da fotossíntese por deficiência hídrica e açúcares em plantas C4 não estão totalmente entendidos. O objetivo deste estudo foi investigar como a deficiência hídrica e perturbações na relação fonte-dreno regulam a fotossíntese em plantas de cana-de-açúcar. Dois estudos foram conduzidos com plantas de cana-de-açúcar com quatro meses de idade cultivadas sob condições de casa de vegetação. No primeiro estudo, plantas de cana-de-açúcar (cv. IACSP94-2094) foram submetidas a deficiência hídrica por 5 dias (WD) com subsequente aplicação de sacarose exógena 50 mM (WD + Suc). Enquanto que no segundo estudo a relação fonte-dreno foi perturbada em duas cultivares de cana-de-açúcar (cv. IACSP94-2094 and cv. IACSP95-5000) pela imposição parcial de sombreamento, aplicação de sacarose exógena 50 mM e por suas combinações por 5 dias. Os efeitos negativos de WD nos parâmetros de trocas gasosas e fotoquímicos foram agravados por sacarose exógena. As reduções na fotossíntese foram relacionadas com limitações estomáticas e bioquímicas, porém a sacarose exógena intensificou as restrições bioquímicas principalmente por reduções na atividade inicial de Rubisco e eficiência quântica do PSII em plantas sob seca. Além disso, o estado de ativação de Rubisco foi inibido por WD + Suc, sugerindo que a atividade inicial dessa enzima foi possivelmente reduzida por inibidores que se ligam fortemente em seu sitio ativo, tais como açúcares fosfato. As enzimas do metabolismo de sacarose e a concentração de açúcares foram modificados diferentemente por WD e WD + Suc em folhas, bainha e colmo. Interessantemente, a relação sacarose/hexose decresceu em folhas e bainha, enquanto que no colmo essa relação aumentou, sugerindo que sacarose e outros açúcares relacionados foram intensamente metabolizados e transportados. Em condições irrigadas a fotossíntese foi inibida pela aplicação de sacarose nos dois genótipos, através de decréscimos da taxa máxima de carboxilação de Rubisco (V_{cmax}), inclinação inicial da curva A-C_i (k), condutância estomática (g_s) e produção de ATP direcionada pelo transporte de elétrons (J_{atp}). A combinação de

sombreamento parcial e sacarose não alterou a fotossíntese em ambos os genótipos. Significantes aumentos em V_{cmax}, g_s, J_{atp} e k foram observados quando sombreamento parcial e sacarose foram combinados em comparação com plantas tratadas apenas com sacarose. Em conclusão, esses resultados sugerem que o impedimento da assimilação de CO_2 é agravada por adição de sacarose exógena em plantas sob estresse hídrico. Essa limitação foi relacionada principalmente com restrições bioquímicas, especialmente associadas com reduções na atividade inicial de Rubisco e eficiência quântica do FSII. Em contraste, a atividade *in vivo* e concentração de PEPCase foram aumentadas em plantas tratadas com sacarose e estresse hídrico, sugerindo que a eficiência do ciclo C4 foi reduzida *in vivo* por inibições do ciclo C3 sob condições de seca. Além disso, o conteúdo de sacarose aumentou no colmo, indicando uma regulação de feedback do colmo para as folhas em plantas sob seca. Nossos dados revelam ainda que aumentos na força do dreno devido ao sombreamento parcial aliviaram os efeitos inibitórios na fotossíntese de cana-de-açúcar causados pela aplicação de sacarose, aumentando o conhecimento na regulação endógena da fotossíntese de cana-de-açúcar através da relação fonte-dreno.

Palavras-chave: Assimilação de CO_2. *Saccharum* spp. Rubisco. PEPCase. Seca.

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INTRODUCTION

Plants, as sessile organisms, are often challenged by abiotic stresses, which are the major constraints to all living organisms (Gupta et al., 2013). Water deficit is the most limiting abiotic stress to plant growth and development in the world and to survive under these conditions many adaptive mechanisms are activated in plants (Chaves et al., 2009). The drought responses depend on stress intensity, duration and specie, but in general the stomatal conductance, transpiration rate, photosynthesis, sugar metabolism and growth are altered in crops cultivated under these conditions (Zhao et al., 2013). CO_2 assimilation and yield are strongly inhibited initially by the stomatal closure which is one of the first response of drought stressed plants. Furthermore, this mechanism could induce photoinhibition, increase sugars amount in the source leaves and down-regulate many genes involved with photosynthesis and plant metabolism (Nouri et al., 2015). The mechanism associated with stomatal closure signalized from roots through ABA is well known, however the biochemical and molecular mechanisms involved with the down-regulation of photosynthetic enzymes remain unclear (Pinheiro and Chaves, 2011).

The increase of leaf carbohydrate content in response to short-term water scarcity could act in the osmoregulation to support water uptake when the soil water content is very low. However, sugar accumulation also might be a consequence of reduced growth, that later could be a source of energy for recovery and rapid growth once water is available (Alam et al., 2010). Moreover, soluble sugars play a central role in plant metabolism and their pool should be continually adjusted to keep the balance between the supply and utilization of carbon at the whole plant level and the cell sucrose-starch partition, which is under control of several factors, including drought (Chaves et al., 1991). In addition, many studies have shown that sugars are important signalling molecules and small changes in carbon status can control many metabolic events, such as gene expression of photosynthetic enzymes (Hanson and Smeekens, 2009; Usadel et al., 2008). Modifications in the enzyme activities and gene expression by intercellular concentration of sugar are also recognized as a consequence of a feedback mechanism (Franck et al., 2006).

In general, source activities such as photosynthesis, nutrient mobilization and exportation are up-regulated under low sugar conditions, as a result of high sink activity, whereas an accumulation of sugars has the opposite effect (McCormick et al., 2008a). Some studies have suggested that some enzymes and/or sugars from sucrose metabolism are involved

with the signaling mechanisms to down-regulate photosynthesis (Araya et al., 2006; McCormick et al., 2008b; Quentin et al., 2013). However, the precise metabolic regulation of source-sink interaction is not yet understood mainly in C4 plants (Watt et al., 2014). Sugarcane plants (*Saccharum* spp.) have a unique source-sink system, as its leaves and stalks exert strong source and sink activity associated with sucrose accumulation, being an interesting model to study the regulation of photosynthesis by sugars (McCormick et al., 2009). The extraordinary accumulation of sucrose in the stalk makes sugarcane a very important crop economically and improve its yield is the goal of many international breeding programs (Rice et al., 2009). Sugarcane CO_2 assimilation varies widely as sugars are accumulated in the stalks and leaves in parallel with the plant aging, regardless the environmental conditions (Allison et al., 1997).

The end-product repression of photosynthesis has encouraged many investigators with the aim of understanding this phenomenon to improve sugarcane yield through increased sucrose content, however the progress has been limited so far (Watt et al., 2014). The elucidation of the mechanisms involved with the inhibition of photosynthesis by sugars and water deficit is a promising target to improve production and tolerance of important crops. Thereby, the object of this study is to investigate how photosynthesis is regulated by source-sink perturbations and drought in two contrasting sugarcane genotypes.

CHAPTER I

GENERAL REVIEW, HYPOTHESIS and OBJECTIVES

1. GENERAL REVIEW

1.1 Drought effects on C4 photosynthesis

The predicted climate change will decrease the resource availability to crop production and yet the sources of water are already scarce in many parts of the world (Gosling and Arnell, 2016). Water scarcity impair many physiological processes in plants and the elucidation of these processes is necessary to provide a selection source for breeders to improve strategies to drought adaptation and water use efficiency (Loka et al., 2011). In general, responses to dehydration in C3 and C4 plants are similar, which includes loss of water content, reduced leaf water potential, stomatal conductance, transpiration rate and photosynthesis (Carmo-Silva et al., 2008). In fact, the drought effects depend on intensity (mild, moderate or severe), duration, rate of progress, genotype, developmental stage of plant and the interaction with other stresses (Vadez et al., 2013). In the mild stress the transpiration rate is not affected by the decrease of water availability, whereas in the moderate phase the transpiration rate and stomatal conductance are reduced and strongly decreased in severe stress (Pimentel, 2004). In advanced phases, is also observed decreases of photosynthesis and nitrogen assimilation and increases of root/shoot ratio and carbohydrates (Pimentel, 2004).

Photosynthesis plays a central role in plant performance, once it is a key process of primary metabolism, and is specially inhibited under drought conditions by diffusive and biochemical limitations (Flexas et al., 2004; Lawlor and Tezara, 2009). However, a long-standing controversy if drought limits photosynthesis due to stomatal closure, metabolic impairment or injuries of photosynthetic apparatus is still under discussion (Flexas et al., 2006). Photosynthesis of C3 and C4 plants share most of the fundamental process such as C3 cycle, light harvesting complexes and electron transport components, but significant differences exist between the two photosynthetic types, which could make their response to water stress differ in many levels (Ghannoum, 2009). These differences are mainly because C4 photosynthesis has a metabolic CO_2 pump that concentrates CO_2 in the vicinity of the main enzyme of carbon dioxide fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Edwards et al., 2004). This mechanism confers several advantages to C4 plants, such as high water use efficiency and CO_2 assimilation even when the stomata are almost closed, limiting flux through the photorespiratory pathway and making C4 photosynthesis much more competitive than C3 in drought-prone areas (Ghannoum, 2009).

However, even with some advantages, the photosynthetic systems of C4 plants are as sensitive to drought-induced inhibition as in C3 plants (Ripley et al., 2010). In both photosynthetic systems the initial stomatal closure decreases intercellular CO_2 concentration (Ci) and during the later stages of drought Ci often increases while photosynthesis continues reducing as a metabolic restriction (Leakey et al., 2004). In parallel, photochemical activity is also inhibited and the excess of light energy could lead to photoinhibition, reducing quantum yield of PSII and inducing photorespiration and reactive oxygen species (ROS) production (Silva et al., 2013). In C4 plants many evidences suggest that the biochemical limitation is, preferentially, a consequence of C3 cycle enzymes inhibition during drought (Ghannoum, 2009). In addition, the differential inhibition between the two cycles could lead to a rise in the CO_2 concentration inside the bundle sheath cells and a build-up of a CO_2 gradient across the bundle sheath membrane that would increase CO_2 leakage (Sage et al., 2014).

Photorespiration pathway in C4 leaves is very reduced, though this process is still required as it plays important role in cellular redox homeostasis and nitrogen metabolism (Foyer et al., 2009). In addition, Carmo-Silva et al. (2008) shown that photorespiration rates do not increase in C4 plants under water stress. The regulation of C4 pathway enzymes is controversial, but some studies have suggested that the activity of phospho*enol*pyruvate carboxylase (PEPCase), malic enzyme and pyruvate orthophosphate dikinase (PPDK) are reduced under water scarcity conditions (Du et al., 1996). The identification of trends in the C4 plants responses to drought is difficult because each C4 subtype [NADP-malic enzyme (NADP-ME), NAD-ME, and phosphoenolpyruvate carboxykinase (PEPCK)] can exhibit different strategies for coping with water deficits (Carmo-Silva et al., 2007). For instance, *Paspalum dilatatum* (NADP-ME) and *Zoysia japonica* (PEPCK) respond to drought by early stomatal closure in order to conserve leaf water status, whereas this response was not apparent in *Cynodon dactylon* (NAD-ME), which lost water rapidly when deprived of water (Carmo-Silva et al., 2007). In drought-stressed sugarcane (NADP-ME) important enzymes from C3 and C4 cycles were inhibited, in special PPDK activity that was closely correlated with CO_2 assimilation rates (Du et al., 1996). In another study on sugarcane, Vu and Allen (2009) proved that water stress inhibit Rubisco activity while PEPCase activity remains unaltered.

In addition to enzymatic limitations, drought trigger a chain of events related to signalling cascades that closely regulates, at protein/gene level, sugars, ROS and hormones pathways, specially ABA (Pinheiro and Chaves, 2011). These metabolic changes, in response to water deficit, down-regulate plant growth and photosynthesis by decline in stomatal

conductance as well as Rubisco activity resulting in lower carbon fixation followed by the over-reduction of the electron transport system components and production of ROS. Nevertheless, even with a massive advance in understanding the events occurring in plants subjected to drought, an integrative picture of the metabolic regulation taking place in drought conditions is still missing particularly in C4 plants (Seki et al., 2007).

1.2 Drought and sugar metabolism

The photoassimilates partitioning is severely altered in dehydrated plants promoting accumulation of reserves accompanied by modifications in the carbon and nitrogen metabolism in different organs (Antonio et al., 2008). Sugars have a dynamic metabolism and regulate many events in plant development in normal and stressful conditions (Rolland et al., 2006). Thus, soluble carbohydrates are key players in the integration, at the whole plant level, of the cellular responses to internal and environmental alterations. They act as substrates and modulators of enzyme activity in carbon-related pathways, control the expression of different genes related to carbon, lipid, and nitrogen metabolism and can interplay with other stress elicitors, such as redox and hormone signals (Gibson, 2005; Rolland et al., 2006; Bolouri-Moghaddam et al., 2010). Usadel et al. (2008) proposed that the supply-use ratio of sugars should be always in balance and small changes in carbon status may signalling to many events in plants. In fact, the expression and activity of photosynthetic enzymes and catabolism pathway are controlled by intracellular sugar concentration through a feedback mechanism (Koch, 1996; Lawlor and Paul, 2014).

The effects of drought in sugar metabolism are very controversial and complex, as sugars can interact in many pathways, and several trends can be found in literature. Regarding to Pinheiro and Chaves (2011) the concentration of soluble sugars in leaves may increase, stay constant or decrease under water deficit. Sucrose and hexose amounts increased, while starch levels decreased in maize leaves under water deficit, suggesting the induction of starch hydrolysis and sucrose synthesis (Pelleschi et al., 1997). Sucrose accumulation in water-stressed cotton leaves has been hypothesized an energy supply to maintain cell survival in high-respiration conditions (Burke, 2007). Iskandar et al. (2011) reported that in drought-stressed sugarcane glucose and fructose levels did not change in the leaves and increased in internodes, while sucrose content was reduced in the leaves and did not change in the stalk. Starch synthesis can be repressed under water deficit or transitorily increase (Chaves, 1991). Soluble sugars

concentration increased in shoot of maize subjected to water stress, whereas the starch amount significantly decreased (Mohammadkhani and Heidari, 2008).

Carbohydrates accumulation are also considered to play a major role in osmotic adjustment to maintain metabolic activity in source leaves. However, sugars may accumulate in leaves likewise because of a decreased demand as a consequence of growth limitation (Hummel et al., 2010). In fact, the modifications in the sugar pool, associated with drought, are supported by changes in the enzyme activities related with carbohydrate pathway, such as α-amylase, sucrose synthase, and invertase (Pinheiro and Chaves, 2011). Trouverie et al. (2003) reported an increase in total acid invertase activity, coinciding with the rapid accumulation of glucose and fructose in water-stressed maize leaves. In long-term drought the activity of sucrose-phosphate synthase, a key enzyme of sucrose synthesis, was down-regulated in coffee leaves (Praxedes et al., 2006). A precise role for each sugar and enzyme associated with its metabolism in plants cultivated under drought conditions still in discussion. However, slight changes in the glucose, sucrose and starch contents and invertase activities seems to be involved with important regulatory network that control plant growth in stressful environments (Sulpice et al., 2009, Ruan et al., 2010).

1.3 Photosynthesis regulation by sugar signalling system

Sugars are synthetized from atmospheric CO_2 assimilation in chloroplasts and are considered the most important end products of photosynthesis since they directly determine plant growth and development (Ruan, 2014). Triose phosphate (TP) is the main product of Calvin-Benson cycle reactions in the light that is exported from chloroplasts by a TP transporter to support sucrose biosynthesis in the cytosol (Wind et al., 2010). Afterwards, sucrose can be transferred to the sinks via the phloem, stored in the vacuole or degraded by invertases to generate glucose and fructose (Roitsch and González, 2004). Another part of the photoassimilates can be used in the starch synthesis inside the chloroplasts during the day, which can be subsequently degraded mainly by the action of β-amylase, debranching enzyme, and disproportionating enzyme (DPE1) to support the night metabolic requirements (Zeeman et al., 2007). Maltose and glucose are the major products from starch degradation that have to be exported from chloroplast by specific transporters and they are the only non-phosphorylated sugars in this organelle (Nittylä et al., 2004).

Beside their roles as temporary storage molecules, long distance transport forms, carbon skeletons and energy source, carbohydrates can act as signalling molecules that modulate a vast array of plant development process (Häusler et al., 2014). Thus, sugars represent an ideal compost to trigger, or to participate in, acclimation in plants and their synthesis and sink utilization should be tightly coordinated (Graf et al., 2010). Usually, photosynthesis decrease when sink activity is decreased, by removing active sinks or introducing nutrient deficiency, and carbohydrates accumulate in leaves (Paul and Pellny, 2003). Similarly, photosynthesis down-regulation was associated with increases of sucrose amount, by cold girdling of petioles or down-regulation of sucrose transporter, in the leaves (Bürkle et al., 1998; Zhang and Turgeon, 2009). In these cases, sugars accumulation in source leaves by decreased sink demand or inhibited sugar transport, enhanced the expression of genes involved in carbohydrate storage and utilization and suppressed photosynthetic gene expression and, subsequent, growth (Paul and Pellny, 2003; Stitt et al., 2010). On the other hand, increased sink demand, through partial defoliation or shading, can enhance photosynthetic activity (McCormick et al., 2006; Ribeiro et al., 2012).

Sugar sensing and downstream signalling components have been described in many crops and strongly indicate that they modulate the expression of nuclear-encoded genes related with photosynthesis (Koch, 1996; Moore, 2005). However, the precise mechanism of how sugars down-regulate photosynthesis is still missing (Smeekens et al., 2010). It is believed that sucrose, glucose, fructose and trehalose 6-phosphate and/or their metabolic process are involved with many signalling mechanisms in source leaves (O'Hara et al., 2013; Ruan et al., 2012). Glucose is considered a prime carbon and energy source and the first enzyme in glucose catabolism, hexokinase, was identified as a genuine glucose sensor with distinguishable catalytic and signalling activities (Jang et al., 1997; Moore et al., 2003). Signalling mechanisms by glucose has been associated primarily with active cell division, respiration, cell wall biosynthesis and feedback regulation of photosynthesis (Bolouri-Moghaddam et al., 2010). Reductions in photosynthesis related to gene expression were correlated with glucose-6-phosphate amount in maize protoplast, supporting a signalling role for hexokinase (Jang and Sheen, 1994).

The hypothesis that hexokinase acts as a glucose sensor was substantiated by the characterization of transgenic Arabidopsis HXK sense and antisense lines (Jang et al., 1997). In general, plants carrying the antisense construct of HXK1 are hyposensitive to glucose repression, while plants overexpressing HXK1 are hypersensitive (Jang et al., 1997). In

addition, some reports have shown that glucose repression of photosynthesis genes and photosynthetic organ development is mediated by hexokinase, which act as a physiological feedback loop in sugar production (Cho et al., 2010; Kelly et al., 2013; Kim et al., 2013). However, sucrose is also mentioned as an important sugar in the photosynthesis down-regulation because it is the major transported sugar and acts as a key carbon source for growth, development and defence (Ruan, 2014). The specific role of signalling by sucrose is difficult to identify since it has an intense metabolism. Sucrose can be easily degraded by invertases or sucrose synthase in the vacuole, cytosol or apoplast, yielding glucose and fructose, or be re-synthetized by sucrose phosphate synthase or sucrose synthase in the cytosol (Ruan, 2014). Although, a number of studies have provided compelling evidence for sucrose-specific regulation in gene expression and growth (Ramon et al., 2008).

To demonstrate the specificity of sucrose in its repression mechanism, several other sugars, such as glucose and fructose, were tested, but none induces repression of translation to the same extend as sucrose (McCormick et al., 2008a; Hill et al., 2011; Lobo et al., 2015). Sucrose may act as a signal, controlling specific developmental processes not affected by hexose sugars (Ramon et al., 2008). A negative correlation between sucrose accumulation and photosynthesis in citrus leaves was reported by Iglesias et al. (2002). Sucrose metabolism, degradation and re-synthesis in cytosol, is mentioned like a futile cycle that can inhibit photosynthetic activity by decreasing the TP/inorganic phosphate (Pi) translocator between chloroplast and cytosol (Paul and Pellny, 2003). The depletion of Pi inside the chloroplast decreases the electron transport rate and the ATP synthesis, though the hypothesis of the sucrose futile cycle causes reduction in photosynthesis is controversial. Jang and Sheen (1994) reported that the replenishing of intercellular phosphate and ATP diminished by sucrose metabolism does not overcome the repression. In other way, Pieters et al. (2001) demonstrated that the Pi recycled by increasing sucrose demand can offset the effects of Pi deficiency on photosynthesis.

More recently, trehalose and trehalose-6-phosphate (T6P) have emerged as regulators of carbon metabolism and development in plants (Ramon et al., 2008). In the cytosol UDP-glucose and glucose 6-phosphate are used to produce T6P catalysed by trehalose phosphate synthase (TPS), then T6P is converted into trehalose by trehalose phosphate phosphatase (TPP). The flux of carbon into trehalose is four orders of magnitude less than into sucrose (Lyu et al., 2013). According to Griffiths et al. (2016), sucrose is sensed by the plant directly through the generation of hexose and sugar signals such as T6P, which relay the sugar status into mechanisms that enable plant acclimation to different environmental conditions. In general,

trehalose act as an inhibitor of plant growth and its accumulation is associated with perturbations of carbohydrate metabolism and decline of sucrose (Wingler et al., 2000).

Trehalose feeding resulted in increased expression and activity of ADP-glucose pyrophosphorylase (AGPase), which is a key enzyme in starch biosynthesis (Wingler et al., 2000). It suggests that growth inhibition by trehalose may be caused by excessive starch accumulation and reduced availability of carbon for export to the growth zones (Lunn et al., 2006; O'Hara et al., 2013). Whereas trehalose is responsible to reduce plant growth, T6P is necessary for normal plant development since the Arabidopsis tps1 mutant, which does not express *TPS1* gene, fails to germinate (Schluepmann et al., 2003). Moreover, the overexpression of a bacterial TPS improved CO_2 assimilation at various CO_2 and light conditions, whereas increased activities of TPP or the hydrolase had an opposite effect in tobacco plants (Pellny et al., 2004).

The regulation of plant metabolism by carbon status is made by different ways and the precise mechanisms of how sugars control plant growth is unknown (Smeekens et al., 2010). The signalling control by sugars is a consequence of modifications in their metabolism, such as amount and activity of enzymes that control carbohydrates flux (Paul and Pellny, 2003). In many cases is hard to identify which sugar is responsible by down-regulating photosynthesis because its concentration is finely regulated and does not change very much, but the flux exercised by any enzyme can be greater than its effects on the concentrations of associated intermediates (Fell, 2005). Some systems that regulate gene expression signalled by sugars or sugar-derived have been described (Eveland and Jackson, 2012; Smeekens et al., 2010). The systems that promote growth are hexokinase, trehalose 6-phosphate signal and the Target of Rapamycin (TOR) kinase system. While the systems that are inhibitory to growth are the Sucrose non-Fermenting Related Kinase 1 (SnRK1) and C/S1 bZIP transcription factor network (see details in Smeekens et al., 2010).

The regulation of photosynthesis, such as changes in biochemistry and stomatal behaviour, by carbohydrates has been well documented at the cellular level (Asao and Ryan, 2015). In general, experimental manipulations that increase sucrose and starch concentrations in leaves result in Rubisco and other Calvin-cycle enzymes down-regulation, and the rates of RuBP regeneration, carboxylation and electron transport decline (Goldschmidt and Huber, 1992; Krapp and Stitt, 1995; Moore, 2005). Stomata may be involved in photosynthesis inhibition by sugars, where accumulating carbohydrates causes stomatal closure, perhaps

through ABA signalling to optimize carbon gain and water use (Nikinmaa et al. 2013). However, more studies comprising crops and the whole plant metabolism to describe better how sugars are involved in photosynthesis inhibition are missing.

1.4 Photosynthesis and source-sink crosstalk in sugarcane

Sugarcane (*Saccharum* sp.) is an important perennial grass cultivated mainly in tropical or subtropical regions for sugar and biofuel production (Wang et al., 2013). As a C4 plant, sugarcane is one of the most efficient crop in converting solar energy into chemical energy (Watt et al., 2014). This plant has a unique source-sink system because, in contrast with other plants, its stem is capable of store high concentrations of sucrose, up to 650 mM or 18% of stem fresh weight in commercial sugarcane varieties (Inman-Bamber et al., 2011). Generally other species store C as insoluble polysaccharides such as starch or cellulose with low amount of sucrose. The storage of sucrose in the stalk parenchyma cells and in both symplast and apoplast compartments are another distinctive feature of sugarcane (Uys et al., 2007). As sugarcane stalk can store huge concentration of sucrose during its maturation, the high sink activity stimulates photosynthesis in source leaves that can converts up to 2% of incident solar energy into biomass (Watt et al., 2014). However, once it reaches the maturity and the sink activity decrease, a feedback mechanism inhibits source activity by metabolic and transcriptional levels (McCormick et al., 2008a).

The first evidence of this phenomenon in sugarcane was the observation that CO_2 assimilation rate is much higher in young leaves and decreases with leaf age, regardless of plant age or environmental conditions (Singh and Lal, 1935). The amount of sucrose accumulated is related with this response in sugarcane plants, supporting the remark that the end-product represses photosynthesis (Park et al., 2005). More recent, van Heerden et al. (2010) have described that the maturity-related end-product repression of photosynthesis has emerged as one of three probable causes along with decreased specific leaf nitrogen content and increased stalk respiration rates. This fact is also supported by sugarcane varieties with low sucrose accumulation, which have 30% higher photosynthesis rate than the commercial hybrids (Irvine, 1975). These statements suggest that sugarcane photosynthetic rates are typically limited by stalk requirements and sucrose accumulation may be regulated by the demand of sink tissues (Watt et al., 2005). However, some studies have suggested that the activity of source is

constrained by accumulation or changes in the sugars profile in the source leaves (Inman-Bamber et al., 2011; Lobo et al., 2015).

It is known that phloem communicates source-sink path and the mechanisms for the responsiveness from source tissues to phloem changes sink demand, but the extent to which source or sink determines fluxes need to be established (Watt et al., 2014). Over the past two decades, sucrose and its constituents (glucose and fructose) have been shown to play an important additional role as signaling molecules that are sensed by both intracellular and extracellular mechanisms and affect a broad range of physiological and molecular processes (Rolland et al., 2006; Hanson and Smeekens, 2009). In addition, there are some reports describing the inhibition of activity and abundance of photosynthetic proteins by sugar concentration in sugarcane leaves (McCormick et al., 2008a; Lobo et al., 2015). Source and sink physiology of sugarcane has been the subject of intense study over many decades and this interest is based on its value as a crop, as well as the physiological features of accumulate massive quantities of sucrose (Inman-Bamber et al., 2011).

According to some theoretical studies sugarcane stalk has the potential to accommodate more sucrose than has been attained to date. However, the conventional breeding programs have not reached such success (Watt et al., 2014). The synthesis of novel sugars in transformed sugarcane represent a promising opportunity to study their source-sink physiology and the role played by the native sugars (Wang et al., 2013). The insertion of bacterial sucrose isomerase gene in sugarcane increased the total sugars amount by converting part of the sucrose in isomaltulose (Wu and Birch, 2007). This study showed that additional metabolic sinks for sucrose could increase sink capacity, and lead to expected enhancement of photosynthesis and overall sugar accumulation (Koch, 2004). Although the transgenic studies conducted in sugarcane have provided valuable information on the biochemistry of component processes of sucrose metabolism, a viable framework for the genetic engineering of higher sucrose amount in the stalk remains elusive (Watt et al., 2014).

The connection between drought, sugar metabolism and photosynthesis inhibition in C4 plants has not been fully understood and the elucidation of the mechanisms involved with down-regulation of photosynthesis by water deficit and sugars are essential to improve crop yield. Therefore, in this study we investigated whether photosynthesis impairment is signalled by sucrose metabolism under drought conditions and how source-sink perturbations regulate photosynthesis in sugarcane plants.

HYPOTHESIS

Photosynthesis impairment in drought-stressed plants is mediated by sugar metabolism and the sink strength modulates CO_2 assimilation through regulations of photosynthetic enzymes in sugarcane plants.

OBJECTIVES

The general objective of this thesis is to investigate how drought and source-sink perturbation regulate photosynthesis in sugarcane plants cultivated under greenhouse conditions.

In order to reach the general objective of this thesis, some specific goals were proposed:

1. Identify if the photosynthesis impairment under drought condition is signalled by sucrose metabolism in sugarcane plants cultivated under greenhouse conditions;

2. Analyse how source-sink perturbations regulate photosynthesis in two contrasting cultivars of sugarcane cultivated under greenhouse conditions;

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CHAPTER II

PHOTOSYNTHESIS REGULATION BY SUCROSE IN DROUGHT-STRESSED PLANTS

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Supplying of exogenous sucrose induces changes in sugar metabolism and intensification of photosynthesis inhibition related to down-modulation of Rubisco under water deficit in sugarcane

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Abstract – Water deficit stress is the major limiting factor for plant growth and to survive in such adverse conditions, many biochemical and physiological changes are triggered by plants. However, the specific mechanisms involved with the photosynthetic regulation in C4 plant under drought conditions still scarcely known. Aiming to investigate whether photosynthesis impairment under drought conditions is related with sucrose signaling routes, an experiment with sugarcane plants subjected to water deficit for 5 days (WD) with concomitant spraying of 50 mM exogenous sucrose (WD + Suc) was performed. Although the relative water content and electrolyte leakage have been slightly modified by WD, suggesting a mild water stress, photosynthesis was strongly reduced over the five days. Exogenous sucrose intensified the negative effects of WD in the gas exchange and photochemical parameters. Photosynthesis was reduced by both stomatal and biochemical limitations, but exogenous sucrose intensified metabolic restrictions mainly through down-regulation of Rubisco initial activity and PSII effective quantum efficiency in drought-stressed plants. Rubisco amount and total activity did not change in WD + Suc, indicating that this enzyme was possibly inhibited by tight-binding inhibitors, such as sugars phosphates. The enzymes from sucrose metabolism and sugars amount in leaves, sheath and stalk were also differently altered by WD and WD + Suc. Interestingly, Sucrose/hexose ratio decreased in both leaf and sheath whereas it was increased in stalk WD + Suc plants, suggesting that sucrose and related sugars were deeply metabolized and transported in the plant. Altogether, these results suggest that CO_2 assimilation impairment is aggravated by exogenous sucrose. This limitation occurs mainly by biochemical restrictions, specially related to Rubisco initial activity and PSII quantum efficiency. In contrast, *in vitro* PEPCase activity and amount were increased in sucrose-treated plants, suggesting that C4 cycle efficiency was reduced *in vivo* by C3 cycle inhibition. Moreover, sucrose amount was increased in the stalk, suggesting the feedback regulation from stalk to source leaves in drought-stressed plants. Nevertheless, further studies still needed, especially regarding the specific signaling steps involved with sucrose-dependent impairment of photosynthetic activity under drought conditions.

Keywords: CO_2 assimilation; PEPCase activity; Photosystem II; drought; *Saccharum* spp.

Highlights

1. Sucrose/hexose ratio decreased in both leaf and sheath, however it increased in stalk
2. Sucrose aggravates photosynthesis impairment under drought
3. Sucrose inhibits photosynthesis mainly by biochemical limitations, specially related to reduction of Rubisco activation state in drought-stressed plants
4. Sucrose induces reduction in PSII efficiency and NPQ triggering under drought
5. Sucrose induces increase in PEPcase abundance and *in vitro* activity in drought-stressed leaves

Introduction

Drought and its adverse effects on photosynthesis represent the major limiting factor for crop productivity worldwide. Although this problem has been amply studied, very little is known on the molecular and biochemical mechanisms involving water deficit, sugar metabolism and photosynthesis regulation (Pinheiro and Chaves, 2011). It has been reported that water deficit can drastically affects photosynthesis and these effects are dependent on water stress intensity (Zhao et al., 2013). Furthermore, several reports have evidenced that drought intensely affect sugar metabolism in C3 and C4 plants but frequently the results are contradictory even for the same plant species (Pinheiro et al., 2001; Ribeiro et al., 2013; Zegada-Lizarazu and Monti, 2013). It has been shown that mild drought induces increases in the total soluble sugar contents at end of day, including sucrose, glucose and fructose whereas the starch response may be variable, including an initial increase in concentration (Bartels and Sunkar, 2005). However, it remains unclear whether exogenous application of sucrose can alleviate or intensify the effects of the drought in the photosynthetic process.

Leaf sugar metabolism is extremely complex and involves several interconnected pathways, including futile cycles (Geigenberger and Stitt, 1993). These pathways are highly regulated at genetic and biochemical levels but the specific mechanisms, especially under drought conditions, are poorly understood (Yu et al., 2015). Several works have evidenced that some sugars and enzymes are direct and/or indirectly involved in the regulation of photosynthetic gene expression under such dry conditions (Chaves and Oliveira, 2004; Muller et al., 2011). Changes in sugar concentration might trigger alterations in the expression of genes related to Calvin-Benson cycle, especially Rubisco (McCormick et al., 2008a; Quentin et al., 2013). In general, low levels of sugars in leaves might act as signals for up-regulation of photosynthetic gene expression (Lawlor and Paul, 2014). Several reports have highlighted that sucrose might act direct or indirectly as a potential signaling molecule for gene expression regulation and photosynthesis control by negative feedback (Koch, 2004; McCormick et al., 2008b; Araya et al., 2010). Moreover, trehalose and the phosphorylation of hexoses (glucose and fructose) by hexokinase could also be involved in signaling in sugarcane as observed in other species (McCormick et al., 2008b; Quentin et al., 2013; Lunn et al., 2014).

In sugar metabolic network in leaves, sucrose has a central position (hub) linking several important pathways and metabolites (Ruan, 2014). In an excellent review on the role of sugars and other compounds in drought response, Albacete et al. (2014) found that sucrose, starch,

soluble acid invertases (SAI) and sucrose-phosphate synthase (SPS) display high connectivity with photosynthesis and expression of important genes such those involved in abscisic acid (ABA) synthesis. Nevertheless, despite these intense efforts, the mechanisms underlying the water deficit effects on biochemical and photochemical components of photosynthesis are lacking. It is largely known that moderate drought induces initially restrictions in CO_2 assimilation by stomatal effects, which are aggravated progressively by restraints in Calvin-Benson cycle and photochemistry activity under severe stress (Lawlor and Tezara, 2009). The central question is elucidating which molecular and biochemical mechanisms are triggered by water deficit to cause impairment in photosynthesis.

We have recently demonstrated that exogenous sucrose supplied in sugarcane leaves was able to alter sugar metabolism and reduce Rubisco activity, inducing strong impairment in photosynthesis under irrigated conditions (Lobo et al., 2015). Intriguingly, in this study the leaf sucrose content at end of the day did not change after exogenous supplying. The majority of the reports have evidenced that the sucrose levels in leaves exposed to water deficit are slightly changed (Inman-Bamber et al., 2009; Pedroso et al., 2014). Therefore, the concentration of this sugar, in a particular time, tissue and physiological condition should reflect a complex balance involving synthesis, transport and consumption in other reactions. Some works have suggested that exogenous supplying of trehalose might be favorable to stress protection and contributing for growth under restrictive sink condition such as water deficit (Yang et al., 2014; Akram et al., 2016). In the case of sucrose, this strategy is apparently contradictory because this sugar induces a negative photosynthetic modulation (Lobo et al, 2015).

Assuming that a mild water deficit induces preponderantly impairment in photosynthesis by diffusional restriction, we argue that the biochemical limitations are signaled by sucrose metabolism in sugarcane plants exposed to moderate drought. This assumption is based in the fact that changes in the sugar pool earlier to water stress would trigger signaling for down-regulation of photosynthetic gene expression, such as Rubisco. In fact, here exogenous sucrose supplied before and during water-deficit in sugarcane leaves induced important changes in sugar metabolism and strongly down-regulated activity, activation state and abundance of Rubisco whereas PEPC activity and abundance were up-regulated, all in comparison with plants grown under single drought. The results suggest that the impairment in photosynthesis induced by exogenous sucrose were predominately by biochemical restrictions in CO_2 assimilation by Rubisco, which were extended for photochemical activity. The

physiological significance involving sugar signaling and negative photosynthesis modulation by changes in sugar metabolism under drought conditions is discussed.

Materials and methods

Plant material and growth conditions

Four-month-old 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 grown in 8 L pots containing a mixture of sand:vermiculite:humus (1:1:1 v/v/v), watered daily with distilled water until reaching the substrate holding capacity. Once a week, the plants were irrigated with Hoagland and Arnon solution (Hoagland and Arnon, 1950) until reaching complete substrate saturation. The pots were exhaustively leached with distillated water to avoid salinization every month. Four-month-old plants (at tillering stage) were initially grown under natural conditions 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.

Water deficit experiment

The experiment was carried out with 4-month-old plants, previously 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 photons m^{-2} s^{-1} and a 12 h photoperiod. After an acclimation period of 24 h inside the growth chamber, the plants were separated in three lots: well-watered (control), water deficit (water withdrawal - WD) and the combination of water deficit and exogenous sucrose (WD + Suc) for five days. Mannitol was supplied in sugarcane leaves from control and WD plants to compensate the osmotic effects caused by 50 mM sucrose. Exogenous sucrose (50 mM) and 50 mM mannitol (control) were dissolved in solution containing 0.01% (v/v) Triton X-100 and sprayed on the shoot until a complete leaf

wetting. Sucrose and mannitol were sprayed on all expanded sugarcane leaves twice a day (10:00 a.m. and 4:00 p.m.) for five consecutive days. The gas exchange measurements were performed in the leaf +1 of each plant over the five days after two hours from the first solution application in the leaves. After five days of supplying of exogenous sucrose, the plant parts (+1 and +2 leaves, sheaths and stalk) were collected at 6:00 p.m. and the samples were washed with 1.5 mM $CaCl_2$ to residue elimination and then they were immediately stored at -80 °C for biochemical analysis.

Gas exchange and chlorophyll a fluorescence measurements

The leaf CO_2 assimilation (A), stomatal conductance (g_S) and intercellular CO_2 partial pressure (C_i) and photochemical activity 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). The conditions inside the IRGA chamber during the measurements were near to those of the growth chamber: PPFD of 1,000 μmol photons m^{-2} s^{-1}, 28 °C, air vapor pressure deficit of 1.0±0.2 kPa, and air CO_2 partial pressure of 38 Pa. The amount of blue light was set to be 10% of the PPFD to maximize stomatal aperture (Flexas et al., 2008). The instantaneous carboxylation efficiency was calculated as A/C_i. The fluorescence parameters were measured using the saturation pulse method in both light and dark-adapted (30 min) leaves (Schreiber et al., 1994). The intensity and duration of the light saturation pulse were 8,000 μmol photons m^{-2} s^{-1} and 0.7 s, respectively. The following photochemical parameters were assessed: maximum quantum efficiency of PSII [Fv/Fm = (Fm-Fo)/Fm], effective quantum efficiency of PSII [ΔF/Fm' = (Fm'-Fs)/Fm'], apparent electron transport rate [ETR = (ΔF/Fm' x PPFD x 0.4 x 0.84)], where 0.4 was used as the fraction of excitation energy distributed to PSII, and 0.84 was used as the fraction of incoming light absorbed by the leaves; and non-photochemical quenching coefficient [NPQ = (Fm-Fm')/Fm']. Fm and Fo are the maximum and minimum fluorescence of dark-adapted leaves, respectively; Fm' and Fs are the maximum and steady-state fluorescence in the light-adapted leaves, respectively (Schreiber et al., 1994).

A quenching analysis during photosynthetic induction in previously dark-adapted leaves was performed, according to Bolhàr-Nordenkampf and Öquist (1993). The measurements were done under a continuous actinic light (1,000 μmol photons m^{-2} s^{-1}), by means of application of 20 sequential saturating light pulses at 25 s intervals, followed by a light saturation pulse. 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, and the CO_2 concentration was changed from 0 to 800 µmol m^{-2} s^{-1} and the curve fitting was performed according to von Caemmerer and Furbank (1999). From A/*Ci* curve the stomatal and metabolic limitations were calculated. 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 Ca = 38 Pa (finite g_S). A_{pot} is the potential leaf CO_2 assimilation or photosynthetic capacity, and A_c is the actual leaf CO_2 assimilation. 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 WD or WD + Suc.

Relative water content and electrolyte leakage (membrane damage)

The leaf relative water content (RWC) was calculated as follows: RCW = [(FW-DW)/(TW-DW)] x 100, where FW is the fresh weight, TW is the turgid weight after 24 h of saturation in deionized water at 25 °C and DW is the dry weight determined after 48 h in an oven at 75 °C (Silveira et al., 2009). Electrolyte leakage (EL) was measured as described previously by Lima Neto et al. (2014). Four leaf segments with 5 cm of length were placed in tubes containing 10 mL of deionized water. Flasks were incubated in a shaking water bath at 25°C for 24 h, and the electric conductive in medium (L1) was measured. Next, the segments were boiled at 95 °C for 1 h and cooled to 25 °C, and the electric conductivity (L2) was measured using the following equation: EL = (L1/L2) x 100.

Measurement of sucrose, glucose, fructose and starch contents

All reagents (chemical and enzymes) used in this study were purchased from Sigma-Aldrich Co. Sucrose was extracted by using the MCW solution (methanol:chloroform:water 12:5:1 v/v/v) and quantified according to van Handel (1968). The starch in the MCW pellet was determined by hydrolyzing with $HClO_4$ (30%, v/v), and the soluble sugars were measured by phenol-sulfuric acid method (Dubois et al., 1956). Glucose and fructose were quantified through an enzymatic method coupled to NADH production monitoring with a

spectrophotometer at 340 nm (Sigma's test kits, Sigma-Aldrich). The contents of all sugars were expressed in μmol g^{-1} DW.

Soluble protein extraction and enzymatic assays

Fresh leaves, sheaths and stalks were ground until obtaining a fine powder in presence of liquid N_2, ice-cold 100 mM K-phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 14000 *g* for 30 min, the supernatant was collected and used as enzymatic extract. All extraction stages were carried out at 4 ºC. The protein amount in the enzymatic extract was determined by Bradford's method (1976).

The activities of soluble acid invertases (SAI, EC 3.2.1.26) and neutral invertases (NI, EC 3.2.1.26) were measured according to Zhu et al. (1997). The crude extracts were incubated with 1 M sodium acetate buffer (pH 4.5 for SAI activity and pH 7.5 for NI activity) and 120 mM sucrose at 37 °C for 30 min. The reactions were stopped after adding 2.5 M Tris and incubating at 90 °C for 2 min. For both SAI and NI assays, the reducing sugars released were enzymatically measured (Sigma's test kits, Sigma-Aldrich). The SAI and NI activities were expressed in μmol glucose g^{-1} FW h^{-1}.

Sucrose synthase (SuSy, EC 2.4.1.13) and sucrose phosphate synthase (SPS, EC 2.4.1.14) activities were evaluated according to Hubbard et al. (1989), with modifications suggested by Zhu et al. (1997) and both were measured towards to sucrose synthesis. To determine the SuSy activity, the crude extracts were incubated with 50 mM HEPES buffer (pH 7.5) containing 15 mM $MgCl_2$, 25 mM fructose and 25 mM UDP-glucose. For the SPS activity, the crude extracts were incubated in 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 4 mM fructose 6-phosphate, 20 mM glucose 6-phosphate, 3 mM UDP-glucose and 1 mM EDTA. Both reactions were incubated at 37 °C for 0 and 60 min and then stopped by boiling for 3 min. After that, the sucrose produced by these reactions was assayed according to van Handel (1968). The SuSy and SPS activities were expressed as μmol sucrose g^{-1} FW h^{-1}.

Rubisco activity was measured spectrophotometrically by the rate of NADH oxidation at 340 nm (Reid et al., 1997). The assay buffer consisted of 100 mM bicine pH 8.0, 25 mM $KHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 80 nkat G-3-P dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, 80 nkat creatine phosphokinase and 0.25 mM NADH. For the initial activity, the extract was added in 900 μL of assay buffer and the reaction

was initiated by adding 0.5 mM RuBP. For the total activity, the extract was incubated at 25 °C in the assay buffer for 15 min to complete activation of Rubisco in the assay solution. Both the initial and total activities were expressed in μmol CO_2 mg protein^{-1} min^{-1}. The Rubisco activation state was calculated by using the initial activity/total activity ratio, and it was expressed as a percentage (%).

Phospho*enol*pyruvate carboxylase (PEPCase, EC 4.1.1.31) activity was measured by using an enzymatic method coupled to NADH oxidation monitored by spectrophotometer at 340 nm according to Degl'innocenti et al. (2002), with minor modifications. PEPCase activity was assayed in a reaction mixture containing the crude extract, 10 mM $NaHCO_3$, 0.3 mM NADH, 5 mM $MgCl_2$ and 33 nkat malate dehydrogenase (MDH) dissolved in 50 mM Tris-HCl buffer (pH 7.8). The reaction was started by adding 20 mM phospho*enol*pyruvate and enzyme activity was measured by recording the decreased absorbance at 340 nm over 300 s. PEPCase activity was expressed as μmol CO_2 mg protein^{-1} min^{-1}.

Western blotting for Rubisco and PEPCase

The same crude extract that was used to measure the enzymatic activities was used for PEPCase and Rubisco western blots. SDS-PAGE electrophoresis was performed with equal amounts of protein (20 μg) per lane. The proteins were denatured using 2% SDS and they were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979). The PEPCase and Rubisco protein abundance were performed by detection of PEPCase subunit and Rubisco large subunit (RLS) using specific polyclonal antibodies (Agrisera Co, Sweden) according to the manufacturer's instructions. The proportion of RLS and PEPCase subunit were detected using peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham PLC, Little Chalfont, UK) and the abundance was calculated using Image Studio Lite version 5.2.5 (LI-COR Biosciences).

Statistical analysis and experimental design

The experiments were arranged in a completely randomized blocks, with four replicates (one plant per pot) per treatment. The data were subjected to the analysis of variance (ANOVA) and the averages were compared by Tukey-test at a confidence level of 5% ($p< 0.05$).

Results

Exogenous sucrose aggravates impairment in CO_2 assimilation, which is related to down-regulation in Rubisco and induction in PEPCase activity in water stressed leaves

Mild water deficit (WD), single or in presence of exogenous sucrose, induced progressive and similar decrease in photosynthesis (A) until the 3rd day of water withdraw but sucrose supplying aggravated this effect afterwards (4th and 5th days). The *Ci* (intercellular CO_2 partial pressure) displayed an opposite trend compared to net photosynthesis: it was higher in WD + Suc than single WD until the 3rd day and was similar in both treatments after this time. The stomatal conductance decreased similarly in both treatments whereas the A/*Ci* (instantaneous carboxylation rate) decreased strongly in both treatments, but single WD presented higher values than WD + Suc during all experimental period. In control plants, all analyzed gas exchange parameters displayed slight changes throughout the experiment (Fig. 1A-D). The sucrose supplying in WD plants induced higher metabolic limitation (56%) in CO_2 assimilation compared to the single WD (42%) whereas the stomatal restriction occurred inversely: it was slightly higher (15%) in WD in comparison with WD + Suc –12% (Table 1). These results indicate that negative effects induced by exogenous sucrose on photosynthesis were more related to biochemical restriction than to stomatal limitation. At the end of the experimental period, the leaf relative water content (RWC) decreased slightly in both WD (86%) and WD + Suc (83%), compared to control – 98% (Table. 1), indicating a mild water deficit. These results are corroborated by the low values of electrolyte leakage (membrane damage) in leaves from plants of both treatments, which reached 13% and 16%, compared to control (10%). Nevertheless, the sucrose supplying induced a slight increase in this stress parameter (Table 1). Exogenous sucrose supplying in water stressed plants did not alter Rubisco initial activity and abundance, while the total activity was increased to control's level. In contrast, WD decreased both Rubisco activities and abundance, proving that Rubisco efficiency was reduced by low amount in WD and by post-translation down-regulations in WD + Suc, since sucrose decreased Rubisco activation state (Fig. 2A-C). In opposition, PEPCase activity was increased in parallel to slight increase in its abundance in WD + Suc plants (Fig. 3A-B).

Sucrose supplying intensifies the negative effects of water deficit on photochemical efficiency of PSII

Water deficit induced a gradual decrease in effective quantum efficiency of PSII – ΔF/Fm' (data not shown) throughout the experimental period, similar to displayed by the electron transport rate – ETR (Fig. 4A). Comparable to the previously noticed for the net photosynthesis, sucrose supplying also aggravated the reduction in PSII efficiency triggered by water deficit. In addition, the non-photochemical quenching (NPQ) increased more prominently in single WD compared to WD + Suc throughout the experimental period (Fig. 4B). The maximum quantum efficiency of PSII (Fv/Fm) remained unchangeable in all treatments until the 4th day but slightly decreased at 5th day in both WD and WD + Suc treatments, compared to control (Fig. 4C). To investigate more accurately the effects of exogenous sucrose on photochemistry of water stressed plants, a photochemical induction study in short-term (five min) involving the two treatments was performed. Sucrose supplying strongly inhibited ΔF/Fm' in comparison with single WD, which reached a peak after 2 min and values 25-fold higher than WD + Suc (Fig. 5A). Control leaves displayed an induction pattern different of WD plants, showing more fast induction of ΔF/Fm' and presenting maximum values highest and earlier (by approx. 1 min), which was followed by a new increase after the 3rd min (data not shown), whereas WD plants-showed a progressive decrease after 2 min. WD leaves supplied with Suc presented a singular trend showing slight initial induction in ΔF/Fm' in the first minutes, followed by unchangeable values from 2 min to 5 min. The photochemical quenching (qP) displayed a similar trend in both WD and WD + Suc compared to ΔF/Fm' (Fig. 5B). NPQ induction during the time-course was decreased by sucrose supplying compared with single WD treatment (Fig. 5C).

Exogenous sucrose triggers changes in the profile and concentration of sugars in leaf, sheath and stalk of water stressed plants

Exogenous sucrose supplying in water stressed plants triggered decrease in sucrose content in both leaves and sheaths whereas single WD and WD + Suc showed higher concentration in stalk compared to control (Fig. 6). The starch content in leaves of single WD and WD + Suc was similarly increased in comparison to control whereas in sheaths this carbohydrate did not change. In stalk, sucrose supplying for water stressed leaves induced significant increase in the starch content, whereas single WD and control displayed similar

values (Fig. 6). Exogenous sucrose also decreased the leaf glucose content in relation to single water stressed plants, which showed higher concentration compared to control, whereas in sheath the glucose content was similar in all treatments. Sucrose supplying in plants exposed to drought significantly increased the glucose concentration in stalk and the content of this sugar also increased in comparison to control. Single WD decreased the glucose concentration in stalk compared to control (Fig. 7). Fructose content in leaves and sheath of water stressed plants also decreased by effect of exogenous sucrose and single WD induced increase in the concentration of this sugar compared to control. In stalk, WD decreased fructose content compared to control and exogenous sucrose intensified this reduction (Fig. 7). Exogenous sucrose supplied to water stressed plants induced decrease in the sucrose/hexoses ratios in leaves and WD decreased this parameter compared to control. In sheath, both WD and WD + Suc displayed similar decrease in sucrose/hexoses ratios in relation to control, whereas in the stalk sucrose strongly increased this ratio in water-stressed plants (Fig. 8).

Sucrose supplying induces changes in the activities of sugar metabolism enzymes in leaf, sheath and stalk of water stressed leaves

To investigate the effects of exogenous sucrose on the sugar metabolism in different parts of water stressed plants, the activities of enzymes involved with sucrose synthesis and hydrolysis were evaluated. Single water deficit induced increase in neutral invertases activity (NI) but exogenous sucrose did not change its activity in leaves, whereas in sheaths it did not change in any treatment, all compared to control. In stalk the activity of this enzyme was increased only in single WD plants (Fig. 9). Soluble acid invertases (SAI) displayed responses different in comparison to NI. In leaf its activity was stimulated by sucrose supplying and remained unchangeable by WD effect while in sheath it was strongly decreased by water deficit and did not change by sucrose effect. In opposition, SAI activity in the stalk was enhanced by exogenous sucrose compared to control and single WD did alter its activity (Fig. 9). Leaf sucrose phosphate synthase activity (SPS) was increased by single drought but sucrose did not affect its activity whereas in sheath and stalk the activity was similar in all treatments (Fig. 10). Leaf sucrose synthase activity (SuSy) was similar in all treatments but in sheath and stalk it was decreased by effect of exogenous sucrose, compared to both control and WD (Fig. 10).

Discussion

In a previous study, we demonstrated that exogenous sucrose supplied to leaves of well-watered sugarcane induced changes in sugar metabolism in parallel to a strong impairment in CO_2 assimilation and photochemical activity (Lobo et al., 2015). Interestingly, these responses were associated with down-regulation of Rubisco and, in a minor extent, to stomatal restriction whilst *in vitro* PEPCase activity and protein abundance were not changed. More recently, our group has expanded these studies evidencing that exogenous sucrose is capable to significantly reduce *in vivo* maximum Rubisco carboxylation rate (Vcmax) whereas *in vivo* PEPcase carboxylation capacity (Vpmax) is reduced in a minor extent in two sugarcane cultivars. This study also demonstrated that exogenous sucrose alters deeply the source-sink relationships and that its inhibitory effects on photosynthesis might be mitigated by increase in leaf source strength (chapter 3). However, although these works have improved our knowledge on the role of sucrose in the regulation of photosynthetic gene expression in sugarcane, remains unclear whether this response also occurs under drought conditions.

Here, we demonstrated that sucrose supplying to sugarcane leaves before and throughout mild water deficit is capable to intensify photosynthetic inhibition. We argue that this negative combination might have involved primarily with an increase in the sugar level and/or changes in the amount or activity of sugar metabolism-related enzymes, which might be involved in signaling for down-regulation of expression of photosynthetic genes (McCormick et al., 2008a; Quentin et al., 2013). Indeed, our results clearly demonstrate a strong down-regulation of Rubisco in parallel to changes in sugar concentration and sugar-related enzyme activities. Interestingly, exogenous sucrose also induced strong inhibition in photochemical activity, especially reduction in $\Delta F/Fm'$, NPQ and qP. These adverse effects could have occurred as a consequence of impairment in CO_2 assimilation, which might induce a negative feedback on photochemical activity, triggering regulatory mechanisms to coordinate an efficient balance between photochemical and Calvin-Benson reactions (Foyer et al., 2012; Lawlor and Tezara, 2009).

Several studies have evidenced that moderate water deficit affects photosynthesis more due to stomatal restriction than by biochemical limitations in Calvin-Benson reactions (Flexas et al., 2002; Galmés et al., 2006; Ghannoum, 2009). On the other hand, severe drought induces great disturbances in sugar metabolism and negative modulation in enzymes involved in CO_2

assimilation and photochemical activities (Ruan et al., 2010; Lopes et al., 2011). Nevertheless, the specific molecular and biochemical mechanisms involved in photosynthesis inhibition by drought, especially in C4 plants, are scarcely known (Pinheiro and Chaves, 2011). The modulation in photosynthetic gene expression triggered by abscisic acid and sugars have been reported in several works (Eveland and Jackson, 2012; Pinheiro et al., 2011). Overall, it has been postulated that an increased sugar pool could act in down-regulation of genes involved with photosynthesis (Smeekens et al., 2010; O'Hara et al., 2013). In C4 plants, such as sugarcane, which have two carboxylation cycles, the mechanisms that control the photosynthetic inhibition by drought should be more complex than in C3 species.

Stomatal limitation is frequently reported as the most important process for photosynthesis impairment under moderate drought conditions (Jones, 1998). However, under this condition the soluble sugar pool in leaves is frequently increased, especially by starch hydrolysis and/or restriction in sink activity in other plant parts (Alam et al., 2010). This circumstance is also related to down-regulation of photosynthetic gene expression, aggravating photosynthesis inhibition by biochemical mechanisms (Quentin et al., 2013; Ye et al., 2011). Overall, plants display high phenotypic plasticity for acclimation and survival in adverse environmental conditions. Comprehension of this homeostasis under water deficit is important to mitigate adverse effects induced by drought on photosynthesis. In some conditions, exogenous supplying of sugars such as trehalose, aiming to increase the source strength of leaves, have been successfully employed (Lyu et al., 2013; Romero et al., 1997). However, the biochemical mechanisms underlying these processes involving photosynthesis modulation by sugars are poorly known.

In this study the leaf sucrose content was decreased in sucrose-treated plants but the starch and glucose contents were increased in stalk, indicating that sucrose was intensely metabolized and transported. These changes were accompanied by a strong increase in the sucrose/hexoses ratios in stalk, reinforcing intense interconversion of these sugars. Water stressed plants displayed a different response in terms of sugar metabolism, compared with water stress and sucrose-treated leaves. This result suggest that the exogenous sucrose was able to trigger great alterations in sugar metabolism and source-sink relationship in a water stress-independent manner, as previously reported for well-watered sugarcane plants (Lobo et al., 2015). These results represent new insights for comprehension of sugar metabolism, source-sink relations and photosynthesis inhibition under drought conditions.

Water deficit induces different responses in plants which involves a complex gene expression network (Nakashima et al., 2014). Unfortunately, the current knowledge concerning the role of sugar metabolism in the regulation of photosynthetic gene expression is fragmented and important issues remain unknown. Moreover, although it has been shown that sucrose can directly regulates the expression of genes related to the photochemical reactions, it remains unclear whether this response extends to drought stress condition. Interestingly, in this study the exogenous sucrose supplying strongly affected the qE (fast NPQ) formation, a complex and important process involved in excess energy dissipation as heat and photosynthetic protection (Ruban, 2016). This photochemical issue is frequently studied in water stressed plants (Medrano et al., 2002; Silva et al., 2015). However, the majority of the studies have neglected the role of sugar metabolism in the regulation of this process.

The photochemical results obtained from the induction kinetic involving ΔF/Fm', NPQ and qP in response to exogenous sucrose treated-leaves were interesting and less investigated so far. Sucrose treatment was able to close PSII reaction centers, inducing strong inhibition in the photochemical efficiency and qE formation. These results could generate deep physiological consequences and they open perspectives for new investigations on photochemical regulation by sugars and its reflexes on the whole photosynthesis. Utilization of exogenous sucrose in Arabidopsis demonstrated that this sugar is capable to directly regulate the electron transport rate, triggering decrease in PSII activity via down-regulation in the amount of photosynthetic electron transport chain (PETC) proteins, such as plastocyanin (Oswald et al., 2001). Thus, our results suggest that the inhibition of photochemical activity could be consequence of direct effect of exogenous sucrose, strengthening the idea that sucrose can be a direct modulator of photochemical reactions most probably by regulating the expression of PETC-related genes (Oswald et al., 2001).

In conclusion, exogenous sucrose supplied to sugarcane leaves combined with moderate water deficit is able to aggravate impairment in photosynthesis, inhibiting photochemical activity and CO_2 assimilation preponderantly by biochemical rather than diffusional limitations. Photosynthetic inhibition was related to strong down-regulation of Rubisco activity, despite its total activity and protein abundance have been increased by sucrose supplying in comparison with water stressed plants. In opposition to Rubisco, *in vitro* PEPCase activity and protein abundance were increased in sucrose-treated plants. It has been recently showed that the accumulation of trehalose-6-phospohate induce a post-translation activation of PEPCase (Figueroa et al., 2016). Taking into account that the effects of exogenous sucrose in leaves were

probably indirect, since this sugar did not accumulate in this organ, we argue that the synthesis of trehalose-6-phosphate following sucrose breakdown could be the signal for the activation of PEPCase in sucrose-treated plants. Nevertheless, further studies using genetic, biochemical and physiological approaches are needed to elucidate the mechanisms involved in photosynthesis inhibition by drought and sugars-related signaling.

Conflicts of interest

The authors have no conflicts of interest to declare.

Contributions

AKML conducted all experiments, performed biochemical determinations, and participated of the interpretation and discussion of results. MOM performed Rubisco and PEPCase westernblot and conducted some assays. FELC performed the photosynthesis measurements. DMD interpreted the results and participated of the manuscript writing. ECM and RVR designed and supervised the experiments determinations and interpreted the results. JAGS was the mastermind of the research and wrote the manuscript.

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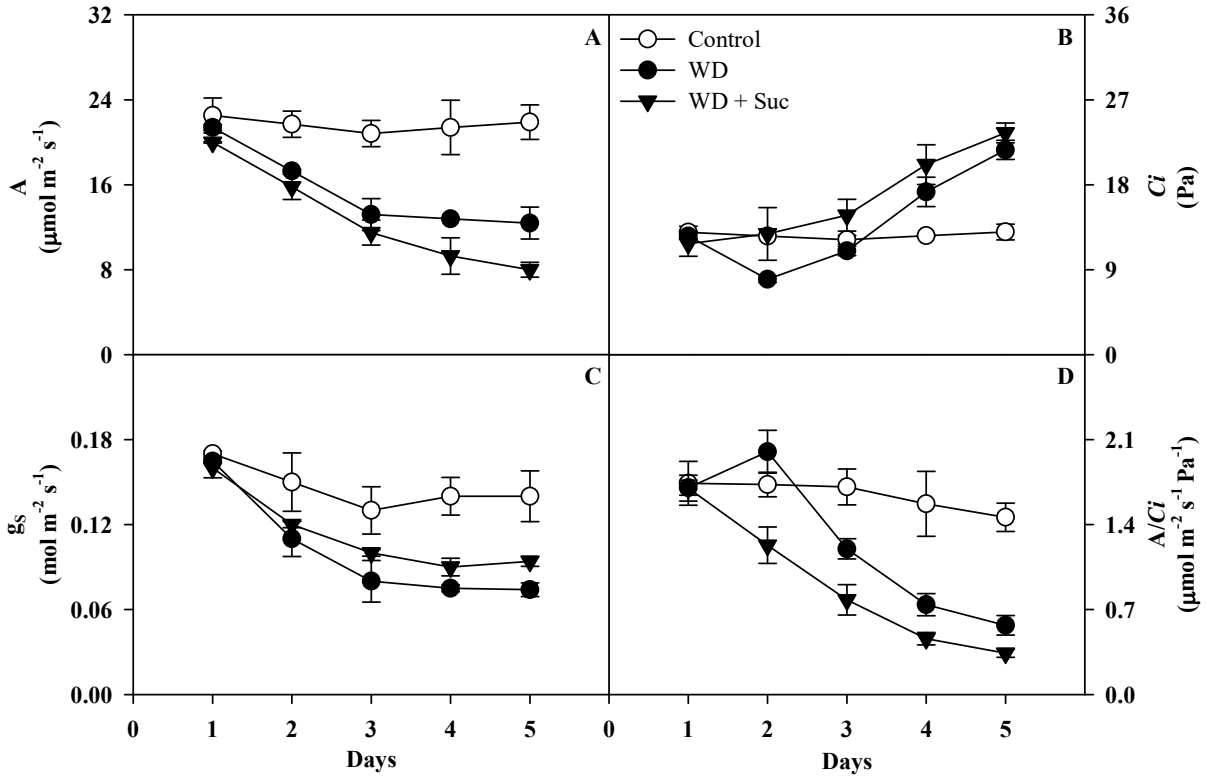

Figure 1: Gas exchange parameters measured in leaves of sugarcane subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Leaf CO_2 assimilation - A (A), intercellular CO_2 partial pressure - *Ci* (B), stomatal conductance g_s (C) and **i**nstantaneous carboxylation efficiency A/*Ci* (D). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each point represents the average of four replicates (± SD).

Table 1: Relative water content (RWC), electrolyte leakage (EL), stomatal (Ls) and metabolic (Lm) limitation in leaves of sugarcane subjected to water deficit (WD) and spayed with 50 mM sucrose (WD + Suc) for five days. A 50 mM mannitol solution was used as control. Each point represents the average of four replicates, different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

Parameters (%)	Control	WD	WD + Suc
RWC	98.52 A	86.42 B	82.73 B
EL	9.72 C	13.46 B	16.31 A
Ls	4.86 B	15.24 A	12.16 A
Lm	-	41.85 B	55.77 A

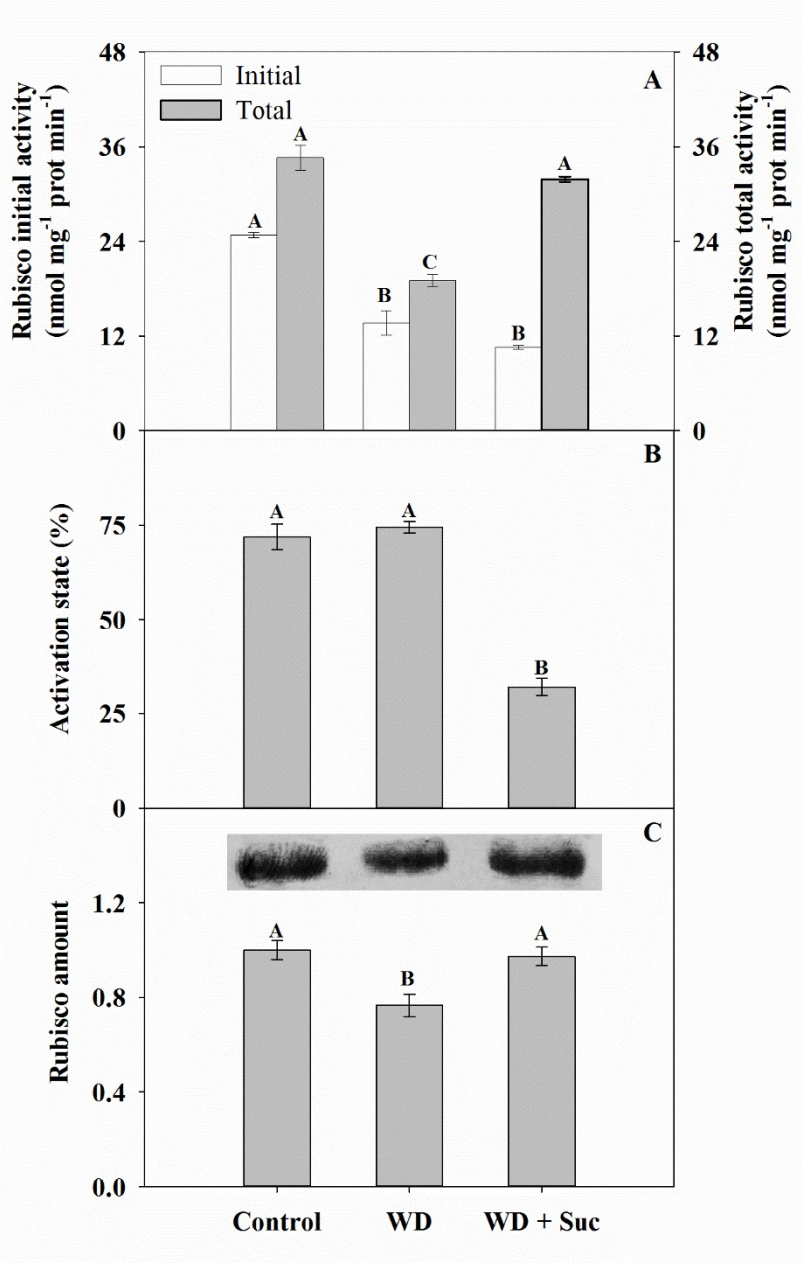

Figure 2: Rubisco activity and protein amount measured in leaves of sugarcane subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Initial and total activity (A), activation state (B) and protein amount (C). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. For westernblot (WB) assays, 20 µg of total soluble protein was loaded in each lane. WB image is the most representative among four independent replicates. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

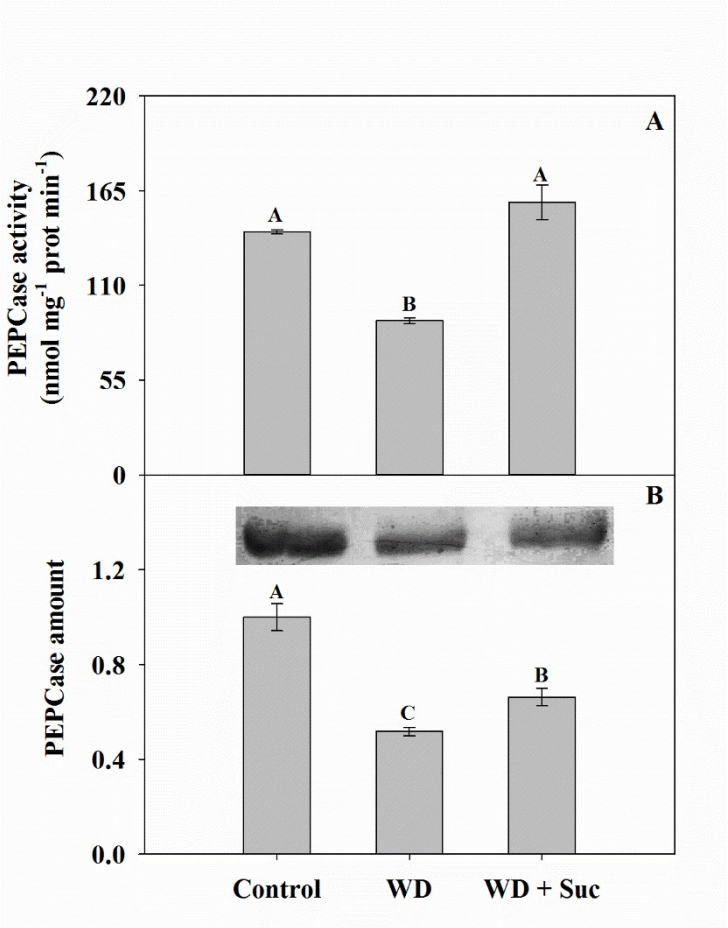

Figure 3: Phospho*enol*pyruvate carboxylase (PEPCase) activity and protein amount measured in leaves of sugarcane subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Total activity (A) and protein amount (B). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. For WB assays, 20 µg of total soluble protein was loaded in each lane and WB image is the most representative among four independent replicates. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

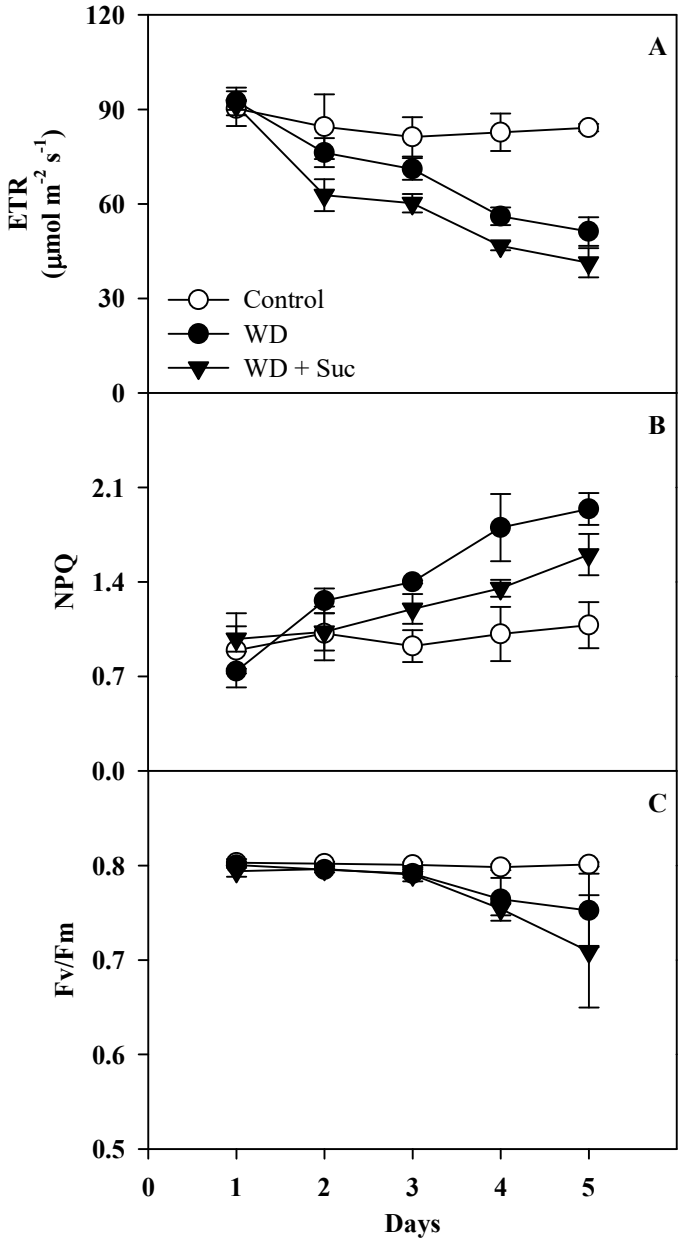

Figure 4: Time-course of photosystem II (PSII) activity parameters measured in leaves of sugarcane subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Apparent electron transport rate of PSII - ETR (A), non-photochemical quenching – NPQ (B) and maximum quantum efficiency of PSII – Fv/Fm (C). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each point represents the average of four independent replicates (± SD).

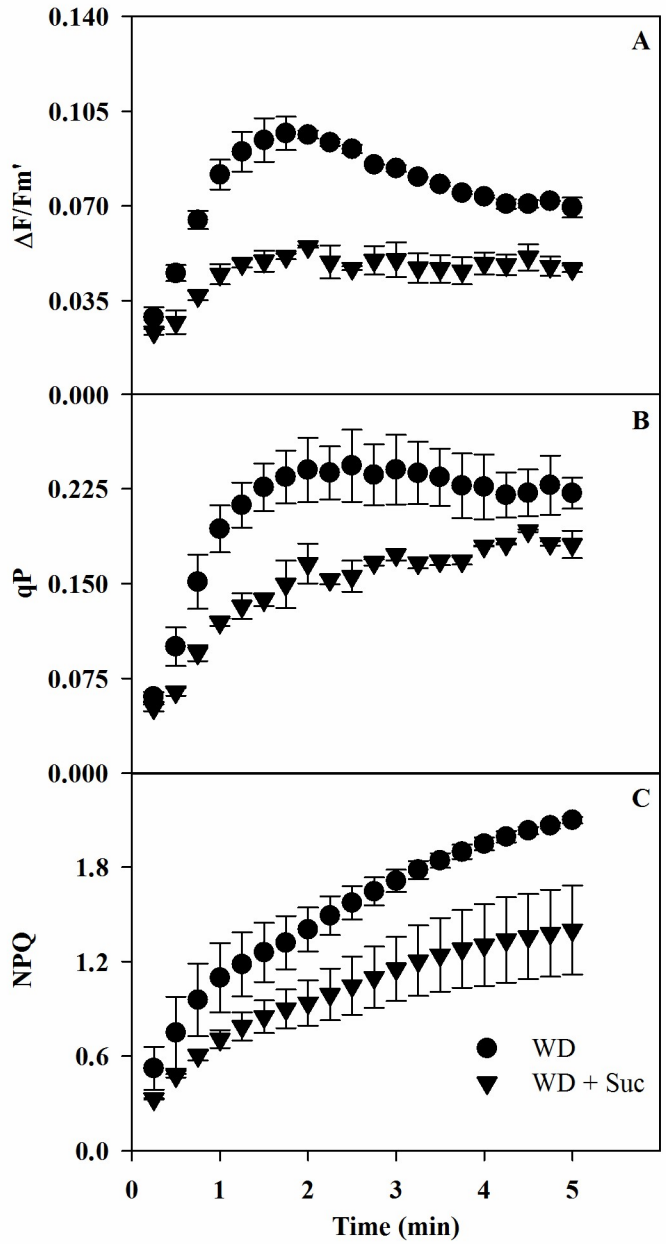

Figure 5: PSII photochemical induction kinetics determined in leaves of sugarcane subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Effective quantum efficiency of PSII – ΔF'/Fm' (A), apparent electron transport rate – ETR (B) and non-photochemical quenching – NPQ (C). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each point represents the average of four independent replicates (± SD).

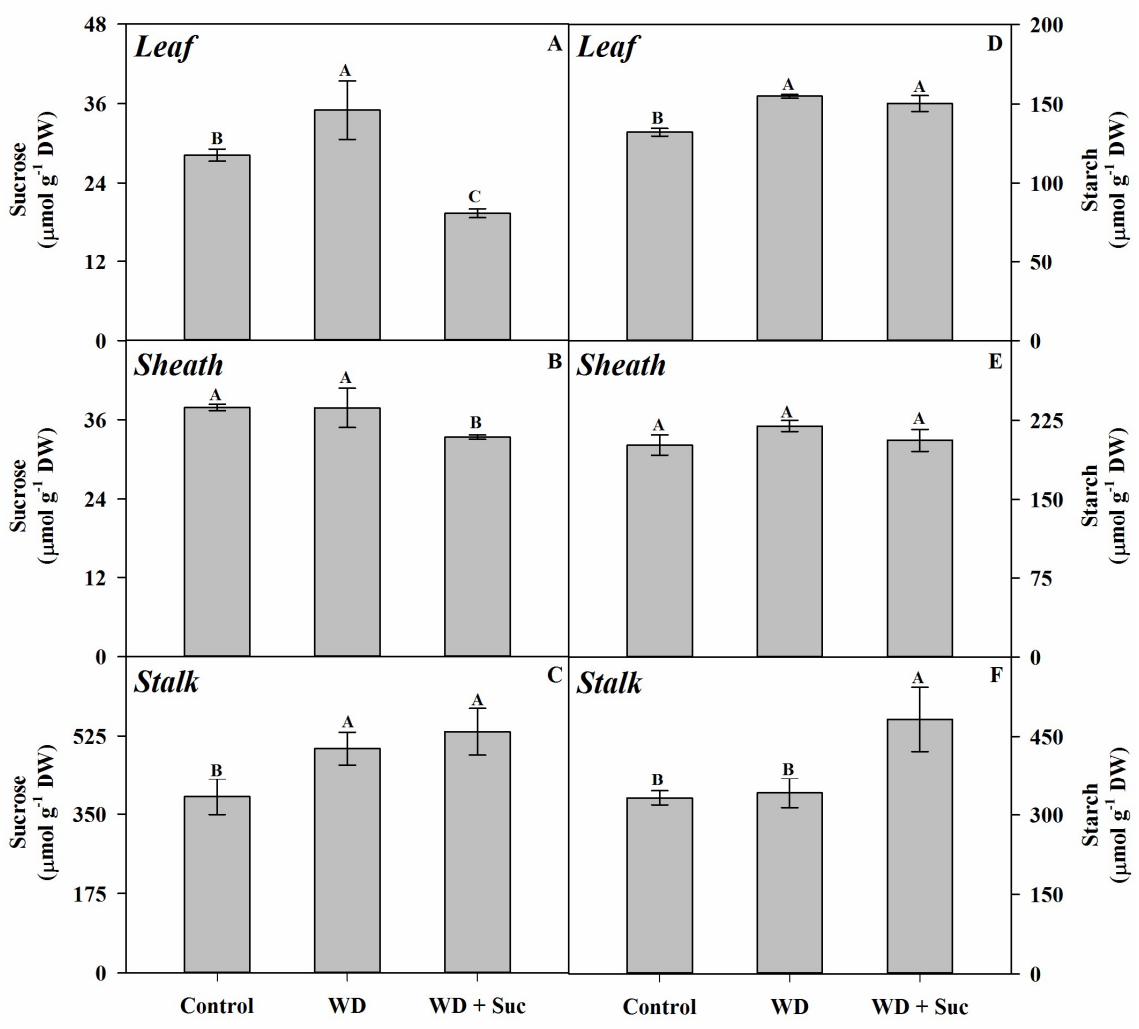

Figure 6: Sugar contents measured in sugarcane plants subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Sucrose and starch in leaves (A, D), sheath (B, E) and stalk (C, F), respectively. In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

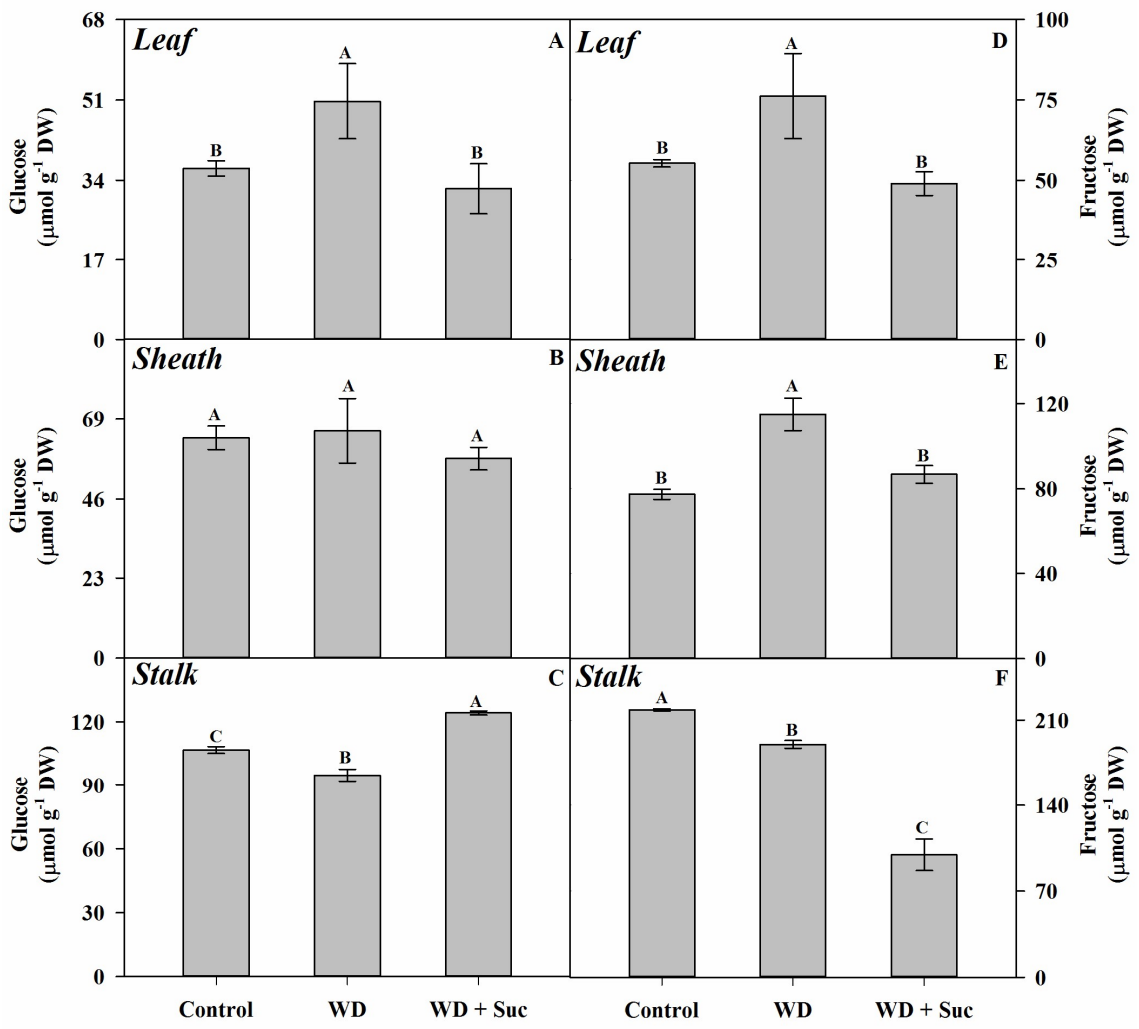

Figure 7: Hexose contents measured in sugarcane plants subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Glucose and fructose in leaves (A, D), sheath (B, E) and stalk (C, F), respectively. In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

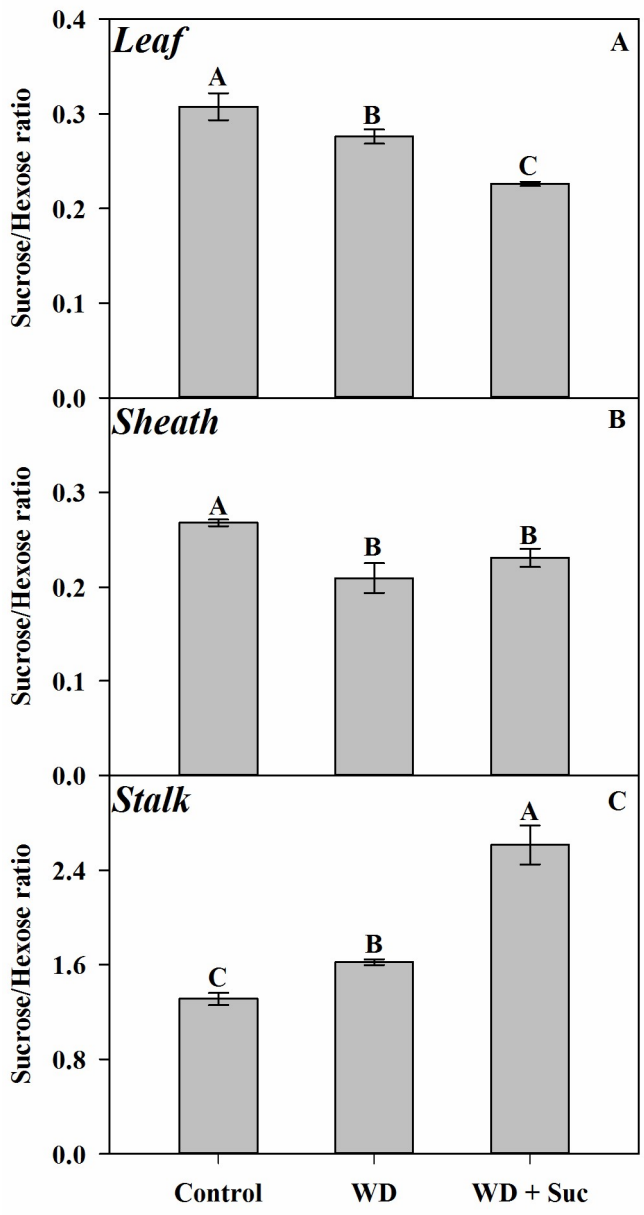

Figure 8: Sucrose/hexose ratios measured in sugarcane plants subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Sucrose/hexose ratios in leaves (A), sheath (B) and stalk (C). In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

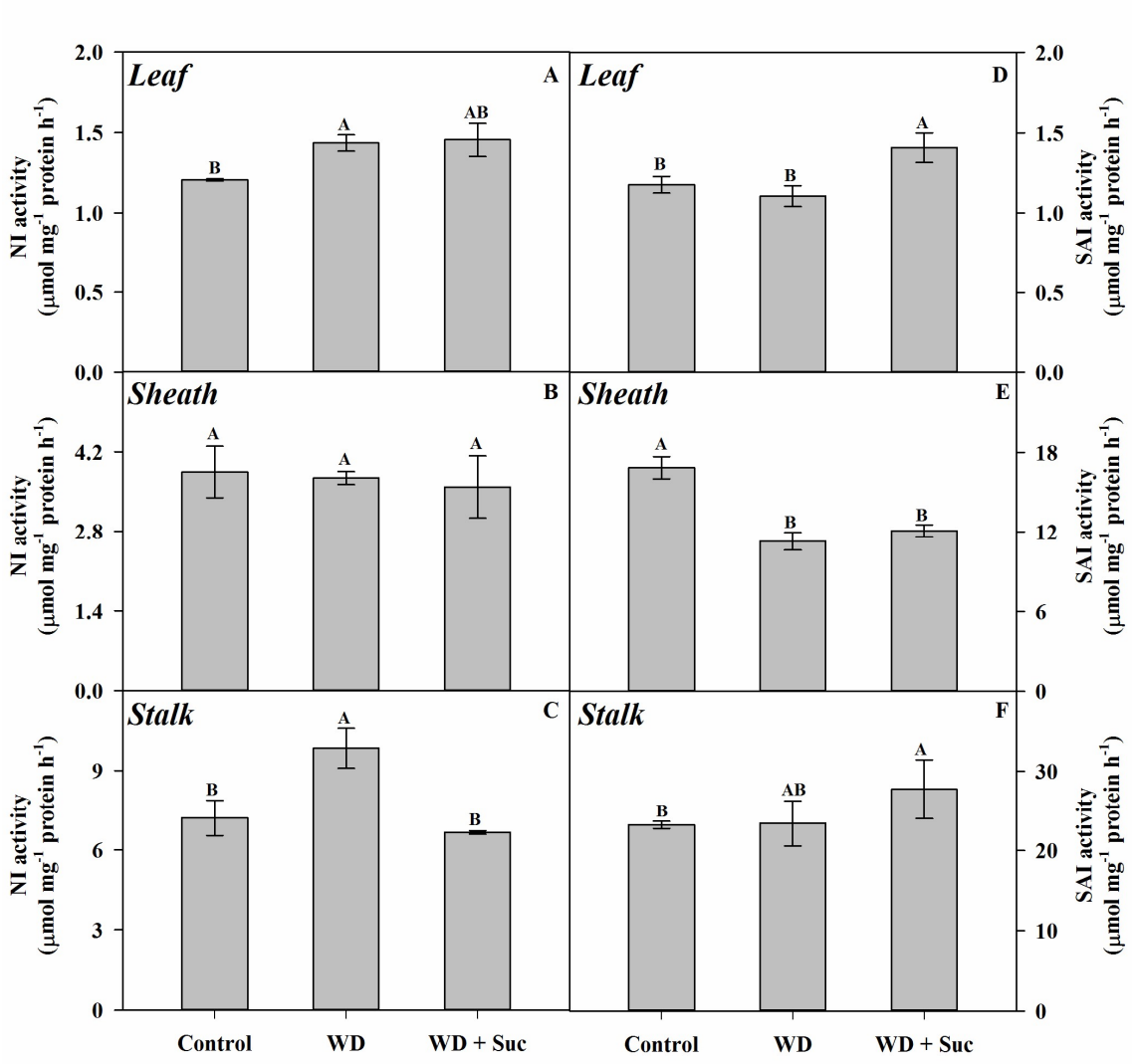

Figure 9: Invertase activities measured in sugarcane plants subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Neutral invertase and soluble acid Invertase activity in leaves (A, D), sheath (B, E) and stalk (C, F), respectively. In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

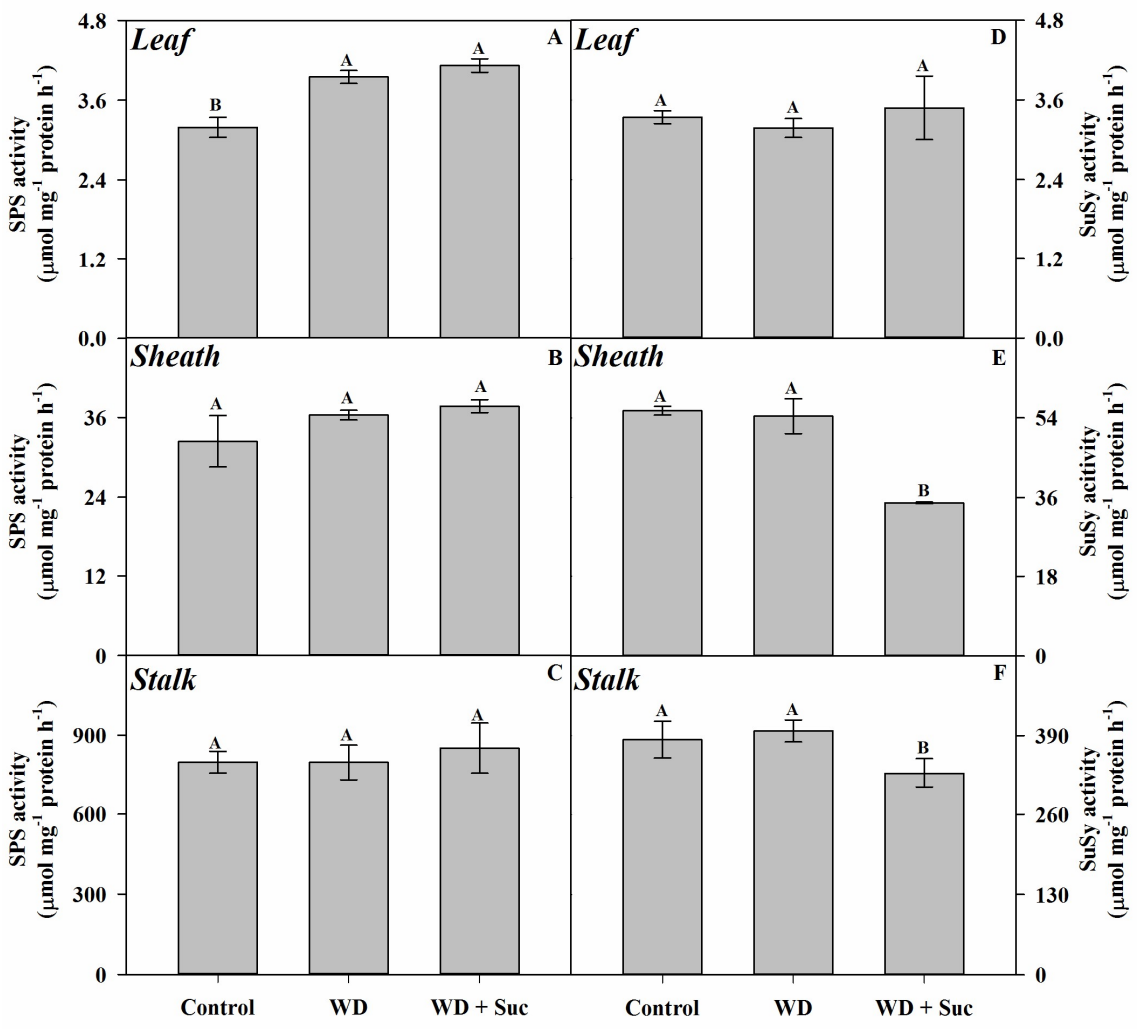

Figure 10: Sugar synthase total activities measured in sugarcane plants subjected to water deficit for five days (WD) and spayed with 50 mM sucrose (WD + Suc). Sucrose phosphate synthase and sucrose synthase activity in leaves (A, D), sheath (B, E) and stalk (C, F), respectively. In order to avoid osmotic differences, 50 mM mannitol solution was used as control. Each vertical bar represents the average of four independent replicates (± SD), different letters represent significant differences between treatments according to Tukey test ($P \leq 0.05$).

INTERCHAPTER

In the chapter II, it was investigated if the photosynthesis impairment is mediated by sucrose metabolism in drought-stressed sugarcane. In this study, we have analysed sucrose metabolism in leaves, sheath and stalk of four-month-old plants to verify the influence of sugars in each plant compartment, since the level of sugars in the sink regulate source activity. This work was performed in growth chamber to keep the environmental parameters more stable and in this condition the photosynthetic saturation light measured from A/PPFD curve was 1,000 μmol m^{-2} s^{-1}. As we have demonstrated how exogenous sucrose modulates photosynthesis in sugarcane plants in our previous work (Lobo et al., 2015) in this current one we just examined the effects of drought and the combination with exogenous sucrose. After five days of experiment the data revealed that exogenous sucrose sprayed in sugarcane leaves combined with mild water deficit intensified photosynthesis impairment through inhibition of Rubisco activity and PSII efficiency. Sucrose metabolism was intensely altered by drought, which the sucrose/hexose ratio was decreased in leaves and sheath and increased in the stalk. Thus, these results suggest that photosynthesis inhibition by drought is mediated by sucrose metabolism in sugarcane plants.

However, it is not fully understood how sugars down-regulates CO_2 assimilation in source leaves of sugarcane plants. Hence, another study to investigate how source-sink perturbations modulate photosynthesis in two contrasting sugarcane cultivars was performed in the next chapter. In this one we have used three-month-old plants without mature stalk to avoid excessive sink activity. Furthermore, the experiment was performed under greenhouse conditions and the saturation light used in the photosynthetic parameters measurements was 2,000 μmol m^{-2} s^{-1}.

CHAPTER III

PHOTOSYNTHESIS REGULATION BY SOURCE-SINK PERTURBATIONS

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Increased sink strength offsets the inhibitory effect of sucrose on sugarcane photosynthesis

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Increased sink strength offsets the inhibitory effect of sucrose on sugarcane photosynthesis

Running title: Source-sink perturbations affect sugarcane photosynthesis

Highlights

Exogenous sucrose down-regulated Rubisco and PEPC activities in sugarcane. Increased sink demand through darkening most of the plant's leaf area nulled inhibitory effect of sucrose on photosynthesis.

Summary

Spraying sucrose inhibits photosynthesis by impairing Rubisco activity, whereas increasing sink demand by partially darkening the plant stimulates sugarcane photosynthesis. We hypothesized that the stimulatory effect of darkness can offset the inhibitory effect of exogenous sucrose. Source-sink relationship was perturbed in two sugarcane cultivars by imposing partial darkness, spraying a sucrose solution (50 mM) and their combination. Five days after the onset of the treatments, the maximum Rubisco carboxylation rate (V_{cmax}) and the initial slope of A-C_i curve (k) were estimated by measuring leaf gas exchange and chlorophyll fluorescence. Photosynthesis was inhibited by sucrose spraying in both genotypes, through decreases in V_{cmax}, k, stomatal conductance (g_s) and ATP production driven by electron transport (J_{atp}). Photosynthesis of plants subjected to the combination of partial darkness and sucrose spraying was similar to photosynthesis of reference plants for both genotypes. Significant increases in V_{cmax}, g_s and J_{atp} and marginal increases in k were noticed when combining partial darkness and sucrose spraying compared with sucrose spraying alone. Our data also revealed that increases in sink strength due to partial darkness offset the inhibition of sugarcane photosynthesis caused by sucrose spraying, enhancing the knowledge on endogenous regulation of sugarcane photosynthesis through the source-sink relationship.

Keywords: photosynthesis, source-sink, sugarcane, sucrose.

Abbreviations

A – leaf CO_2 assimilation in a given condition; A_{380} – leaf CO_2 assimilation under 380 μmol CO_2 mol^{-1} in the air and a level of photosynthetically active irradiance of 2,000 μmol m^{-2} s^{-1}; Dark – partial darkness treatment; D+S – treatment combining partial darkness and spraying a sucrose solution; g_S – stomatal conductance; J_{atp} – rate of ATP production driven by electron transport; k – the initial slope of the A-C_i curve; *F'* – steady-state fluorescence; *Fm'* – maximum fluorescence; NI – neutral invertase; PEPC – phosphoenolpyruvate carboxylase; R_d – day respiration or leaf respiration in the light; Ref – reference treatment; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; *s*' – calibration factor for converting electron flux to ATP flux; SAI – soluble acid invertase; SPS – sucrose-P synthase; Suc – treatment with spraying a sucrose solution; SuSy – sucrose synthase; V_{cmax} – maximum Rubisco carboxylation rate; ϕ_{PSII} – apparent operating quantum efficiency of photosystem II.

Introduction

Photosynthetic regulation by environmental changes has been extensively studied in sugarcane (*Saccharum* spp.), with plants showing large seasonal variation in leaf CO_2 uptake. For instance, cool temperatures and low water availability are commonly found during the winter season and they represent two of the most important factors leading to low photosynthetic rates in sugarcane plants growing in subtropical regions (Sales *et al.*, 2012; 2013 Machado *et al.*, 2013). Besides the exogenous regulation, photosynthesis is also affected by endogenous factors, with the source-sink relationship affecting photosynthetic rates of sugarcane (McCormick *et al.*, 2008a, b; 2009). While an increase in sink demand due to active growth is able to stimulate photosynthesis in source leaves, the opposite happens when plants are dormant or when sucrose loading in phloem and export from leaves are reduced (McCormick *et al.*, 2006; 2008a; Inman-Bamber *et al.*, 2011).

Increases in sugarcane photosynthesis induced by increased sink demand was associated with over-expression of genes encoding for PEPC, Rubisco, and hexokinase, as well as some components of the mitochondrial metabolism and sugar transport (McCormick *et al.* 2008b). At post-translational level, our knowledge about photosynthesis regulation by sugar remains limited (Lobo *et al*., 2015). In general, source-sink imbalances may change the leaf levels of inorganic phosphate (Pi), triose phosphates, sucrose and hexoses, which compose the sugar sensing and signalling mechanisms in plants and then could affect photosynthesis through the regulation of Calvin-Benso cycle reactions and sugar metabolism (Paul and Foyer, 2001; Rolland *et al.*, 2002; 2006).

Sugarcane is among the most important crop species for studying source-sink relationships due to its high accumulation of sucrose in culms and its C_4 photosynthetic metabolism. In fact, sucrose concentration in culms may reach 0.7 M in sugarcane (Chandra *et al.*, 2011), with this species being very sensitive to the manipulation of source-sink relationship (Inman-Bamber *et al.*, 2008; 2009; 2010; 2011; McCormick *et al.*, 2006; 2008a, b, c; 2009). As sugarcane shows high photosynthetic rates, it has a great potential for producing and exporting sucrose from leaves to culms during ripening, when culms are the main sinks. However, ripening happens when environmental conditions (i.e., cool temperature and drought) are limiting for sugarcane photosynthesis. If plants are able to maintain source activity under unfavourable conditions, we would expect higher sucrose yield in field-grown plants as photo-

assimilates would be partitioned to storage in culms rather than to vegetative growth (Inman-Bamber *et al.*, 2008; 2009; 2010; 2011; McCormick *et al.*, 2006; 2008a, b, c; 2009).

Sugarcane photosynthesis is sensitive to sucrose and/or derivative sugars: spraying a sucrose solution significantly impairs *in vitro* Rubisco activity, reduces Rubisco activation state and abundance, and decreases photosynthetic rates in four-month old plants (Lobo *et al.*, 2015). We also know that increases in sink demand stimulate photosynthesis through increases in both carboxylation efficiency and photochemical activity (McCormick *et al.*, 2006; 2008a; 2009) and changes in sugar concentration of immature culms have controversial effects on leaf photosynthesis (McCormick *et al.*, 2006; Inman-Bamber *et al.*, 2011; Lobo *et al.*, 2015). In fact, the underlying processes driving the source-sink relationship are still poorly understood for C_4 species like sugarcane (McCormick *et al.*, 2008a; Lobo *et al.*, 2015).

As there is a close cooperation between C_3 and C_4 cycles, we may argue that both *in vivo* Rubisco and PEPC enzymes could be down-regulated by spraying sucrose solution on leaves. In fact, we have recently proposed such an effect of exogenous sucrose on PEPC activity based on the instantaneous ratio between CO_2 assimilation and intercellular CO_2 concentration in sugarcane plants (Lobo *et al.*, 2015). In addition, we have at least one open question regarding the endogenous regulation of photosynthesis by source-sink perturbation: Is the increase in sink demand able to offset the inhibitory effect of spraying sucrose solution in sugarcane leaves? Previously, the inhibitory effect of hexoses on sugarcane photosynthesis was reversed by darkening leaf segments (McCormick *et al.*, 2008a); however, such approach using leaf segments incubated with hexoses impedes any signalling between plant tissues/organs and also the transport of nutrients, which would occur in intact plants.

Herein, we report the inhibition of *in vivo* Rubisco activity and provide evidence towards inhibition of PEPC activity due to sucrose spraying on two sugarcane varieties with differential sucrose yield. In addition, the relative importance of sink demand for sugarcane photosynthesis was revealed through the perturbation of source-sink relationship, a neglected issue in regulation of sugarcane photosynthesis (McCormick *et al.*, 2009). Our data confirm the inhibition of both key photosynthetic enzymes by spraying a sucrose solution and demonstrate that increased sink demand is able to offset the inhibitory effect of sucrose spraying on sugarcane photosynthesis. Such results are discussed taking into account the underlying mechanisms regulating photosynthesis and also highlighting the relevance of such findings for crop production.

Materials and Methods

Plant material and growth conditions

Sugarcane (*Saccharum* spp.) varieties IACSP94-2094 and IACSP95-5000 were grown in pots filled with 12 L of commercial substrate (Carolina Standard, Carolina Soil of Brazil, Vera Cruz RS, Brazil). IACSP94-2094 is a drought-tolerant genotype with lower biomass production and culm yield than IACSP95-5000 (Ribeiro *et al.*, 2013; Silva *et al.*, 2016). Each pot containing one plant was fertilized with 3.00 g urea, 7.50 g of superphosphate and 1.95 g potassium chloride at sowing. Fifty days after sowing, each pot received additional fertilization of 1.35 g urea, 1.35 g superphosphate and 1.17 g potassium chloride. Plants were maintained under well-watered conditions through daily irrigation during the experimental period. Sugarcane varieties were grown for 90 days under greenhouse conditions, where air temperature (day/night) was set to 31.5±1.0 / 21.5±1.5 °C. The maximum photosynthetic active irradiance measured inside the greenhouse was 1,100 μmol m^{-2} s^{-1} and photoperiod was around 13 h.

Source-sink perturbation

Source-sink perturbation was initiated when plants were three-months old. Four groups of plants (with four plants each) for each variety were formed and then subjected to partial darkness and/or spraying of a sucrose solution as follows. In one group of plants all leaves were sprayed with sucrose 50 mM solution (Suc) and another group was subjected to partial darkening by enclosing plant shoots in black plastic bags for five days (Dark). In the Dark treatment, only the youngest fully expanded leaf with visible ligule (leaf +1) was maintained under light conditions and therefore those plants had only one source leaf. As the third treatment, sucrose solution spraying and partial darkening were combined, with the leaf +1 receiving sucrose solution spraying (D+S). The sucrose solution was composed of sucrose 50 mM, water and Triton X-100 (0.01%, v/v). We have previously shown that plants supplied with sucrose at 50 mM presented a significant inhibition of photosynthesis (Lobo *et al.*, 2015). As reference (Ref), a solution of water and Triton X (0.01% v/v) was sprayed in all leaves of the fourth group of plants. The water solution was also sprayed in leaf +1 of plants subjected to partial darkening. Both sucrose and water solutions were sprayed on leaves twice a day during

five consecutive days, with physiological measurements done at the first day after ending treatments in leaf +1.

Gas exchange and chlorophyll fluorescence measurements

Measurements of gas exchange and chlorophyll fluorescence were taken simultaneously from leaf +1, with an infrared gas analyser LI-6400XT (Li-Cor Inc., Lincoln NE, USA) and a modulated fluorometer model 6400-40 (Li-Cor Inc., Lincoln NE, USA). Photosynthetic responses to varying light and air CO_2 concentration were evaluated under 21% and 1.2% O_2. During measurements, leaf temperature was maintained at 31 ºC and leaf-to-air vapour pressure difference was kept between 1.5 and 2.0 kPa. Air CO_2 partial pressure was varied as suggested by Long and Bernacchi (2003) and then leaf gas exchange and chlorophyll fluorescence were measured at 380, 200, 100, 90, 80, 60, 50, 380, 600, 1,300 and 2,000 μbar, under constant incident photosynthetically active irradiance (I_{inc}) of 2,000 μmol m^{-2} s^{-1} and two [O_2] levels of 21% and 1.2%. Measurements of leaf CO_2 assimilation (A) were started after 15 min of acclimation to high light and were taken after reaching steady-state (~ 6 min) in each CO_2 concentration. To estimate the mitochondrial day respiration (R_d) and better convert chlorophyll fluorescence measurements to useful physiological fluxes (see Data analyses), we additionally measured a part of light response curves by varying I_{inc} as follows: 400, 200, 100, 75, 50 and 25 μmol m^{-2} s^{-1}, while maintaining air CO_2 partial pressure at 1,000 μbar combined with [O_2] of 1.2% in which photorespiration is inhibited. All these measurements were done at the same position in sugarcane leaves. Measurements under low [O_2] were done with a mixture of gas containing 1.2% O_2 and 98.8% N_2 (White Martins Praxair Inc., São Paulo SP, Brazil), which was humidified and supplied through the air inlet of the LI-6400XT. We corrected all leaf gas exchange measurements for any CO_2 leakage by using the method of heat-killed leaves proposed by Flexas *et al.* (2007).

All measurements were taken under low coefficient of variation and high stability over time, with chlorophyll fluorescence being measured after the leaf gas exchange reached steady-state. The steady-state fluorescence (F') was recorded and then the maximum fluorescence (F_m') signal was measured after a light saturation pulse (~8,000 μmol m^{-2} s^{-1}, 0.6 s). With those signals, the apparent operating quantum efficiency of photosystem II (ϕ_{PSII}) was calculated according to Baker (2008).

Leaf nitrogen content

Total leaf nitrogen concentration was determined in lyophilized leaves after digestion with sulphuric acid by the colorimetric method of Baethgen and Alley (1989).

Western blotting of Rubisco and PEPC

Fresh leaf samples were ground until obtaining a fine powder in presence of liquid nitrogen and 100 mM K-phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 14,000 *g* for 30 min, the supernatant was collected and used as crude extract. All extraction stages were carried out at low temperature (0 to 4 ºC) and the protein amount in the enzymatic extract was determined following Bradford (1976). SDS-PAGE electrophoresis was performed with equal amounts of protein (20 µg) per lane. Soluble proteins were denatured using 2% SDS and they were electrophoretically transferred to a nitrocellulose membrane (Towbin *et al.*, 1979). PEPC and Rubisco protein abundances were measured by detection of PEPC subunit and Rubisco large subunit (RLS) using specific polyclonal antibodies (Agrisera Co, Sweden) according to the manufacturer's instructions. The proportion of RLS and PEPCase subunit were detected using peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham PLC, Little Chalfont, UK) and the abundance was calculated using Image Studio Lite version 5.2.5 (LI-COR Biosciences).

Carbohydrate metabolism

All reagents (chemicals and enzymes) used in this study were purchased from Sigma-Aldrich Co. Sucrose was extracted by a MCW solution (methanol: chloroform: water 12:5:1 v/v/v) and quantified according to van Handel (1968). Starch was extracted and hydrolyzed from the MCW pellet with $HClO_4$ (30%, v/v) and measured by the phenol-sulphuric acid method (Dubois et al., 1956). Glucose and fructose were quantified through an enzymatic method coupled to NADH production at 340 nm (Sigma's test kits, Sigma-Aldrich).

The same crude extract mentioned previously for photosynthetic enzymes was used to evaluate the activities of enzymes involved in carbohydrate metabolism. The activities of

soluble acid invertases (SAI, EC 3.2.1.26) and neutral invertases (NI, EC 3.2.1.26) were measured according to Zhu *et al.* (1997), in presence of 120 mM sucrose at 37 °C for 30 min. For SAI activity the reaction was stopped by adding 2.5 M Tris and incubating at 90 °C for 2 min. NI assay reaction was also stopped by incubating at 90 °C for 2 min. For both SAI and NI assays, the reducing sugars released were enzymatically measured (Sigma's test kits, Sigma-Aldrich). Sucrose synthase (SuSy, EC 2.4.1.13) and sucrose-P synthase (SPS, EC 2.4.1.14) activities were evaluated according to Hubbard *et al.* (1989), with modifications suggested by Zhu *et al.* (1997). SuSy can either synthetize or hydrolyze sucrose and its activity was measured towards sucrose synthesis. To determine the SuSy activity, the crude extracts were incubated with 50 mM HEPES buffer (pH 7.5) containing 15 mM $MgCl_2$, 25 mM fructose and 25 mM UDP-glucose. For the SPS activity, crude extracts were incubated in 100 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 4 mM fructose 6-phosphate, 20 mM glucose 6-phosphate, 3 mM UDP-glucose and 1 mM EDTA. Both reactions were incubated at 37 °C for 60 min and then stopped by boiling for 3 min. Sucrose produced by these reactions was assayed according to van Handel (1968).

Data analyses

Experiments were arranged in random blocks and the sources of variation were defined by the treatments of source-sink perturbation. Data was subjected to analysis of variance and the mean values (n=3) compared by the Tukey test when statistical significance was detected ($p<0.05$).

Mitochondrial day respiration (R_d) was estimated as the intercept of the linear regression of A against $I_{inc}*\phi_{PSII}/3$ under limiting light conditions (Yin *et al.*, 2011). The same linear regression also including data obtained under low $[O_2]_{air}$ and high $[CO_2]_{air}$ from A-C_i curves was used to estimate the calibration factor s' (see Supplementary Fig. S1). This calibration using data under non-photorespiratory conditions is considered necessary because ϕ_{PSII} from fluorescence measurements is an apparent PSII efficiency. The obtained factor s' was used to calculate the rate of ATP production driven by electron transport: $J_{atp}=s'I_{inc}\phi_{PSII}/(1-x)$ (Yin *et al.*, 2011), in which x is the fraction of ATP allocated to the C_4 cycle and equal to 0.4 (von Caemmerer and Furbank, 1999).

The calculated J_{atp} was used as input to the C_4 model as described by Yin *et al*. (2011) for fitting all photosynthesis measurements. This resulted in an *in vivo* estimation of Rubisco carboxylation capacity (V_{cmax}). Probably because J_{atp} was used as input, most points of measured A were shown being electron transport limited; as a result, we were not able to estimate the PEPC carboxylation capacity (V_{pmax}) with the model. Then, the initial slope of the A-C_i curve (k) until a C_i of 100 μbar was calculated, as it is related to V_{pmax} (von Caemmerer and Furbank, 1999). For all model fitting, we used the least-squares non-linear regression in SAS Software (SAS Institute Inc., Cary NC, USA).

Results

Photosynthesis under source-sink perturbation

Exogenous sucrose spraying reduced leaf CO_2 assimilation (A_{380}) in both sugarcane varieties and there was no significant response to partial darkness treatment (Fig. 1A). Interestingly, plants showed A_{380} similar to reference conditions when partial darkness and sucrose spraying were combined (Fig. 1A). Stomatal conductance (g_S) followed a similar pattern of response as compared to A_{380}, with the lowest values found in plants sprayed with sucrose solution. The combination of partial darkness and exogenous sucrose supplying induced g_S values similar to the ones found in reference plants of both varieties (Fig. 1B).

In general, s' and R_d were not significantly affected by source-sink perturbation in both varieties (Fig. 2). However, IACSP94-2094 showed higher R_d than IACSP95-5000, regardless the source-sink conditions (Fig. 2B). While J_{atp} was not changed by darkness in IACSP94-2094, stimulation of J_{atp} by darkness was found in IACSP95-5000 (Fig. 2C). The imposition of darkness alone or combined with exogenous sucrose caused the highest J_{atp} in IACSP95-5000 (Fig. 2C). In IACSP94-2094, reference plants and those subjected to darkness combined with sucrose supplying presented similar J_{atp} values (Fig. 2C).

The responses of leaf CO_2 assimilation and quantum efficiency of PSII to intercellular CO_2 concentration (A-C_i) evaluated under low and high air O_2 concentration are shown in Supplementary Figs. S2 and S3. By using the Yin *et al.* (2011) model, we were able to estimate the Rubisco carboxylation capacity (V_{cmax}), with good agreement between measured and predicted leaf CO_2 assimilation for both sugarcane varieties (Supplementary Fig. S4).

Plants sprayed with sucrose solution showed decreases in *k*, regardless the sugarcane variety (Fig. 3A). The combination of partial darkness and exogenous sucrose supplying caused increases in *k* when compared with sucrose-sprayed plants. However, the value of *k* in IACSP94-2094 after combining partial darkness and exogenous sucrose did not reach the values found in reference plants. IACSP94-2094 also showed decreases in *k* after imposing partial darkness, being more sensitive to source-sink perturbation than IACSP95-5000. Spraying with sucrose reduced V_{cmax} and the combination of partial darkness and sucrose spraying caused full recovery of V_{cmax} in both varieties (Fig. 3B). Herein, we used the term recovery when the combination of partial darkness and sucrose supplying resulted in values of a given trait similar to those found in reference plants.

Immunoblots of PEPC and Rubisco proteins revealed that the amount of PEPC increased under both partial darkness and combination of partial darkness with sucrose spraying in IACSP95-5000 (Fig. 4). Exogenous sucrose decreased the abundance of PEPC only in IACSP95-5000. This variety also showed increases in Rubisco abundance when partial darkness was imposed alone or in combination with sucrose spraying. On the other hand, IACSP94-2094 did not show any change in PEPC abundance after source-sink perturbation (Fig. 4). Decreases in Rubisco abundance were found when IACSP94-2094 was subjected to the combination of partial darkness and exogenous sucrose (Fig. 4).

Leaf nitrogen concentration

Leaf N concentration was significantly changed by partial darkness, with sugarcane varieties showing increases of 1.4 (in IACSP94-2094) and 1.6 times (in IACSP95-5000) in relation to the reference conditions (Fig. 5). While spraying of sucrose solution alone or in combination with partial darkness did not change leaf N concentration in IACSP95-5000, it significantly reduced leaf N concentration in IACSP94-2094 (Fig. 5). Correlations between leaf N concentration on area basis and other photosynthetic variables were studied and significant correlations were found only for *s*' (R^2=0.97, p=0.011) and R_d (R^2=0.96, p=0.013) in IACSP95-5000 (data not shown).

Leaf carbohydrate concentrations and sucrose metabolism

Source-sink perturbation did not change leaf sucrose concentration in both sugarcane varieties (Fig. 6A). However, both varieties showed reductions in leaf starch content when subjected to the combination of partial darkening and sucrose spraying, with IACSP95-5000 being more sensitive than IACSP94-2094 (Fig. 6B). Both leaf glucose and fructose concentrations were not changed by source-sink perturbation in IACSP94-2094 (Fig. 6C, D). On the other hand, IACSP95-5000 showed the highest glucose and fructose levels when sprayed with the sucrose solution (Fig. 6C, D).

Regarding the enzymes of sucrose metabolism, non-significant changes were found for activities of both neutral invertases (NI) and soluble acid invertases (SAI) in both sugarcane varieties (Table 1). Regardless the sugarcane variety the activity of sucrose synthase (SuSy) was increased by spraying with the sucrose solution (Table 1). While IACSP94-2094 did not show any significant change in the activity of sucrose-P synthase (SPS) due to source-sink perturbation, IACSP95-5000 tended to show higher SPS activity when sprayed with the sucrose solution (Table 1).

Discussion

The underlying mechanisms leading to the inhibition of sugarcane photosynthesis by exogenous sucrose supplying

Our results revealed that sugarcane photosynthesis was inhibited by spraying with a sucrose solution through reductions in both PEPC and Rubisco carboxylation capacities as well as in stomatal aperture (Figs. 1 and 3). As an additional limitation to photosynthesis, IACSP94-2094 showed a reduced photochemical activity, as indicated by decreases in ATP production (Fig. 2C). While inhibitory effects of sucrose on *in vitro* Rubisco activity and electron transport rate were already described by Lobo *et al.* (2015), our data also revealed that both *in vivo* Rubisco and PEPC carboxylation rates were impaired by exogenous sucrose. Such reductions in the activities of key photosynthetic enzymes were likely caused by decreases in PEPC and Rubisco abundances in IACSP95-5000, as indicated by the immunoblots (Fig. 4). For IACSP94-2094, reduction in leaf N content would be another component leading to the impairment of photosynthesis after sucrose spraying (Fig. 5). Although most leaf N is invested in photosynthetic proteins such as PEPC and Rubisco (Lawlor, 1987), there were non-

significant changes in the amount of both enzymes in IACSP94-2094 (Fig. 4). Our data revealed that the activity rather than the abundance of those enzymes were affected by source-sink perturbation in IACSP94-2094. In fact, both Rubisco and PEPC are highly modulated at post-translational level by several mechanisms and these regulatory processes are directly involved in *in vivo* enzymatic activities (Lobo *et al*., 2015).

As plants used herein had no culms accumulating sucrose, we can ascribe the sucrose-induced inhibition of photosynthesis to changes in the physiological state of leaf tissues. As suggested by Lobo *et al.* (2015), such reductions in sugarcane photosynthesis would be a consequence of reduction in Rubisco gene expression in IACSP95-5000 as well as of post-translational regulation of both PEPC and Rubisco in both varieties. Besides changes in activation state (Ludwig, 2013), enzymatic inhibition induced by metabolites can occur. For instance, accumulation of malate, aspartate and glutamate is able to down-regulate PEPC activity (Izui *et al.*, 2004; O'Leary *et al.*, 2011). Although there are several reports on the repression of photosynthetic genes induced by sugars (McCormick *et al.*, 2008b; Aranjuelo *et al.*, 2009), the molecular basis of such phenomenon are not yet completely understood. Degradation of both enzymes due to exogenous sucrose cannot be ruled out in IACSP95-5000, as suggested by decreases in Rubisco and PEPC abundances (Fig. 4).

Interestingly, stomata seem to mediate the inhibitory effect of sucrose on photosynthesis through limiting CO_2 availability at carboxylation sites, a response found in both sugarcane varieties (Fig. 1B). In previous studies in which hexoses were responsible for impairing sugarcane photosynthesis, the role of stomata in photosynthesis regulation was not relevant (McCormick *et al.*, 2006; 2008a, b). As reported previously, sucrose can reduce the osmotic potential of guard-cell apoplast through its accumulation, which may lead to reductions in stomatal aperture (Talbott and Zeiger, 1998; Kang *et al.*, 2007). As guard cells were not evaluated herein, we were not able to conclude about any accumulation of sucrose in leaf epidermis. However, we should consider this possible effect of sucrose spraying on sugarcane stomatal apparatus as accumulation of this sugar in guard cells is currently a hypothesis for stomatal aperture control (Daloso *et al*., 2016).

Considering both varieties, significant correlations between A_{380} *vs.* k (R^2=0.60, p=0.024), A_{380} *vs.* V_{cmax} (R^2=0.78, p=0.001), A_{380} *vs.* g_s (R^2=0.87, p<0.001), and A_{380} *vs.* J_{atp} (R^2=0.80, p=0.003) were found (data not shown). Therefore, we surmise that sucrose spraying

impaired sugarcane photosynthesis through stomatal (Fig. 1B), photochemical (Fig. 2C) and biochemical (Figs. 3 and 4) limitations.

Sucrose metabolism as affected by source-sink perturbation

Sucrose content in leaves of both sugarcane varieties was not affected by source-sink perturbation (Fig. 6A), even with plants sprayed with sucrose solution showing inhibition of photosynthesis (Fig. 1A). In principle, we would expect increases in leaf sucrose concentration and/or changes in glucose and fructose concentrations due to sucrose degradation when plants were sprayed with the sucrose solution. However, significant increases in leaf concentration of reducing sugars such as glucose and fructose were found only in IACSP95-5000 (Fig. 6C, D). This response seems to be a result of sucrose cleavage by increases in SuSy activity and such assumption is also supported by co-occurrence of high hexoses levels and no significant changes in NI and SAI activities in plants sprayed with sucrose solution (Fig. 6C, D and Table 1). On the other hand, increases in leaf content of reducing sugars likely caused a slight stimulation of SPS activity (Zhu *et al.*, 1997), with this response being genotype-dependent (Fig. 6C, D and Table 1).

As sucrose is rapidly metabolized in sugarcane leaves (Rolland *et al.*, 2006; McCormick *et al.*, 2008a, b), we may argue that the leaf pool of soluble sugars is maintained relatively constant due to photo-assimilate exportation with partial darkness combined or not with sucrose spraying. The large reduction in leaf starch content when plants were subjected to partial darkness combined with spraying sucrose solution (Fig. 6B) indicates a significant reduction in carbon flux (Du and Nose, 2002). As product of starch degradation, a build-up of soluble sugars in leaves of both varieties would be expected. However, such response was not found and this is another evidence towards the increased exportation of photo-assimilates for supplying the energetic demand of tissues in darkness. According to Du *et al.* (2000), sugarcane plants are able to export more than 80% of recently fixed carbon during the day, revealing a fascinating ability in exporting photo-assimilates from source to sink organs.

Starch concentration in leaves of plants subjected to partial darkness and spraying with sucrose solution was lower than in plants subjected to partial darkness alone (Fig. 6B). As changes in leaf metabolism were not evaluated during source-sink perturbation, we can only suggest that starch was degraded to support the energetic demand of darkened shoots when

photosynthesis was repressed by spraying with the sucrose solution during the first days of treatments. In fact, our data clearly show that photosynthesis has attained similar levels in both reference plants and darkened/sucrose-supplied ones; however, we cannot discard a possible photosynthetic impairment during the spraying with the sucrose solution. As compared to the reference plants, the partial recovery of *k* is evidence of such photosynthetic inhibition in plants subjected to partial darkness and spraying with the sucrose solution (Fig. 2A).

In general, variation in leaf concentrations of non-structural carbohydrates such as sucrose, glucose, fructose and starch induced by changes in source-sink relationships did not have a clear correlation with sugarcane photosynthesis (Figs. 1A and 6). Although we found evidence of changes in overall carbon flux as revealed by decreases in starch concentration (Fig. 6B) and sucrose synthesis (Table 1), punctual evaluations of sucrose metabolism did not uncover the complexity of source-sink relationship in plants. Most likely, such regulation of photosynthesis by sugars is based on carbohydrate dynamics rather than on carbohydrate concentration itself. In other words, the influence of daily variation in leaf sugar concentration determined by exportation/consumption of photo-assimilates would be more correlated to the photosynthetic regulation than absolute sugar concentration (Ribeiro *et al.*, 2012). For instance, transient changes in inorganic phosphate and triose-P may affect photosynthesis through regulation of the Calvin cycle enzymes (Paul and Foyer, 2001). Accordingly, Paul and Pellny (2003) suggested that sugars derived from hexose cycling would be more important in regulating the expression of photosynthetic genes than sugar concentration itself.

Balance between stimulation and inhibition of sugarcane photosynthesis: the relative importance of sink demand

We were able to perturb the source-sink relationships and then photosynthesis in both sugarcane varieties (Fig. 1A). Our idea with partial darkness was to increase the demand for photo-assimilates by increasing respiration of the entire plant canopy, with exception of leaf +1 used for photosynthetic measurements. Based on previous reports (McCormick *et al.*, 2006; 2008a, b), we would expect a stimulatory effect of increased sink demand on photosynthesis of plants subjected to partial darkness. However, significant effects were found only when partial darkness was combined with spraying sucrose solution (Fig. 1A). In principle, our data suggest that sugarcane varieties should be in a permissive physiological state for reacting to increases in sink strength. In our case, responsiveness to increased sink demand was found only when

sugarcane photosynthesis was limited by spraying a sucrose solution (Fig. 1A). Previous studies on source-sink relationships of sugarcane have tested the inhibition of photosynthesis caused by hexoses or its stimulation due to shading or defoliation (McCormick *et al.*, 2006; 2008a, b, c). In those studies, photosynthetic rates in control plants were low (13 to 25 μmol m^{-2} s^{-1}) for a C_4 species like sugarcane that reaches maximum photosynthetic rates close to 60 μmol m^{-2} s^{-1} under field conditions (Magalhães Filho, 2014), which suggests some kind of limitation to photosynthesis or that this process was not maximized.

Herein, the photosynthetic rates of reference plants were relatively high and varied between 35 and 40 μmol m^{-2} s^{-1} (Fig. 1A). When partial darkness was imposed to such plants for increasing sink strength, we did not find any significant stimulation of photosynthesis. However, increase in sink strength was able to null the inhibition of photosynthesis caused by spraying with the sucrose solution in both sugarcane varieties (Fig. 1A). Taking into account the physiological bases of such response, our data revealed that partial darkness and then increased sink demand has reverted the inhibitory effect of sucrose spraying on photosynthesis, which was dependent on stomatal aperture (Fig. 1B), partial recovery of PEPC activity and full recovery of Rubisco carboxylation capacity (Fig. 3). Increases in PEPC carboxylation capacity (given by *k*) were associated with increases in PEPC abundance in IACSP95-5000 (Fig. 4), with the combination of partial darkness and sucrose spraying inducing increases in the amount of this key photosynthetic protein.

Another interesting aspect of our study is the analysis of leaf N content, an important variable not evaluated in other studies of source-sink relationships (McCormick *et al.*, 2006; 2008a, b, c; Lobo *et al.*, 2015). The increase in leaf N concentration in plants subjected to partial darkness (Fig. 5) is a consequence of N translocation from darkened tissues to the illuminated leaf, with both varieties showing this response. Considering the immunoblots of photosynthetic enzymes (Fig. 4), we would argue that N was driven to the synthesis of PEPC and Rubisco in IACSP95-5000 subjected to partial darkness alone. However, such investment did not have any significant effect on leaf CO_2 assimilation (Fig. 1A) and was not found in IACSP94-2094 (Fig. 4). Indeed, Rubisco and PEPC represent important N sources in leaves, mainly under periods of reduced growth. In IACSP94-2094, N was likely invested in other proteins or amino acids not directly related to photosynthesis, representing a conservative strategy to preserve N in active photosynthetic leaves when plants were subjected to partial darkness. Interestingly, such N translocation was inhibited when partially darkened plants were sprayed with the sucrose solution (Fig. 5). Further studies on the interaction between sucrose and nitrogen metabolisms

would reveal how source-sink imbalance changes N allocation and also the importance of such response for sugarcane photosynthesis.

Regarding the sensitivity to the source-sink perturbation among genotypes, we studied herein two sugarcane varieties with differential culm production under field conditions (Silva *et al.*, 2016). While the high-yielding genotype IACSP95-5000 uses energy and carbon skeletons to produce more biomass, IACSP94-2094 is known as a drought tolerant genotype with lower biomass production than IACSP95-5000 (Ribeiro *et al*., 2013; Silva *et al.*, 2016). Herein, IACSP94-2094 was negatively affected by partial darkness, while IACSP95-5000 tended to increase PEPC and Rubisco activities under the same conditions (Fig. 3). When partial darkness and spraying with sucrose solution were combined, only IACSP95-5000 showed recovery of PEPC activity (Fig. 3A). Such differential sensitivity to source-sink perturbation was also found in leaf carbohydrates, with IACSP95-5000 showing increases in glucose and fructose concentrations when the sucrose solution was sprayed and decreases in starch concentration when partial darkness was combined with spraying the sucrose solution (Fig. 6). Enzymes involved in sucrose synthesis were also responsible to spraying the sucrose solution in IACSP95-5000 (Table 1).

Taken together, our data suggest that a high-yielding variety was more sensitive to source-sink manipulation than a less-productive one. In other words, a given variation in leaf carbohydrate concentration will have a greater impact on carbon metabolism in those varieties with higher demand for photo-assimilates. Actually, Singels *et al.* (2005) and Inman-Bamber *et al.* (2008) reported that any perturbation in source-sink relationship has a higher effect when there is high sucrose accumulation. Then, one may argue that genotypes with distinct resource allocation as the two studied herein would be affected differentially by source-sink perturbation. Despite the differential sensitivity of sucrose metabolism (Fig. 6; Table 1) and abundance of key photosynthetic enzymes between genotypes (Fig. 4), overall photosynthetic responses to source-sink perturbation was similar in both genotypes (Figs. 1 to 3). Based on these findings, we may assume a general pattern of response to increasing sink demand by partial darkness and to sucrose-induced inhibition in sugarcane, as discussed previously. Now, we have made one step more towards the understanding of (i) the physiological basis of the sensitivity to source-sink imbalance in sugarcane, and (ii) the endogenous regulation of photosynthesis in field-grown plants, where temperature and water deficit also affect the source-sink relationship (Inman-Bamber *et al.*, 2010).

Conclusions

The inhibitory effect of exogenous sucrose on sugarcane photosynthesis is mediated by inhibition of stomatal conductance and impairments of *in vivo* Rubisco and PEPC activities, and not only reduced Rubisco activity as previously known. This finding reinforces the coupling between C_3 and C_4 cycles, which should be well-orchestrated to improve CO_2 uptake under limiting conditions. In addition, we have revealed that increases in sink demand exert more influence on sugarcane photosynthesis than spraying with a sucrose solution. As result, the inhibitory effect of sucrose on photosynthesis was offset by partial darkness in sugarcane genotypes.

Supplementary data

Figure S1. Leaf CO_2 assimilation (A) as a function of $I_{inc}\phi_{PSII}/3$ in sugarcane genotypes subjected to source-sink manipulation.

Figure S2. Measured and modelled responses of leaf CO_2 assimilation (A) to increasing intercellular CO_2 concentration (C_i) in sugarcane genotypes subjected to source-sink manipulation.

Figure S3. Responses of the quantum efficiency of PSII (ϕ_{PSII}) to increasing intercellular CO_2 concentration (C_i) in sugarcane genotypes subjected to source-sink manipulation.

Figure S4. Correlation between measured (A) and modelled (Yp) leaf CO_2 assimilation in sugarcane genotypes subjected to source-sink manipulation.

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Table 1. Leaf sucrose metabolism as affected by source-sink manipulation: neutral invertase (NI), soluble acid invertase (SAI), sucrose synthase (SuSy) and sucrose-P synthase (SPS). Reference plants were sprayed with water and not subjected to partial darkness (Ref); plants were sprayed with water and subjected to partial darkness (Dark); plants were sprayed with sucrose solution and not subjected to partial darkness (Suc); or plants were subjected to partial darkness and sprayed with sucrose solution (D+S).

Activities	IACSP94-2094				IACSP95-5000			
(μmol g^{-1} h^{-1})	Ref	Dark	Suc	D+S	Ref	Dark	Suc	D+S
NI	5.4±2.7 a	5.8±3.9 a	5.2±0.7 a	6.8±2.1 a	4.6±0.7 a	4.7±0.1 a	3.7±0.9 a	2.6±0.7 a
SAI	2.1±0.7 a	3.0±1.2 a	2.9±1.8 a	3.6±1.3 a	3.8±1.2 a	3.5±0.9 a	2.9±0.4 a	3.0±1.8 a
SuSy	23.9±8.7 ab	18.8±7.5 b	37.2±6.9 a	25.2±10.4 ab	20.5±6.3 b	21.6±5.5 b	33.4±4.9 a	14.6±2.2 b
SPS	29.3±11.7 a	24.7±7.4 a	36.6±4.1 a	28.1±10.5 a	30.8±14.1 ab	20.0±7.8 b	41.4±4.3 a	26.3±5.8 ab

Different letters in the same row for a given genotype mean statistical difference (*t*-test, $p<0.05$) among treatments within the genotype. Mean values ± SD (*n*=3).

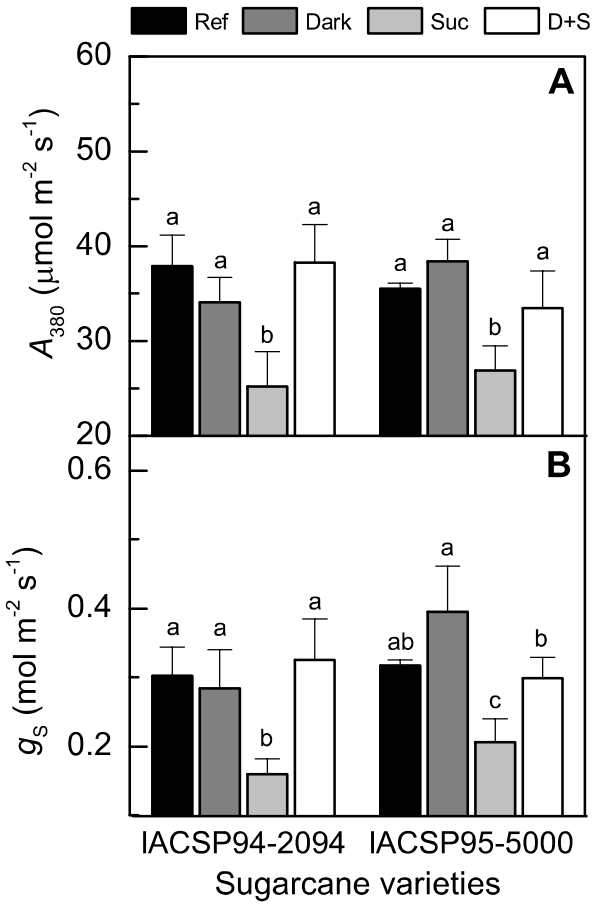

Figure 1. Leaf CO_2 assimilation (A_{380}, in A) and stomatal conductance (g_S, in B) of two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Different letters mean statistical difference (*t*-test, $p<0.05$) among treatments within a variety. Mean values ± SD (n=3). Measurements taken under an I_{inc} of 2,000 µmol m^{-2} s^{-1} and a $[CO_2]_{air}$ of 380 µbar.

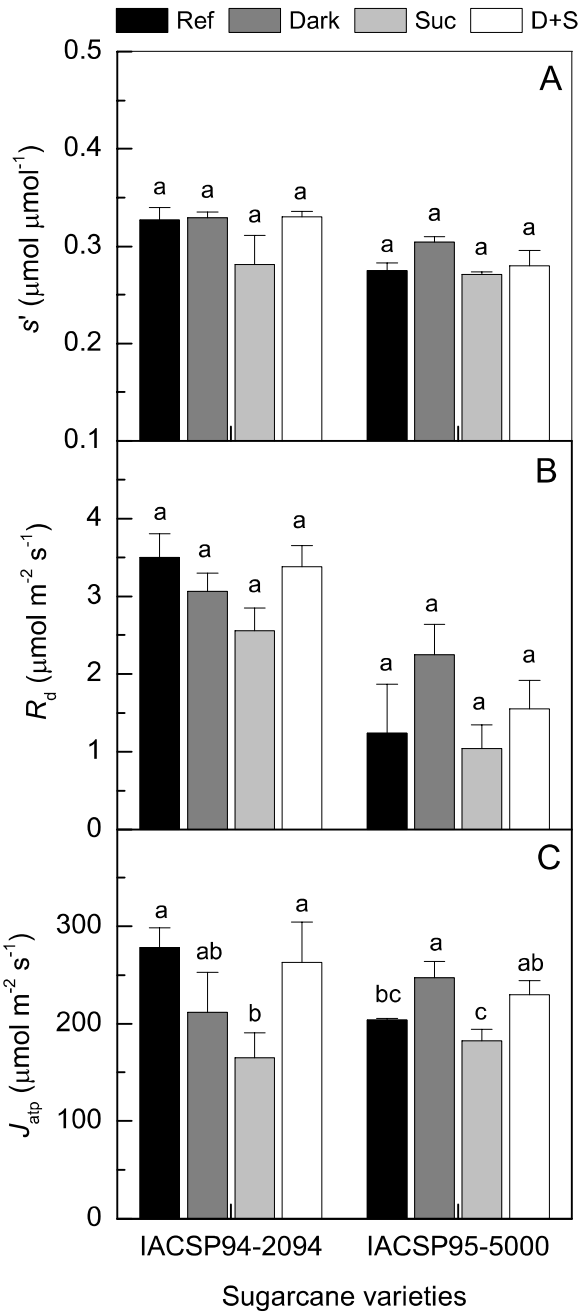

Figure 2. Calibration factor for converting electron flux to ATP flux (*s*', in A), day respiration (R_d, in B) and ATP production rate driven by electron transport (J_{atp}, in C) of two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Different letters mean statistical difference (*t*-test, $p<0.05$) among treatments within a variety. Mean values ± SD (n=3).

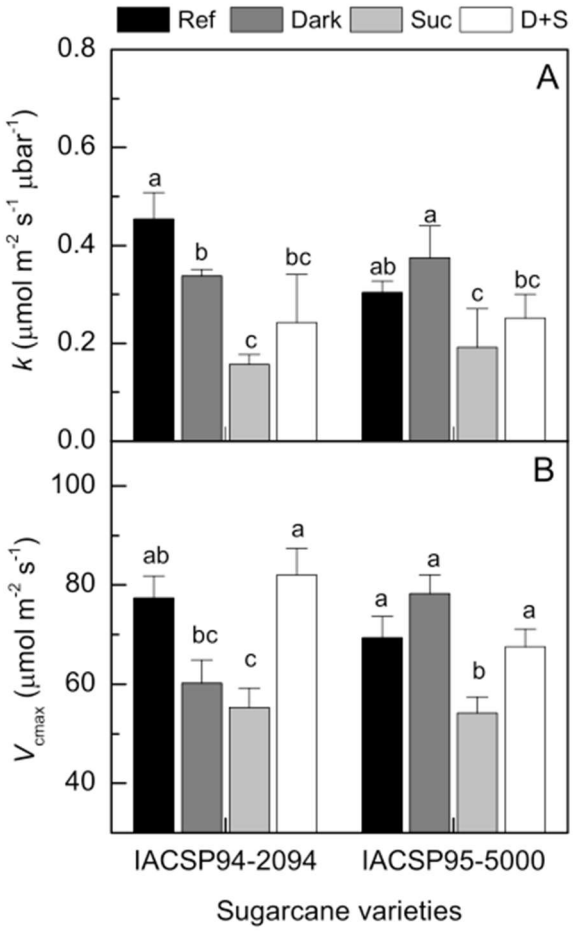

Figure 3. Initial slope of A-C_i curve (k, in A) and Rubisco carboxylation capacity (V_{cmax}, in B) of two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Different letters mean statistical difference (t-test, $p<0.05$) among treatments within a variety. Mean values ± SD (n=3). Measurements taken under an I_{inc} of 2,000 µmol m^{-2} s^{-1}.

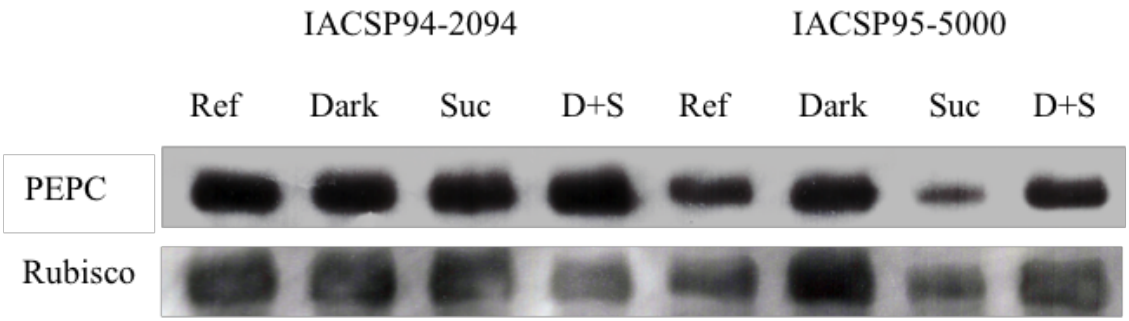

Figure 4. Immunoblots of total leaf proteins probed with antisera raised against PEPC and Rubisco in two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Representative of four runs.

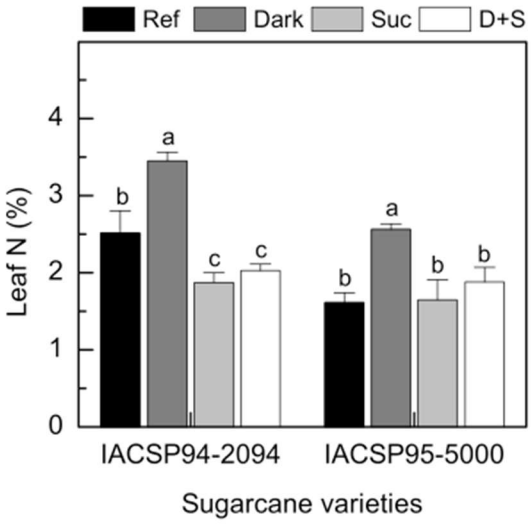

Figure 5. Leaf nitrogen concentration in two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Different letters mean statistical difference (*t*-test, $p<0.05$) among treatments within a variety. Mean values ± SD (n=3).

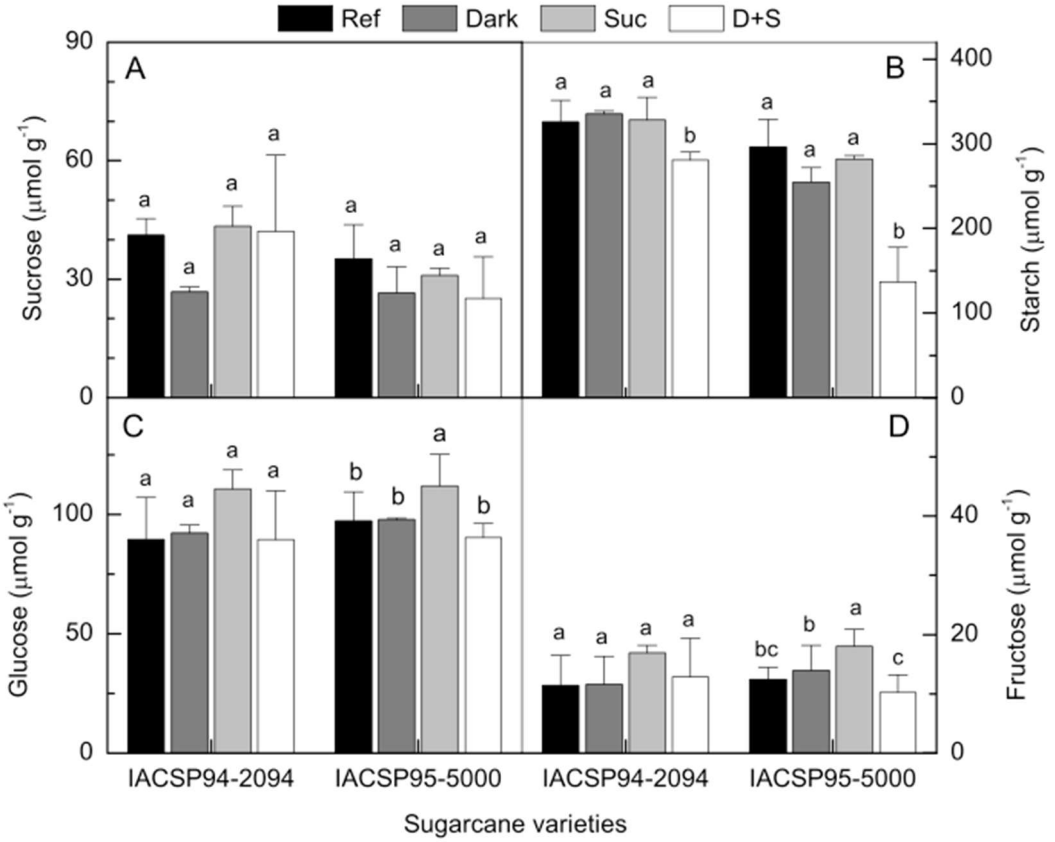

Figure 6. Leaf concentration of sucrose (A), starch (B), glucose (C) and fructose (D) in two sugarcane genotypes as affected by source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to partial darkness; Dark - plants were sprayed with water and subjected to partial darkness; Suc - plants were sprayed with sucrose solution and not subjected to partial darkness; D+S - plants were subjected to partial darkness and sprayed with sucrose solution. Different letters mean statistical difference (*t*-test, $p<0.05$) among treatments within a variety. Mean values ± SD (n=3).

Supplementary material

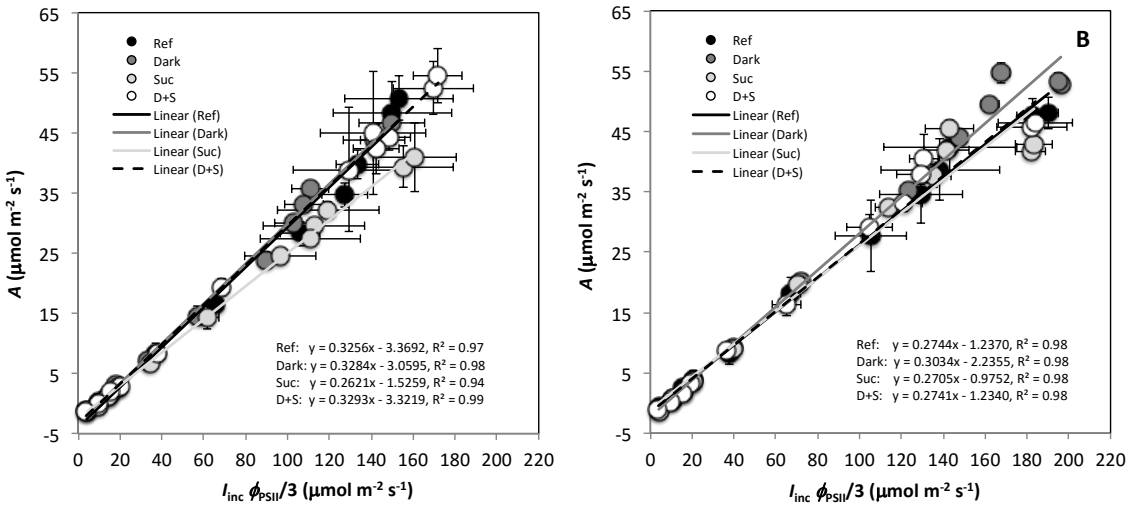

Figure S1. Leaf CO_2 assimilation (A) as a function of $I_{inc}\phi_{PSII}/3$ in two sugarcane genotypes IACSP94-0294 (A) and IACSP95-5000 (B) subjected to source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to darkness (circles and continuous line in black); Dark - plants were sprayed with water and subjected to darkness (circles and continuous line in gray); Suc - plants were sprayed with sucrose solution and not subjected to darkness (circles and continuous line in light gray); D+S - plants were subjected to partial darkness and sprayed with sucrose solution (white circles and dashed line). Measurements were taken under non-photorespiratory conditions (1.2% O_2 and 1000 µmol CO_2 mol^{-1}). Each point is the average of three replications taken in different plants ± SD.

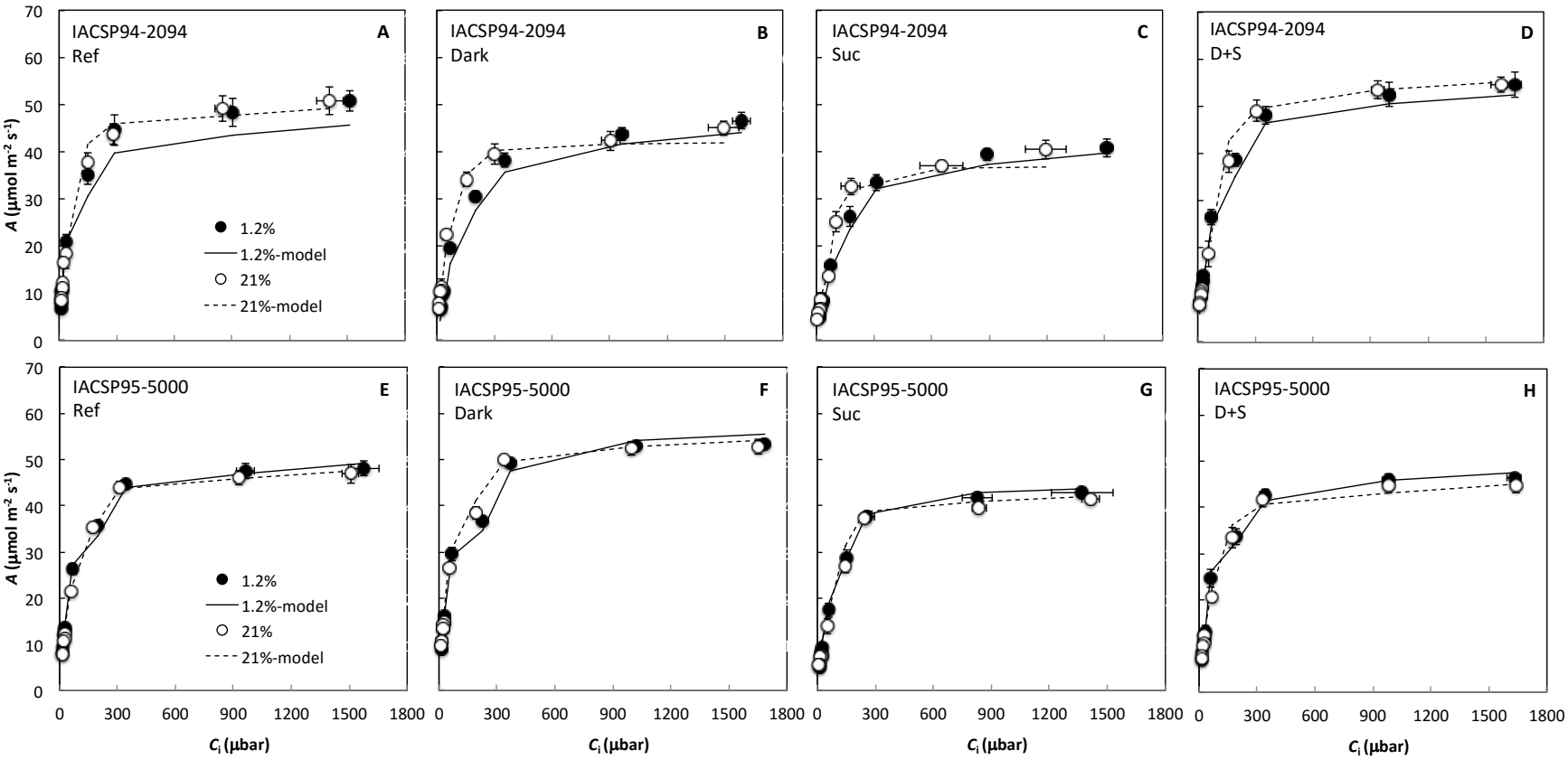

Figure S2. Measured and modelled responses of leaf CO_2 assimilation (A) to increasing intercellular CO_2 concentration (C_i) in two sugarcane genotypes subjected to source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to darkness (A, E); Dark - plants were sprayed with water and subjected to darkness (B, F); Suc - plants were sprayed with sucrose solution and not subjected to darkness (C, G); D+S - plants were subjected to partial darkness and sprayed with sucrose solution (D, H). Measurements were taken under 1.2% O_2 (closed symbols and continuous lines) or 21% O_2 (open symbols and dashed lines) under I_{inc} of 2,000 µmol m^{-2} s^{-1}. Mean values ± SD (n=3).

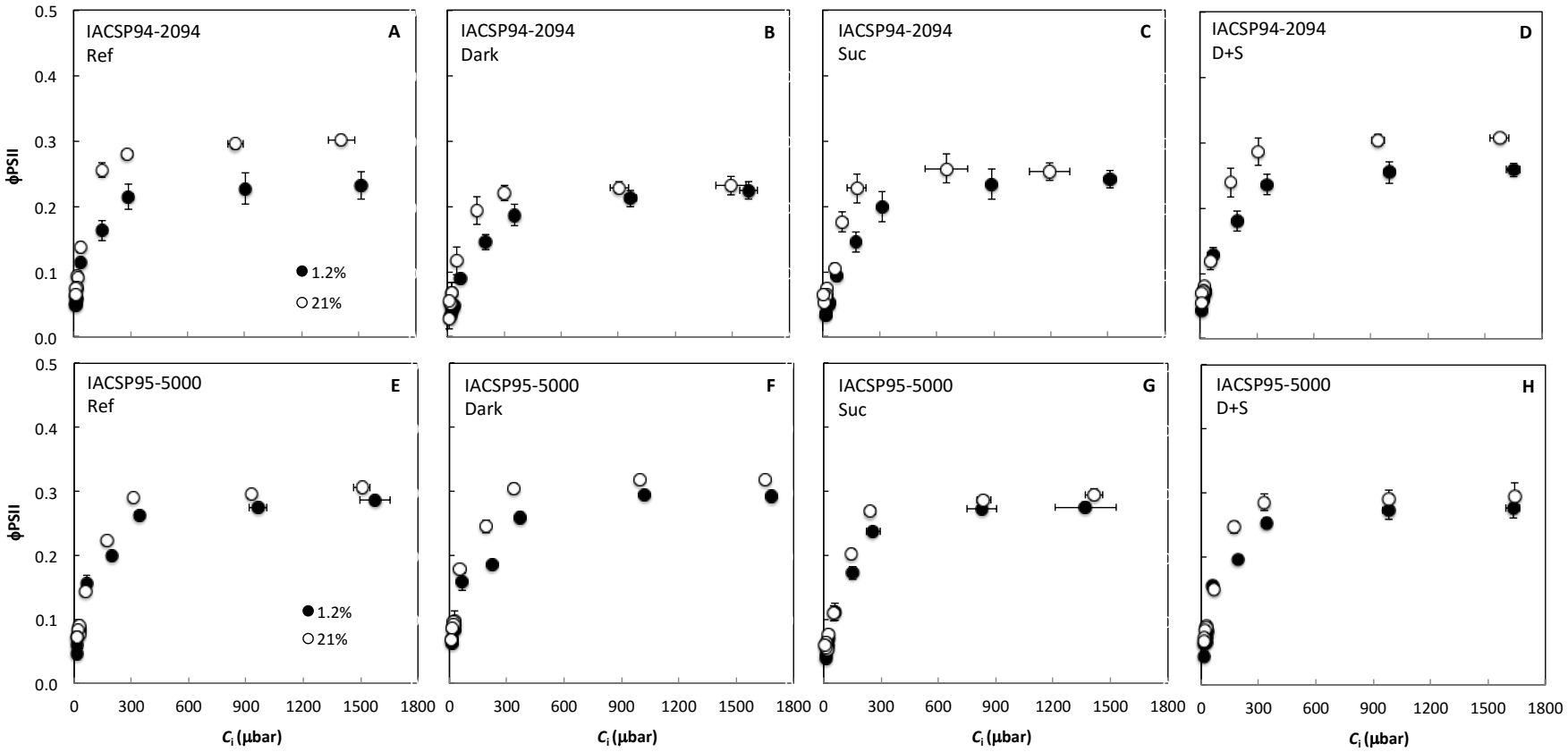

Figure S3. Responses of the quantum efficiency of PSII (ϕPSII) to increasing intercellular CO_2 concentration (C_i) in two sugarcane genotypes subjected to source-sink manipulation: Ref - reference plants were sprayed with water and not subjected to darkness (A, E); Dark - plants were sprayed with water and subjected to darkness (B, F); Suc - plants were sprayed with sucrose solution and not subjected to darkness (C, G); D+S - plants were subjected to partial darkness and sprayed with sucrose solution (D, H). Measurements were taken under 1.2% O_2 (closed symbols) or 21% O_2 (open symbols) under I_{inc} of 2,000 µmol m^{-2} s^{-1}. Mean values ± SD (n=3).

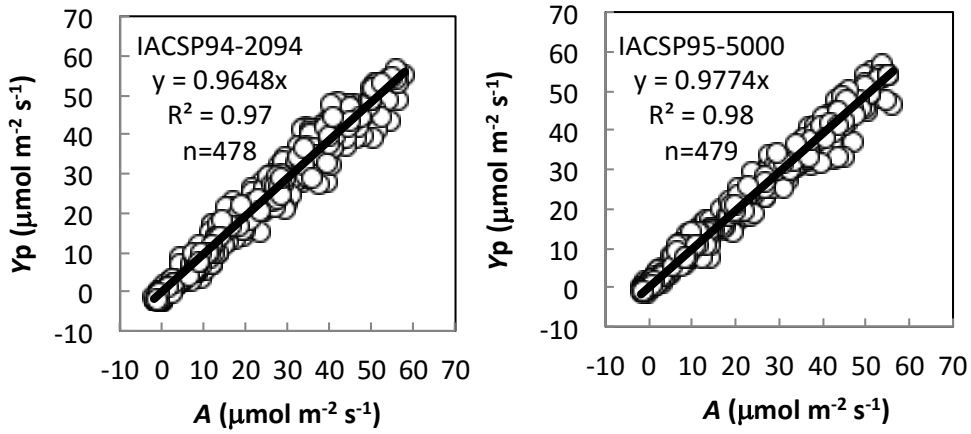

Figure S4. Correlation between measured (*A*) and modelled (*Y*p) leaf CO_2 assimilation in two sugarcane genotypes subjected to source-sink manipulation.

GENERAL CONCLUSION AND PERSPECTIVES

Sugarcane photosynthesis was severally affected by exogenous sucrose and source-sink modifications. Furthermore, the combination of exogenous sucrose and mild water deficit aggravated photosynthesis impairment, while the partial shading mitigated the negative effects of exogenous sucrose in sugarcane photosynthesis. The restrictions in CO_2 assimilation by exogenous sucrose were associated mainly to down-regulations in Rubisco initial activity and PSII efficiency in drought-stressed plants. In well-watered conditions exogenous sucrose inhibited photosynthesis by stomatal and metabolic limitations in both genotypes. The water stress sensible genotype (IACSP95-5000) seems to be more sensible to modulations by exogenous sucrose than the tolerant one (IACSP94-2094), once in IACSP95-5000 plants the abundance of Rubisco and PEPCase were decreased. In fact, these results show that drought and exogenous sucrose down-regulate photosynthesis by similar ways, with stomatal and metabolic restrictions. However, the limitations caused by exogenous sucrose are related mainly to biochemical reductions, in special Rubisco, whereas drought limits photosynthesis first by stomatal closure and after by negative modulations in C3 and C4 cycles.

Although the involvement of sugar metabolism in down-regulation of photosynthesis under drought conditions and/or exogenous sucrose application is clear, we could not identify the specific sugar and/or mechanism involved with this regulation. Therefore, more studies with whole plants associating genetic, biochemical and physiology approaches are essential to explain in a better way how drought and sugars inhibit photosynthesis. These elucidations are a promising target to increase crop yield and the tolerance of plants to drought.

APENDDIX

APPENDIX A – Published paper related to this thesis:

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Physiology

Exogenous sucrose supply changes sugar metabolism and reduces photosynthesis of sugarcane through the down-regulation of Rubisco abundance and activity

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ABSTRACT

Photosynthetic modulation by sugars has been known for many years, but the biochemical and molecular comprehension of this process is lacking. We studied how the exogenous sucrose supplied to leaves could affect sugar metabolism in leaf, sheath and stalk and inhibit photosynthesis in four-month old sugarcane plants. Exogenous sucrose 50 mM sprayed on attached leaves strongly impaired the net CO_2 assimilation (P_N) and decreased the instantaneous carboxylation efficiency (P_N/Ci), suggesting that the impairment in photosynthesis was caused by biochemical restrictions. The photosystem II activity was also affected by excess sucrose as indicated by the reduction in the apparent electron transport rate, effective quantum yield and increase in non-photochemical quenching. In leaf segments, sucrose accumulation was related to increases in the activities of soluble acid and neutral invertases, sucrose synthase and sucrose phosphate synthase, whereas the contents of fructose increased and glucose slightly decreased. Changes in the activities of sucrose hydrolyzing and synthesizing enzymes in leaf, sheath and stalk and sugar profile in intact plants were not enough to identify which sugar(s) or enzyme(s) were directly involved in photosynthesis modulation. However, exogenous sucrose was able to trigger down-regulation in the Rubisco abundance, activation state and enzymatic activity. Despite the fact that P_N/Ci had been notably decreased by sucrose, *in vitro* activity and abundance of PEPCase did not change, suggesting an *in vivo* modulation of this enzyme. The data reveal that sucrose and/or other derivative sugars in leaves inhibited sugarcane photosynthesis by down-regulation of Rubisco synthesis and activity. Our data also suggest that sugar modulation was not exerted by a feedback mechanism induced by the accumulation of sugars in immature sugarcane stalk.

ANNEX

ANNEX A – Published and submitted papers during the PhD:

Lima Neto, M.C., Lobo, A.K.M., Martins, M.O., Fontenele, A.V, Silveira, J.A.G., 2014. Dissipation of excess photosynthetic energy contributes to salinity tolerance: a comparative study of salt-tolerant *Ricinus communis* and salt-sensitive *Jatropha curcas*. J. Plant Physiol. 171, 23–30. doi:10.1016/j.jplph.2013.09.002

Silva, R.G.G., Vasconcelos, I.M., Martins, T.F., Varela, A.L.N., Souza, P.F.N., Lobo, A.K.M., Silva, F.D.A., Silveira, J.A.G., Oliveira, J.T.A., 2016. Drought increases cowpea (*Vigna unguiculata* [L.] Walp.) susceptibility to Cowpea severe mosaic virus (CPSMV) at early stage of infection. Plant Physiol. Biochem. *accepted manuscript*. doi: 10.1016/j.plaphy.2016.09.010

Paiva, A.L., Passaia, G., Jardim-Messeder, D., Lobo, A.K.M., Silveira, J.A.G.; Margis-Pinheiro, M., 2016. The mitochondrial glutathione peroxidase (*OsGPX3*) has a crucial role in rice protection against salt stress. Environ Exp Bot, *submitted manuscript*.