ZULMA CATHERINE CARDENAL RUBIO

SUGARCANE PHOTOSYNTHESIS AND ASSOCIATED LEAF TRAITS IN RESPONSE TO HIGH [CO_2] AND DROUGHT

LAVRAS-MG
2022

ZULMA CATHERINE CARDENAL RUBIO

SUGARCANE PHOTOSYNTHESIS AND ASSOCIATED LEAF TRAITS IN RESPONSE TO HIGH [CO_2] AND DROUGHT

Thesis presented to the Federal University of Lavras, as part of the requirements of the Postgraduate Program in Agronomy / Plant Physiology, area of concentration in Plant Ecophysiology, to obtain the title of Doctor

Prof. Dr. Paulo Eduardo Ribeiro Marchiori
Advisor

Prof. Dr. Rafael Vasconcelos Ribeiro
Co-Advisor

LAVRAS-MG
2022

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Cardenal-Rubio, Zulma Catherine.
Sugarcane photosynthesis and associated leaf traits in response to high [CO_2] and drought / Zulma Catherine Cardenal Rubio. - 2022.
78 p. : il.

Orientador(a): Paulo Eduardo Ribeiro Marchiori

Co-orientador (a): Rafael Vasconcellos Ribeiro

Tese (doutorado) - Universidade Federal de Lavras, 2022.
Bibliografia.

1. Cana-de-açúcar. 2. Metabolismo fotossintético C_4. 3. Mudanças climáticas. I. Ribeiro Marchiori, Paulo Eduardo. II. Vasconcellos Ribeiro, Rafael. III Título.

ZULMA CATHERINE CARDENAL RUBIO

SUGARCANE PHOTOSYNTHESIS AND ASSOCIATED LEAF TRAITS IN RESPONSE TO HIGH [CO_2] AND DROUGHT

FOTOSSÍNTESE DA CANA-DE-AÇÚCAR E CARACTERISTICAS ASSOCIADAS EM RESPOSTA À ALTA [CO_2] E À SECA

Thesis presented to the Federal University of Lavras, as part of the requirements of the Postgraduate Program in Agronomy / Plant Physiology, area of concentration in Plant Ecophysiology, to obtain the title of Doctor

APROVED on February 22, 2022
Prof. Dr. Paulo Eduardo Ribeiro Marchiori - UFLA
Dr. Eduardo Caruso Machado - IAC
Dra. Cristina Rodrigues Gabriel Sales – UC - Inglaterra
Prof. Dr Eduardo Gusmão Pereira - UFV
Prof. Dr João Paulo Rodrigues Alves Delfino Barbosa - UFLA

Prof. Dr. Paulo Eduardo Ribeiro Marchiori
Advisor

Prof. Dr. Rafael Vasconcelos Ribeiro
Co-advisor

LAVRAS-MG
2022

Dedico

A Dios que nunca soltó mi mano; a Isabellita de mi corazón con todo mi amor; a mis dos hijitos (Cris y Sergi), la alegría de mis días; a mis padres que son luz en mi camino; A mi Johis, mi alma gemela; A mi abuelito (Pastor) mi puerto de llegada; a H mi compañero de vida.

ACKNOWLEDGEMENTS

To God for never letting go of my hand, for giving me the health, wisdom, and courage to face this challenge, for giving comfort to my heart in moments of anguish. Thank you for the blessings you have bestowed and especially for the life of my daughter. To Isabellita of my heart, thank you for coming in moments of darkness to give me light, strength and meaning to my dreams, for you and for your happiness I will never give up.

To my Family for having accompanied me on this path, even from afar, my heart felt your company at all times, thank you for making me feel loved and brave, you are my safe haven always.

To my parents Luz Amanda and Hector for their unconditional love, for the teachings, for always being willing to listen to me and support my decisions, for you i am here today. To my sister Johis, for her love, patience, and for always giving me strength. To my babies (Cris and Sergi), for the smiles, for giving me happiness and filling my heart with love. To my grandfather Pastor for his patience, for waiting for me each year, gratitude for the fun conversations and for always being present in my life. To my love H, for all the support, understanding, patience and love offered, for dreaming with me and taking my hand in the pursuit of our goals.

To Antonia Almeida and Paulo Cássio, friends and brothers that i met on my path and that i will carry with me forever. I thank them for all their help and dedication in the elaboration and execution of the work done during the doctorate. They were always with me in all stages. To my family in Lavras, Mayra Alejandra, Andrea, Janet, Dayana, Viviana, Robert Karen and Dawison for the support, advice, care and company in these 4 years, thanks for the Friday dinners that eased the pandemic years, gratitude for letting me into your lives, I will carry you in my heart always (Partners 4ever). To Layane Silva, Melina Arantes, Daniel Vieira, Hélio Andrade, Joyce Alvarenga, Ludmila Bastos, and Paulo César, friends that I gained in the doctorate and that I will take with me for life.

I wish to extend my special thanks to Prof. Dr. Paulo Eduardo Ribeiro Marchiori, my advisor who became a true friend. I thank him for his orientation, teachings, dedication, for all the support, and for the trust he placed in me. All this contributed to my growth and maturation as a person and as a professional during my doctorate. Thank you very much.

I would like to thank the Prof. Dr. Rafael Vasconcelos Ribeiro my co-advisor for the time dedicated. Thank you for always being available to solve my doubts.

To everyone at the Laboratory of Biochemistry and Molecular Physiology of Plants, especially Paulo Cássio, Antonia, Paulo César, Ludmila, Marua Eduarda, Patrícia and Ketlen for their collaboration in the analyses performed.

To Prof. Dr. Guilherme Vieira Pimentel for his collaboration in obtaining the materials studied here. To Dr. Claudia Rita de Souza for the support, and loan of the IRGA Li-6800.

To Dr. Eduardo Caruso Machado, Dr. Cristina Rodrigues Gabriel Sales, Dr. Eduardo Gusmão Pereira and Prof. Dr. João Paulo Rodrigues Alves Delfino Barbosa for accepting the invitation to participate in this moment so important to me, I thank them for their contributions to the improvement of the work.

This study was carried out with the support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior -Brasil (CAPES)- Funding Code 001. Therefore, I thank CAPES for granting the scholarship to carry out the research and for the support in maintaining the PPGFV.

To the National Council for Scientific and Technological Development (CNPq, Edital Universal, Process 402950/2016-0) and the Foundation for Research Support of Minas Gerais (FAPEMIG, Process PPM 00285-18) for the research financial support.

To the Organization of American States and the Coimbra Group of Brazilian Universities (GCUB) for the scholarship conception through the program “ Alianzas para la Educación y la Capacitación Becas Brasil PAEC OEA-GCUB 2017”.

To the Federal University of Lavras, for the opportunity to participate in one of its programs, for the structure offered, as well as the faculty and administration that contributed in some way to my education.

Thank you all!

A goal is a dream with a deadline
Napoleon Hill

ABSTRACT

Expected changes in climate, such as increased atmospheric CO_2 concentration and variation in rainfall, will affect the photosynthetic metabolism of plants and, therefore, crop productivity. The increase in CO_2 can benefit plants as it increases photosynthetic rate and water use efficiency by reducing stomatal conductance. In this regard, it is widely accepted in the scientific community that the positive effects of CO_2-enriched atmospheres are more marked in plants with C_3 metabolism as a consequence of the inefficiency in the photosynthetic process, associated with the desaturation of the Rubisco enzyme in the current CO_2 levels and with photorespiration. Thus, the elevated concentration of CO_2 [CO_2] improves the efficiency of the photosynthetic process by limiting photorespiration and increasing the concentration of CO_2 near the Rubisco active site. On the other hand, C_4 plants theoretically do not respond to the increase in [CO_2], since they already have a concentration mechanism in the vascular bundle sheath cells. However, it has been reported that sugarcane plants (C_4) show increases in photosynthesis, changes in growth and increases in biomass. Therefore, it was suggested that the C_4 metabolism in these plants does not show maximum efficiency under the current CO_2 condition and that the limitation in the expression of the maximum efficiency of the photosynthetic process may be caused by failures, either in the carboxylation or decarboxylation phase.Thus, the aim was to identify the key processes of C_4 metabolism involved with the limitation in the mechanism of CO_2 concentration in sugarcane. The study was conducted in open-top chambers using two sugarcane varieties (RB867515 and RB855536). These were grown under two CO_2 environments (400 and 680 µmol CO_2 mol^{-1} of air) and exposed to water restriction in order to potentiate failures in metabolism. Biometric, anatomical, biomass partitioning and A/Ci and A/Q curves under 21% and 2% O_2 were analyzed. Overview, it was found that the variety most responsive to high CO_2 was RB855536, and it was concluded that in the current CO_2 condition the photosynthetic process of sugarcane plants may be limited by the elevated carboxylation rate of PEPC in relation to Rubisco which leads to leakage of CO_2 from the bundle sheath cells to the mesophyll cells, resulting in lower quantum carboxylation efficiency. At the same time, we indicate that plants are able to modulate their metabolism in favor of water conservation at the leaf level, since in this study, they superimposed the efficient use of water over the gain in C by eCO_2, which is a typical acclimation response under atmospheres enriched with this gas.

Key words: Photosynthetic metabolism. C_4 photosynthesis. Climate change. *Saccharum* spp.

RESUMO

As mudanças previstas no clima, como aumento da concentração de CO_2 ([CO_2]) atmosférico e variação no regime de chuvas afetarão o metabolismo fotosintético das plantas e portanto, a produtividade das culturas. O aumentodo CO_2 pode beneficiar as plantas ao estimular a fase difusiva da fotossíntese, pelo fato de aumentar a diferença no gradiente de concentração entre a folha e o ar, o que, teoricamente permite maior entrada de CO_2 no interior do mesofilo foliar; e a bioquímica, uma vez que ao fornecer maior quantidade de CO_2 para as enzimas responsáveis pela carboxilação, sua atividade seria estimulada, resultando em incrementos nas taxas fotossintéticas. Neste sentido, é amplamente aceito na comunidade científica que os efeitos positivos de atmosfera enrriquecida com CO_2 são mais marcados em plantas com metabolismo C_3 como consequência da ineficiência no processo fotossintético, associada à insaturação da enzima Rubisco e à fotorrespiração. Por outro lado, teoricamente plantas C_4 não respondem ao aumento na [CO_2], visto que já apresentam mecanismo de concentração de CO_2 nas células da bainha do feixe vascular. Contudo, tem sido relatado que plantas de cana-de-açúcar (C_4) crescidas sob alta [CO_2] apresentam aumentos em fotossíntese, crescimento e biomassa. Diante disso, estudos sugerem que o metabolismo C_4 em cana-de-açúcar não apresenta máxima eficiência sob a condição atual de [CO_2] e possivelmente por falhas nas fases de carboxilação ou de descarboxilação. Assim, no presente trabalho objetivou-se identificar os processos-chave do metabolismo C_4 envolvidos na limitação no mecanismo de concentração de CO_2 em cana-de-açúcar. O estudo foi conduzido em câmaras de topo aberto utilizando duas variedades de cana-de-açúcar (RB867515 e RB855536). As plantas foram crescidas em ambientes com duas concentrações de CO_2 (400 e 680 μmol mol^{-1}), irrigadas ou expostas a restrição hídrica. O déficit hídrico foi aplicado a fim de potencializar falhas no metabolismo fotossintético. Avaliações biométricas, anatômicas, partição de biomassa e curvas de resposta ao CO_2 e luz (A/Ci e A/Q, respetivamente) sob 21% e 2% de O_2 foram realizadas. De forma geral, a variedade mais responsiva à elevada [CO_2] foi a RB855536. Concluiu-se que na condição atual de [CO_2] o processo fotossintético de plantas de cana-de-açúcar pode estar sendo limitado pela maior velocidade de carboxilação da PEPC em relação à Rubisco o que resulta em vazamentos de CO_2 das células da bainha para as células do mesofilo, tendo como consequência menor eficiência quântica de carboxilação. Por outro lado, os resultados sugerem que as plantas são capazes de modular seu metabolismo em favor da conservação de água, uma vez que neste estudo houve aumento na eficiência do uso da água, sendo esta uma resposta típica de aclimatação sob atmosferas enriquecidas com CO_2.

Palavras-chave: Metabolismo fotossintético. Fotossíntese C_4. Mudanças climáticas. *Saccharum* spp.

LIST OF FIGURES

LIST OF TABLES

FIRST PART

SECOND PART-MANUSCRIPT

SUMMARY

FIRTS SECTION: BIBLIOGRAPHY REVIEW

1 INTRODUCTION

The yield of a crop is a function of the amount of biomass produced x harvest index (HI), the latter beingthe physiological efficiency and capacity of a crop to convert total dry matter into economic yield. Photosynthesis is the process by which solar energy is transformed into chemical energy and stored in carbon compounds. This energy is used by plants to grow and develop. Although photosynthesis converts only ~5% of the sun's energy into biomass, it is a key process in crop productivity (BUCHANAM; JONES, 2015). In this sense and considering that HI is at the theoretical limit, the study of photosynthesis should be the focus of research aimed at improving crop yields to meet the demand for food production for a rapidly growing population (ASEFA, 2019).

In this regard, research has been developed in order to obtain significant increases in photosynthesis,focusing on three optimization strategies:: (1) light capture and conversion, (2) CO_2 diffusion and supply, and (3) Calvin-Benson cycle (YAMORI, 2013; NÖLKE; SCHILLBERG, 2020; BRESTIC et al., 2021; SALES et al., 2021). Many of these studies have used biotechnology as a powerful tool to achieve increases in net CO_2 uptake and/or productivity of crops such as rice, soybean, corn, and tobacco (HUSSAIN et al., 2021). However, to ensure food security in 2050, which requires increasing food production by ~70%, studies aimed at improving the photosynthetic capacity of plants must consider future climate scenarios. This, since photosynthesis is influenced by numerous environmental factors such as temperature, water availability, quantity and quality of lightmineral nutrition and atmospheric CO_2 concentration (ALLAKHVERDIEV, 2020; BRESTIC et al., 2021; RAMAZAN et al., 2021).

During the past 50 years, carbon dioxide concentration and atmospheric temperature have increased at a rate of 2.5 μmol mol^{-1} each year and 0.2 °C per decade, respectively (WALIA et al., 2022). By the end of the century carbon dioxide concentration ($[CO_2]$) is predicted to reach 750 μmol mol^{-1} and there will be an increase in global warming of 3 to 5°C. The CO_2 increasing concentration is of particular interest because plant growth, productivity, photosynthetic capacity and other biochemical functions necessary for normal metabolic function will be affected by increasing levels of CO_2 (eCO_2) in the atmosphere (LEAKEY; LAU, 2012; BHARGAVA; MITRA, 2020). Several studies have reported on the effect of eCO_2 on photosynthesis, respiration, nutritional quality and stress responses of plants (De SOUZA et al., 2008; BHARGAVA; MITRA, 2020; DOMICIANO et al., 2020 CLEMENS et al., 2022).

Among the changes due to eCO_2, studies have reported increases in plant productivity, leading to biomass increases of more than 40% compared to plants grown in [CO_2] (DE SOUZA et al., 2008). Increases in leaf area (DE SOUZA et al., 2008), decrease in leaf nitrogen content (~15%), increases in Carbon (C) content (~6%), alterations in plant growth and phenology (early blooming, short vegetative periods) and carbohydrate accumulation in sink organs were also observed (DOMICIANO et al., 2020; BHARGAVA; MITRA, 2020). Additionally, anatomical changes such as decrease in stomatal density and increase in cell wall thickness have been reported (OKSANEN; SOBER; KARNOSKY, 2001). Regarding gas exchange, De Souza et al. (2008) found increases of about 30% in photosynthesis, while Cohen et al. (2022) observed decreased stomatal conductance, reduced transpiration rates, and increased water use efficiency (WUE). These responses to eCO_2 in plants differ according to their phenological state, genotype, and interaction with other biotic and abiotic factors (LEAKEY; LAU, 2012; DOMICIANO et al., 2020). However, in the literature it is widely accepted that plants with C_3 photosynthetic metabolism respond more strongly to CO_2 increment when compared to C_4 plants (BHARGAVA; MITRA, 2020). This can be explained by the fact that the enzyme ribulose-1,5-biphosphate carboxylase-oxygenase (Rubisco) - responsible for CO_2 fixation via ribulose-1,5-biphosphate carboxylation (RuBP) in photosynthesis - is limited by the substrate under [CO_2] ambient conditions, and also exhibits oxygenase activity, which further limits the CO_2 fixation process. Thus, increases in CO_2 concentration restrict the oxygenase activity of the enzyme and at the same time stimulate carbon assimilation rates (HUSSAIN et al., 2021).On the other hand in C_4 plants, theoretically the increase in [CO_2] does not have a significant effect, since these species already have a mechanism for concentrating CO_2 near the active site of Rubisco. Furthermore a fosfo-enol privutao carboxilase (PEPC), the enzyme responsible for the first carboxylation in C_4 plants, is insensitive to changes in pCO_2/pO_2 since PEPC uses bicarbonate (HCO_3^-) to catalyze the carboxylation of PEP (KUNDU; GOEL; ZINTA, 2022).

Some work on the topic described above carried out on C_4 plants observed an increase in CO_2 assimilation (GHANNOUM et al., 2000; COUSINS et al., 2003; De SOUZA et al., 2008; HABERMANN et al., 2020), while other studies have reported increased phytomass accumulation without increasing photosynthesis per unit area, or reduced photosynthesis (VU; ALLEN Jr, 2009; PRINS et al., 2011; SELIM et al., 2021). Thus, the responses in C_4 plants are controversial and there is still no consensus in the scientific community about the effects of eCO_2 on C_4 plants. Added to this, few research has been done with C_4 species, making it difficult to understand how C_4 plants respond to eCO_2. In this sense, in the present work we we hypothesize thatthat sugarcane plants (C_4) respond to eCO_2 possibly by the inefficiency of C_4 photosynthetic metabolism. Therefore, the aim of this work was to identify the key processes of C_4 metabolism involved in limiting the CO_2 concentration mechanism in sugarcane. For this the thesis was organized in two parts, the first in which the theoretical framework is presented, highlighting the main topics that will be addressed during the discussion of the results, and the second, in which the results of the experiment carried out to test the hypothesis mentioned here are presented.

2 LITERATURE REVIEW

2.1 Climate change

Changes in rainfall patterns and frequency and extreme weather events such as high temperatureshave become more frequent due to climate change. Many of these recorded changes are linked to increasing levels of carbon dioxide and other greenhouse gases in the atmosphere (IPCC, 2021). Global monitoring indicates that [CO_2] increased from 315 µmol mol^{-1} in 1960 to 416.8 µmol mol^{-1} in December 2021 (NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION – NOAA, 2022) and climate prediction models suggest that the increase will continue over the coming decades, with increases up to 750 µmol mol^{-1} over the next 100 years. Similarly, forecast indicate increases in global average air temperature (2 to 5 °C) and increases in droughts and fires, which will continue to affect agriculture, water systems, and ecosystems. (IPCC, 2021).

Climate change has a direct influence on the development of plants and ecosystems, since it is capable of modulating the photosynthetic capacity of plants and, as a consequence, their productivity (LEAKEY; LAU, 2012; REHMAN et al., 2021). Baslam et al. (2020) for example, reported that increases in atmospheric temperature caused yield losses of between 6 and 25 percent for different crops. The study also pointed out that drought is the abiotic stress that causes the greatest loss of agricultural productivity, and has led to an estimated loss of 1.8 billion tons of cereals over the past four decades. Regarding the responses to eCO_2, it is known that they vary depending on the species, the presence and intensity of stressors (LEAKEY; LAU, 2012), because under limiting developmentconditions the internal availability of CO_2in the plant can be reduced. Therefore, studies have been conducted with the aim of increasing the understanding of how plants detect, respond, acclimate, and adapt to environmental variations (GHANNOUM et al., 2000; WANG et al., 2012; DOMICIANO et al., 2020).

2.2 Effect of high CO_2 on plants

It is known that eCO_2 can be considered beneficial to plants since CO_2 is one of the substrates in the photosynthetic process. CO_2 diffuses through the stomata into the intercellular cavity of the leaves, proceeding to the stroma of the chloroplasts where, in the presence of light, it reacts with RuBP, in a reaction catalyzed by Rubisco. After a sequence of biochemical reactions occurs the synthesis of phosphate trioses, as well as the regeneration of RuBP in the C_3 photosynthetic cycle, the Calvin-Benson-Bassham Cycle (BASSHAM et al., 1954; LUDWIG, 2013).

Free Air CO_2 Enrichment (FACE) experiments or experiments that using Open Top Chamber (OTC) have shown that elevated atmospheric CO_2 concentration results in increased stromal CO_2 concentration and favors net CO_2 assimilation rate (*A*). Added to this effec,t has been reported improvement in water relations along with higher carbohydrate accumulation (BHARGAVA; MITRA, 2020; ZHANG et al., 2021; DOMICIANO et al., 2020). Experimental results with double the current CO_2 concentration show increases in dry biomass and yield of some crops. However, the increase varies by crop, with the average increase being 32, 42, 54 and 52% for fruits, C_3 cereals, leafy vegetables and root crops, respectively (WALTER; TELLES; STRECK, 2015). Despite the increase in photosynthetic rates observed during short-term exposure to eCO_2 in some crops, it has been observed that long-term acclimation occurs, resulting in a decrease in photosynthetic rates mainly in C_4 species (BHARGAVA; MITRA, 2020).

In this context, it has been reported that the capacity of some photosynthetic enzymes of plants grown in eCO_2 showed acclimation. In sorghum (*Sorghum bicolor* L. Moech) grown in FACE, for example, Rubisco and PEPC activities decreased under atmospheres of 600 μmol mol^{-1} (COUSINS et al., 2003). In maize (*Zea mays* L.) grown at 1100 μmol mol^{-1} and under high photosynthetically active radiation (PAR; 2000 μmol m^{-2} s^{-1}), the activities of several C_3 and C_4 cycle enzymes (Rubisco, PEPC) were negatively regulated (MAROCO et al., 1999). Sorghum plants grown in OTC's with 700 μmol mol^{-1} show a 49% reduction in leaf PEPC protein concentration, but no change in Rubisco protein content, even after eight weeks of exposure to eCO_2 (WATLING et al., 2000). Variations in photosynthetic enzyme responses suggest that among C_4 plants, species-specific differences will be found as a result of increases in [CO_2], in addition to differences in plant growth conditions.

Anatomical changes have also been reported. Under CO_2 enriched atmospheres increased leaf thickness and lacunate parenchyma were observed in some species (OKSANEN; SOBER; KARNOSKY, 2001; HABERMANN et al., 2020). Likewise, it has been observed that greater cell wall thickness improves stomatal functionality and reduces stomatal density (MASLE, 2000; LIN; JACH; CEULEMANS, 2001). Many of these changes have been used to explain the increase in *A* and WUE of plants grown under eCO_2. Thus, assessments of leaf structure in conjunction with gas exchange analyses may provide a more complete understanding of the effects of CO_2 on biophysical and biochemical aspects of stomatal function (PRITCHARD et al., 2001).

The vast majority of research of this nature is conducted with C_3 species, since it is widely accepted that C_4 species do not respond to increasing $[CO_2]$. This supposed absence of response is based on the fact that C_4 plants have a CO_2 concentration mechanism around Rubisco and by the insensitivity of PEPC to changes in pCO_2/pO_2, because this enzyme does not have oxygenase activity (GHANNOUM et al., 2000; LEAKEY; LAU, 2012). Considering the publications indexed in major databases (Scopus, Web of Science, AGRIS, Agriculture Science Database (ProQuest)) with the central theme "plants * high CO_2", only 18% of the studies conducted used C_4 species. This disparity between the amount of studies that assess the responses of C_3 or C_4 species to eCO_2 occurs for two reasons: (i) more than 90% of plant species are C_3, and (ii) there is common sense that C_4 plants do not respond to eCO_2. A meta-analysis study reviewed plant responses to increasing $[CO_2]$ in 156 publications, of which 130 corresponded to C_3 and 18 to C_4 plants. Thus, it was noted that in 44% of the cases C_3 plants (57 species) showed an increase in growth. However, only 22% (four species) of the results indicated increased biomass in C_4 species. (POORTER, 1993). Therefore, there is considerable variation among these studies depending on the group of plants used, with mainly plant species with C_3 metabolism being evaluated (POORTER, 1993; WANG et al., 2022).

2.2.1 Effect of increasing atmospheric $[CO_2]$ on C_4 plants

C_4 plants represent only ~4% of all terrestrial species, but their primary production is approximately 20% of global production (SAGE, 2004; SAGE et al., 2012). Crops such as corn, sugarcane and sorghum represent the basis for human and/or animal food and bioenergy production (GHANNOUM et al., 2000). Thus, any change in the carbon balance caused by environmental variables must be very well understood for correct assessments and forecasts of the productive potential of these species in the climate change scenario, ensuring greater food and energy security (KROMDIJK et al., 2010; KANT et al., 2012; ZHANG et al., 2021).

The C_4 photosynthetic metabolism is characterized by anatomical and biochemical changes in the leaves that allow the maintenance of high CO_2 concentrations around the catalytic site of Rubisco, close to 50 to 70 mol L^{-1}, minimizing the occurrence of photorespiration (GHANNOUM et al., 2000; VON CAEMMERER; FURBANK, 2003; SAGE, 2004). The CO_2 diffused into the mesophyll cells is converted to bicarbonate by the action of the enzyme carbonic anhydrase (BADGER, 1994). Bicarbonate and phosphoenolpyruvate (PEP) are converted to oxaloacetic acid (OAA) by the action of PEPC. OAA is converted to malate or aspartate, which diffuses from the mesophyll cells to the vascular bundle sheath (BSC) cells where it is decarboxylated by the action of decarboxylation enzymes releasing CO_2 in the proximities of Rubisco. Thereby, CO_2 is refixed in the Benson-Bassham Calvin Cycle to produce triose-phosphate (SAGE, 2004). There are three types of decarboxylation enzymes: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and PEP-carboxykinase (PCK). C_4 species are historically classified according to the types of enzymes decarboxylating malate or aspartate. However, there are reports of coexistence between decarboxylation types in C_4 (WANG et al., 2012; FURBANK, 2011; SALES et al., 2017).

The theory explained that C_4 plants do not show responses to elevated CO_2 due to the high efficiency in the mechanism of concentrating CO_2 in the active site of Rubisco (STOKEN et al., 2016). However, responses of C_4 plants to increasing [CO_2] are controversial. Some studies indicate an increase in phytomass accumulation without an increase in photosynthesis per unit area, while other studies report a reduction in photosynthesis (Table 1), which can be attributed to the CO_2 acclimation process. CO_2 acclimation can occur by reducing the content of photosynthetic metabolism enzymes and also by reducing stomatal density that arises in an environment with higher [CO_2]. (AINSWORTH; ROGERS, 2007). Reduced Rubisco (V_{max}) and PEPC (V_{pmax}) contents and activities in maize and sorghum plants was report (COUSINS et al., 2003). In other studies, however, no changes in Rubisco content were detected in sorghum and Panicum antidotale (GHANNOUM et al., 2000; WATLING, 2000). Therefore, there is no consensus in the scientific community about the effects of high [CO_2] on photosynthesis and growth of C_4 plants.

Table 1 - Responses$^{(1)}$ of growth (G) and photosynthesis (*A*) in species with C_4 photosynthetic metabolism exposed to increasing partial pressure of CO_2 .(Continue)

Species	**CO_2 Exposure**		**G**	***A***	**References**
	Time	Pa			
Brachiaria ischaemum	2 years	55	n.a.	+	Anderson et al. (2001)
Pannicum conjugatum	90 days	71	-	-	Ziska et al. (1991)
F. trinervia	40 days	70	+	+	Ziska et al. (1999)

P. annicum miliaceum	40 days	70	=	+	Ziska et al. (1999)
P. annicum maximum	30 days	60	-	+	Habermann et al. (2020)
Sorghum bicolor	60 days	70	n.a	-	Watling et al. (2000)
	60 days	57	=	+	Cousins et al. (2001)
	79 days	57	-	n.a	Derner et al. (2003)
Saccharum spp.	1 year	72	+	+	Souza et al. (2008)
Saccharum officinarum	70 days	72	+	=	Vu et al. (2006)
	120 days	72	n.a	=	Vu e Allen Jr. (2009a)
	90 days	72	=	=	Vu e Allen Jr. (2009b)
Zea mays	30 days	110	+	+	Maroco et al. (1999)
	180 days	62	+	+	Selim et al. (2021)
	56 days	72	-	-	Prins et al. (2011)
Seven C_4 grasses	180 days	66	+	+	Wand et al. (1999)

(1) '+' means increase; '-' means decrease; '=' means no answer; and 'n.a.' means not evaluated. (End)

Source: From the author (2022).

2. 3 Effect of drought on plants

Water relations perform a fundamental role in plant growth and development. The inadequate availability of water affects nutritional aspects and essential metabolic processes such as photosynthesis. Thus, drought stress is widely discussed because of its importance in limiting crop productivity. Drought, defined as a period of water restriction in which the availability of this resource in the soil becomes limited (VITAL et al., 2017), was the cause of losses for more than 34% of the productive systems, with a cost of more than $37 billion (FAO, 2021). Only considering the production of cereals, about 1.8 billion tons have been lost due to drought in the last four decades (BASLAM et al., 2020).

The responses of plants to this type of abiotic stress have been characterized, being that, in general, studies show that during the drought event plants present a series of strategies and physiological and biochemical adaptations in order totolerate, avoid, or escape the drought. Increase in the absorptive capacity, modification in root morphology, stomatal closure, and increase in the amount of smaller vessel diameter to improve water transport from roots to leaves are some of the typical drought avoidance responses (PANDEY et al., 2015). Tolerance strategies involve osmotic adjustment, induction of the antioxidant system, and increased cell wall elasticity. The escape strategies are associated mainly with a decrease in the metabolic activities of the plant and the induction of a state of latency (DING et al., 2018).

During a drought event, the hydraulic conductance of the whole plant is affected (root and leaf conductance), with factors such as genotype, restriction time, and stress level influencing the response. For example, an increase in root hydraulic conductance was evidenced for species such as rice (*Oriza sativa* L) and corn (*Zea mays* L.) under water stress, contrary to that shown in cucumber (*Cucumis sativus* L). Already in the leaves in general, it is observed that the water potential (ψ) decreases along with the hydraulic conductivity, although the values are different between species (BERTOLLI, 2012). Considering the level of stress, responses to drought can also vary. In the literature three levels of stress are reported based on stomatal conductance (g_s): (i) mild: $g_s > 0.15$ mol H_2O m^{-2}-s^{-1}, (ii) moderate: 0.05 mol H_2O $m^{-2}s^{-1}$ $< g_s <$ 0.15 mol H_2O $m^{-2}s^{-1}$ (iii) severe: $g_s < 0.05$ mol H_2O $m^{-2}s^{-1}$ (DING et al., 2018). g_s is important because it is a factor that affects important metabolic activities such as photosynthesis in C_4 species.

Drought has been associated with various aspects of carbon metabolism, with stomatal closure being one of the first responses observed, followed by a decrease in growth. Stomatal closure limits the entry of CO_2 into the leaf resulting in decreased *A* as a consequence of increased resistance to CO_2 diffusion. On the other hand, there is reduced water vapor exit from the leaf and consequently less transpiration rate (*E*). Stomatal or diffusive limitations due to drought occur during the early stages of stress, along with osmotic adjustment, hydraulic changes, and transport of signaling molecules (AHLUWALIA; SINGH; BHATIA, 2021). With increasing severity or prolongation of stress, biochemical limitations can occur. Biochemical limitations are mainly characterized by the uncoupling between the photochemical and biochemical phase of photosynthesis. Due to decreased stomatal conductance, which limits carboxylation efficiency by decreasing substrate CO_2. If the duration of stress is sufficiently prolonged, the excitation energy may become greater than the energy used in biochemical processes, leading to a decrease in the rate of reduction of adenosine diphosphate (ADP) and nicotiamide adenine dinucleotide phosphate (NADP) in the electron transport chain (BUCHANAN; JONES, 2015). Consequently, the occurrence of over-reduction of the electron transport chain leads to increased formation of reactive oxygen species (ROS), which in turn will further compromise the formation of adenosine triphosphate (ATP), thus culminating in an uncoupling of the photochemical and biochemical phases. During biochemical limitation the internal CO_2 concentration may increase as a consequence of respiration processes, but CO_2 assimilation will not recover, this being an important characteristic of this type of limitation (MARTINEZ et al., 2015).

Restrictions in the photosynthetic process as a consequence of drought affect crop production. For example, in sugarcane a reduction in stem growth and content of soluble sugars due to low CO_2 assimilation has been observed. (LOBO, 2016). On the other hand, work addressing the interaction between drought and elevated [CO_2] suggests that crops may benefit, mainly by increasing WUE. C_4 forage plants such as *Echinochloa crus-galli* (L.) Beauv., *Eleusine indica* (L.) Gaertn., *Digitaria ciliaris* (Retz.) Koel, as well as *Glycine max* (L.) Merr. 'Ransom' grown under 675 μmol mol^{-1} and drought showed less deleterious effects of drought when compared to plants grown under 400 μmol mol^{-1} (PATTERSON, 1986). In this sense, research has shown a decrease in the deleterious effects caused by drought in plants under eCO_2. However, the responses are genotype-dependent and vary according to the level of stress (LEAKEY; LAU, 2012).

2.4 C_4 mechanism limitation

The C_4 metabolism favors the saturation of CO_2 near the site of action of Rubisco, decreasing the photorespiration process and providing a high of CO_2 assimilation rate. In C_4 plants, several strategies are proposed to increase CO_2 assimilation per unit leaf area, such as increasing the activity of the enzymes PEPC and Rubisco, together with improving the processes involved in CO_2 influx into the mesophyll cells (MC) (VON CAEMMERER; FURBANK, 2016).

Although Rubisco has a lower affinity for O_2 than for CO_2 (specificity of Rubisco for CO_2 is 82 times higher than for O_2), the concentration of CO_2 in the mesophyll in a C_3 plant is low (UBIERNA; SUN; COUSINS, 2011). Due to the low CO_2 concentration, the oxygenase activity of Rubisco competes with the carboxylase activity, and the synthesis of a 2-phosphoglycolate (2-PG) molecule occurs. To prevent the accumulation of 2-PG triggers a series of reactions that involve the coordination of three organelles and results in the release of a CO_2 molecule that was previously fixed by photosynthesis. Therefore, photorespiration promotes a reduction in the carboxylation efficiency of Rubisco (BAUWE, 2010; MORONEY et al., 2013).

Besides photorespiration, the reduction in photosynthetic efficiency may be caused by other factors. Although mesophyll and BSC are separated by suberized cell wall, which reduces CO_2 leakage by directly interfering with vascular bundle sheath conductance (FARQUHAR, 1983; YIN et al., 2011), the higher rate of carboxylation by PEPC in the mesophyll compared to carboxylation by Rubisco in the BSC may favor increased CO_2 leakage (WATLING, 2000). CO_2 leakage (ϕ) is defined as the fraction of CO_2 that after being bound by PEPC and decarboxylated in the BSC escapes to MC (FARQUHAR, 1983). Given that C_4 photosynthetic metabolism has an additional energy cost of two moles of ATP per mol of fixed CO_2, the increase in ϕ will require more energy for PEP regeneration and for PEPC to refix CO_2 (KROMDIJK et al., 2016). Thus, ϕ can be considered a key parameter for determining the efficiency of the C_4 process (VON CAEMMERER; FURBANK, 2003). In addition, ϕ tends to increase as the Calvin Benson-Bassham cycle operation is reduced, which is aggravated in stress situations (KROMDIJK et al., 2014).

In this regard, an increase in ϕ was observed in *Aristida glabrata*, a C_4 grass of the NADP-ME type, when plants were submited to drought and nitrogen deficiency (FRAVOLINI et al., 2002). However, a study of four species and four hybrids of sugarcane grown in ambient CO_2 reported no relationship between leaf nitrogen, ϕ and photosynthetic efficiency (MEINZER; ZHU, 1998). GHANNOUM et al. (2000) identified that the interactions between high [CO_2], water relations and photosynthesis of C_4 plants are areas that urgently need research. Even after several years, such plant-environment interactions have not yet been fully elucidated indicating the need for experiments to improve the understanding of the mitigationof water stress effects and the possible increase in productivity of C_4 species due to the increase in [CO_2] (LEAKEY; LAU, 2012).

2.5 Sugarcane like a study species

Sacharum spp, represents an important target of study because of its economic importance for countries such as Brazil, China, Indonesia and Thailand since they are responsible for 70% of the world's sugarcane production (DE MATOS et al., 2020). Brazil is the leading producer of sugarcane, producing approximately 40% of all sugarcane in the world. Around 23% of the sugar consumed in the world is produced from sugarcane planted in Brazil, positioning this country not only as the largest producer with 597,590 tons in the 2020/2021 season, but also as the world's largest exporter, with 49% of this amount (UNICA, 2022).

This species besides having a fundamental role in Brazil's economy, also presents itself as a suitable plant in studies that evaluate C and drought metabolism due to several factors. Sugarcane is one of the main representatives of plants with C_4 photosynthetic metabolism, and they show better WUE when compared to C_3 species. However, the main factor limiting the productivity of this crop is drought (VU; ALLEN, 2009). Sugarcane plants subjected to drought for prolonged periods show a decrease in internal carbon (C_i) due to increased resistance to CO_2 diffusion in the stomata and mesophyll (g_s, g_m, respectively) resulting in metabolic alterations associated mainly with photosynthesis and nitrogen metabolism (DING et al., 2018). However, variations in drought response within species are genotype-dependent. Some studies reported, for example, that plants of the variety IACSP95-5000 subjected to drought showed a reduction in *A* under severe stress. Therefore, *A* was not limited by diffusive aspects but rather by aspects associated with carboxylation rate, enzyme activity, and photochemical activity (MARCHIORI et al., 2017).

2.5.1 Varieties studied

The two sugarcane varieties used in the study were RB867515 and RB855536, from the Sugarcane Genetic Improvement Program of RIDESA (Interuniversity Network for the Development of the Sugarcane Industry). The variety RB867515 (cross between parental lines RB72454 × unknown) is currently the most widely planted in Brazil and shows higher yield and better performance than RB855536 (cross between parental lines: SP70-1143 × RB72454) under drought stress conditions (VITAL et al., 2017). Although both have the characteristics of fast growth, tall size and high sucrose content (RIDESA, 2017).

A study of molecular responses of these two genotypes under drought conditions found that differences exist between cultivars at different levels (transcriptome, proteome and metabolome), suggesting that the genetic differences between them may explain the unique physiological mechanisms that seem to help cv. RB867515 to cope with water stress (VITAL et al., 2017). This was corroborated by Junior et al. (2018), as it was observed that the cultivar RB867515 when grown in rainfed system showed increased intrinsic water use efficiency (WUE_i) and reduced *E*, g_s , *A* and carboxylation efficiency (*CE*). It can therefore be considered as a drought resistance mechanism.

REFERENCES

AHLUWALIA, O.; SINGH, P. C.; BHATIA, R. A review on drought stress in plants: Implications, mitigation and the role of plant growth promoting rhizobacteria. **Resources, Environment and Sustainability**,v.5. 2021. doi:10.1016/j.resenv.2021.100032

ALLAKHVERDIEV, S. I. Editorial for the special issue on photosynthesis and hydrogen energy research for sustainability—*2019*. **Photosynthesis Research**, v.146, p. 1-3. 2020. doi: 10.1007/s11120-020-00764-5. 2020.

ASEFA. G. The Role of Harvest Index in Improving Crop Productivity: A Review. **Journal of Natural Sciences Research**, v. 9, n. 6. p.24-28. 2019. doi: 10.7176/JNSR

BADGER MR The role of carbonic anhydrase in photosynthesis. **Annual Review in Plant Physiology** l 45: 369–392. 1994

BASSHAM, J.A *et al*. The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. **Journal of the American Chemical Society**, v. 76, p. 1760–1770.1954

BERTOLLI, S. C.; SOUZA, G.M.The level of environmental noise affects the physiological performance of *Glycine max* under water deficit. **Theoretical and Experimental Plant Physiology.** v. 25, n. 1, pp. 36-45. 2013.

BASLAM, M *et al.* Photosynthesis in a changing global climate: Scaling up and scaling down in crops. **Frontiers in Plant Science**, v. 11, p. 882. 2020. doi:10.3389/fpls.2020.00882

BHARGAVA, S.; MITRA, S. Elevated atmospheric CO_2 and the future of crop plants. **Plant Breeding**,v. 140, n. 1, p. 1-11. 2021.

BRESTIC, M *et al.* Crop photosynthesis for the twenty-first century. **Photosynthesis r Research**,v. 150, n. 1, p. 1-3. 2021.

BUCHANAN B, G. W.; JONES R. Biochemestry & molecular biology of plants (L. John Wiley & Sons Ed. Wiley-blackwell. ed.). UK. 2015.

CLEMENS, M. E.; ZUNIGA, A.; OECHEL, W. Effects of elevated atmospheric carbon dioxide on the vineyard system of vitis vinifera: A Review. **American Journal of Enology and Viticulture**,v. 73, n. 1, p. 1-10. 2022. doi: 10.5344/ajev.2021.21029

COHEN, I *et al.* Leaf coordination between petiole vascular development and water demand in response to elevated CO_2 in tomato plants. **Plant Direct**,v. 6, n. 1, p.371. 2022.

COUSINS, A. B *et al.* Development of C_4 photosynthesis in sorghum leaves grown under free-air CO_2 enrichment (FACE). **Journal of Experimental Botany**,v.54, n.389, p. 1969-1975. 2003. doi: 10.1093/jxb/erg197

DE MATOS, M.; SANTOS, F.; EICHLER, P. Sugarcane world scenario. Sugarcane Biorefinery, **Technology and Perspectives**, p. 1–19. 2020. doi: 10.1016/b978-0-12-814236-3.00001-9

DE SOUZA, A. P *et al.* Elevated CO_2 increases photosynthesis, biomass and productivity, and modifies gene expression in sugarcane. **Plant, Cell & Environment**,v. 31, n. 8, p.1116-1127. 2008. doi: 10.1111/j.1365-3040.2008.01822.x

DING, L *et al.* Is nitrogen a key determinant of water transport and photosynthesis in higher plants upon drought stress? **Frontiers in Plant Science**. v. 9. 2018. doi: 10.3389/fpls.2018.01143

DOMICIANO, D *et al.* Nitrogen sources and CO_2 concentration synergistically affect the growth and metabolism of tobacco plants. **Photosynthesis research**, v. 144. n.3, p. 327-339.2020.

FARQUHAR G.D. On the nature of carbon isotope discrimination in C_4 species. **Australian Journal of Plant Physiology**, v.10, n. 2, p. 205–226. 1983.

Food and Agriculture Organization of the United Nations Rome-FAO. 2021. The impact of disasters and crises on agriculture and food security. Rome. 2021. Available in: https://www.fao.org/3/cb3673en/cb3673en.pdf , Access in January 30 2022.

FRAVOLINI, A.; WILLIAMS, D. G.; THOMPSON, T.L. Carbon isotope discrimination and bundle sheath leakiness in three C_4 subtypes grown under variable nitrogen, water and atmospheric CO_2 supply. **Journal of Experimental Botany**, v.3, n. 378, p. 2261–2269. 2001. doi: 10.1093/jxb/erf084

FURBANK, R.T. Evolution of the C_4 photosynthetic mechanism: are there really three C_4 acid decarboxylation types?. **Journal of Experimental Botany**, v.62, n.9, p. 3103–3108. 2011.

GHANNOUM, The growth response of C_4 plants to rising atmospheric CO_2 partial pressure: a reassessment. **Plant, Cell and Enviroment**, v. 23, n. 9 , p. 931-942. 2000.

GU, D.; ANDREEV, K.; DUPRE, M.E. Major trends in population growth around the world. **China CDC weekly**,v. 3, n. 28, p.604. 2021.

HABERMANN, E *et al*. Increasing atmospheric CO_2 and canopy temperature induces anatomical and physiological changes in leaves of the C_4 forage species *Panicum maximum*. **Plos one,** v. 15, n. 8. 2020. doi:10.1371/journal.pone.0238275

HOANG, T. D *et al.* Photosynthetic response and nitrogen use efficiency of sugarcane under drought stress conditions with different nitrogen application levels. **Plant Production Science**, v. 20, n. 4, p. 412-422. 2017.

HUSSAIN, S. *et al.* Photosynthesis research under climate change. **Photosynthesis Research**, v. 150, n. 1, p. 5-19. 2021. doi:10.1007/s11120-021-00861-z

JUNIOR, F. R *et al.* Biometric responses of third ratoon sugarcane varieties under variable water regime. **Comunicata Scientiae**, v.9, n.1, p. 81-92. 2018

KANT, S. Understanding nitrate uptake, signaling and remobilisation for improving plant nitrogen use efficiency. **Seminars in Cell & Developmental Biology**, v. 74, p. 89-96, 2018

KRÄMER, K *et al.* Acclimation to elevated CO_2 affects the C/N balance by reducing de novo N-assimilation. **Physiologia Plantarum**, v. 13615, p. 1-13. 2021.

KROMDIJK, J.; GRIFFITHS, H.; SCHEPERS, H.E. Can the progressive increase of C_4 bundle sheath leakiness at low PFD be explained by incomplete suppression of photorespiration?. **Plant, Cell & Environment**, v. 33, p. 1935–1948. 2010.

KROMDIJK, J *et al.* Bundle-sheath leakiness in C_4 photosynthesis: a careful balancing act between CO_2 concentration and assimilation. **Journal Experimental Botany,** v. 65, p. 3443–3457. 2014.

KROMDIJK, J.; LONG S.P.One crop breeding cycle from starvation? How engineering crop photosynthesis for rising CO_2 and temperature could be one important route to alleviation. **Proceedings of the Royal Society B: Biological Sciences, v.** 283, n. 1826. 2016. doi:10.1098/rspb.2015.2578

KUNDU, P.; GOEL, K.; ZINTA, G. Chapter 20 - Nutritional imbalance in plants under rising atmospheric CO_2, Editor(s): Vinay Kumar, Ashish Kumar Srivastava, Penna Suprasanna,Plant Nutrition and Food Security in the Era of Climate Change, Academic Press, 2022. Pages 513-536,ISBN 9780128229163. doi:10.1016/B978-0-12-822916-3.00006-8.

LEAKEY, A. D.; LAU, J. A. Evolutionary context for understanding and manipulating plant responses to past, present and future atmospheric [CO_2]. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v. 367. p. 613-629. 2012.

LIN, J.; JACH, M.; CEULEMANS, R. Stomatal density and needle anatomy of Scots pine (*Pinus sylvestris*) are affected by elevated CO_2. **New phytologist**,v. 150, n. 3, p. 665-674. 2001.

LOBO, A. K. M. Photosynthesis regulation by sucrose metabolism under water deficit and source-sink alterations in sugarcane. 118 f. Tese (Doutorado em Bioquímica)-**Universidade Federal do Ceará,** Fortaleza, 2016.

LUDWIG, M. Evolution of the C_4 photosynthetic pathway: events at the cellular and molecular levels. **Photosynthesis research**, v.117, n. 1, p.147-161. 2013.

MARCHIORI, P. E.; MACHADO, E. C.; RIBEIRO, R. V. Photosynthetic limitations imposed by self-shading in field-grown sugarcane varieties. **Field Crops Research**, v.155, p. 30-37. 2014.doi: 10.1016/j.fcr.2013.09.025

MARCHIORI, P. E. R *et al.* Physiological plasticity is important for maintaining sugarcane growth under water deficit. **Frontiers in Plant Science**, v.8, n.2148 2017. doi:10.3389/fpls.2017.02148.

MARIN, F.R.; RIBEIRO, R.V.; MARCHIORI, P.E.R. How can crop modeling and plant physiology help to understand the plant responses to climate change? A case study with sugarcane. **Theoretical and Experimental Plant Physiology**, v.26, p. 49–63, 2014.

MAROCO, J.; EDWARDS, G.; KU, M. Photosynthetic acclimation of maize to growth under elevated levels of carbon dioxide. **Planta**, v. 210, p. 115–125. 1999. doi: 10.1007/s004250050660

MEINZER, F. C.; ZHU, J. Nitrogen stress reduces the efficiency of the C_4 CO_2 concentrating system, and therefore quantum yield, in *Saccharum* (sugarcane) species**. Journal Experimental Botany**, v. 49, p. 1227–1234. 1998.

MORONEY, J. V *et al.*. Photorespiration and carbon concentrating mechanisms: two adaptations to high CO_2, low CO_2 conditions. **Photosynthesis research**, v. 117, n. 1, p. 121-131. 2013.

MASLE, J. The effects of elevated CO_2 concentrations on cell division rates, growth patterns, and blade anatomy in young wheat plants are modulated by factors related to leaf position, vernalization, and genotype. **Plant Physiology**, v. 122, n. 4, p.1399-1416. 2000.

NEUPANE, D *et al.* Does climate change affect the yield of the top three cereals and food security in the world? **Earth**, v. 3. n. 1, p. 45-71. 2022.

NÖLKE, G.; SCHILLBERG, S. Strategies to enhance photosynthesis for the improvement of crop yields. **Climate Change, Photosynthesis and Advanced Biofuels**, p. 143-157. 2020.

NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION – NOAA. Trends in Atmospheric Carbon Dioxide Mauna Loa Observatory, Hawaii. Available in: https://gml.noaa.gov/ccgg/trends/mlo.html. Access in: 02 fev. 2022. 2022https://gml.noaa.gov/ccgg/trends/

OKSANEN, E.; SOBER, J.; KARNOSKY, D. Impacts of elevated CO_2 and/or O_3 on leaf ultrastructure of aspen (*Populus tremuloides*) and birch (*Betula papyrifera*) in the Aspen FACE experiment. **Environmental Pollution**, v.115, n. 3, p.437-446. 2001.

PACKER, A. P *et al.* Challenges in a Free Air CO_2 Enrichment (FACE) coffee crop experiment to prospect strategies for mitigation and adaptation to climate change. In: **INTERNATIONAL PLANT NUTRITION COLLOQUIUM,** v.18, Copenhagen. 2017.

PATTERSON, D. Responses of soybean (*Glycine max*) and three C_4 grass weeds to CO_2 enrichment during drought. **Weed Science**, v.34, n.2, p. 203-210. 1986. doi:10.1017/S0043174500066686

POORTER, H. Interspecific variation in the growth response of plants to an elevated ambient CO_2 concentration. **CO_2 and Biosphere**, p.77-98. 1993

.

PANDEY, R *et al.* Physiological and molecular alterations in plants exposed to high [CO_2] under phosphorus stress, **Biotechnology Advances**, v.33, n. 3, p. 303. 2015. doi: 10.1016/j.biotechadv.2015.03.011.

PRITCHARD, S. G *et al.* Elevated CO_2 and plant structure: a review. **Global Change Biology**, v. 5, n. 7, p. 807-837. 1999.

RAMAZAN, S *et al.* Combined gas exchange characteristics, chlorophyll fluorescence and response curves as selection traits for temperature tolerance in maize genotypes. **Photosynthesis Research**, v. 150, n. 1, p. 213-225. 2021.doi:10.1007/s11120-021-00829-z.

REHMAN, A *et al.* How do climatic change, cereal crops and livestock production interact with carbon emissions? Updated evidence from China. **Environmental Science and Pollution Research**, p. 1-12. 2021.

SAGE, R. F. and MCKOWN, A. D. Is C_4 photosynthesis less phenotypically plastic than C_3 photosynthesis? **Journal of experimental botany**, 57. 2,303-317. 2006.

SAGE, R. F. The evolution of C_4 photosynthesis. **New phytologist**,v. 161, n. 2, p. 341-370. 2004.

SAGE, R. F.; SAGE, T.L.; KOCACINAR, F. Photorespiration and the Evolution of C_4 Photosynthesis. **Annual Review of Plant Biology,** v. 63, p.19-47. 2012 doi: 10.1146/annurev-arplant-042811-105511

SALES, C. R *et al.* Improving C_4 photosynthesis to increase productivity under optimal and suboptimal conditions. **Journal of Experimental Botany**, v. 72. n. 17, p. 5942-5960. 2021.

SELIM, A *et al.* Elevated CO_2 differently suppresses the arsenic oxide nanoparticles-induced stress in C_3 (*Hordeum vulgare*) and C_4 (*Zea maize*) plants via altered homeostasis in metabolites specifically proline and anthocyanin metabolism. **Plant Physiology and Biochemistry,** v. 166. 2021. doi: 10.1016/j.plaphy.2021.05.036

SHAMS, K. Physiological, biochemical and yield responses of wheat cultivars to deficient water stress. **Modern Phytomorphology**. v.12, p.124-130. 2018.

STOKES, J. C *et al.* Measuring and modelling CO_2 effects on sugarcane. **Environmental Modelling & Software**, v.78, p. 68 -78. 2016.

SUN, W *et al.* Effects of elevated CO_2 and temperature on soybean growth and gas exchange rates: A modified GLYCIM model. **Agricultural and Forest Meteorology**, v. 312, n. 15. 2022.

The Intergovernmental Panel on Climate Change (IPCC) IPCC. Summary for Policymakers. In: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change DELMOTTE, V; P. ZHAI, A; PIRANI, S.L; CONNORS, C; PÉAN, S; BERGER, N. CAUD, Y. CHEN, L. GOLDFARB, M.I. GOMIS, M. HUANG, K. LEITZELL, E. LONNOY, J.B.R. MATTHEWS, T.K. MAYCOCK, T. WATERFIELD, O. YELEKÇI, R. YU, AND B. ZHOU. 2021.

UBIERNA, N.; SUN, W.; COUSINS, A. B The efficiency of C_4 photosynthesis under low light conditions: assumptions and calculations with CO_2 isotope discrimination. **Journal of Experimental Botany**. v.62, n. 9, p. 3119–3134. 2011. doi: 10.1093/jxb/err073

UNICA (Brazilian Sugarcane Industry Association). **Observatorio de cana- Harvest update of South-Central region Bi-weekly Bulletin HARVEST 2021/2022**. Available in:https://observatoriodacana.com.br/listagem.php?idMn=63. Access in: 16 January 2022.
VITAL, C. E *et al.* An integrative overview of the molecular and physiological responses of sugarcane under drought conditions. **Plant molecular biology**, v. 94, n. 6, p. 577-594. 2017.

VON CAEMMERER, S.; FURBANK, R. T. The C_4 pathway: an efficient CO_2 pump. **Photosynthesis research**,v. 77. n. 2, p. 191-207.2003.

VU, J. C.; ALLEN JR, L. H. Growth at elevated CO_2 delays the adverse effects of drought stress on leaf photosynthesis of the C_4 sugarcane. **Journal of Plant Physiology**, v. 166, n. 2, p. 107-116. 2009. doi:10.1016/j.jplph.2008.02.009

WANG, W *et al.* Improving the accuracy of meta-analysis for datasets with missing measures of variance: Elevated [CO_2] effect on plant growth as a case study.**Science of The Total Environment**, v. 806, n. 2. 2022. doi:10.1016/j.scitotenv.2021.150669.

WALIA, S.; RATHORE, S.; KUMAR, R. Elucidating the mechanisms, responses and future prospects of medicinal and aromatic plants to elevated CO_2 and elevated temperature. **Journal of Applied Research on Medicinal and Aromatic Plants**, v. 261. 2022. doi: 10.1016/j.jarmap.2021.100365

WALTER, L.C.; TELLES, H.R E.; STRECK, N. A.Mecanismos de aclimatação das plantas à elevada concentração de CO_2. **Ciência Rural**, v. 45, n. 9, p. 1564-1571. 2015. doi: 10.1590/0103-8478cr20140527.

WANG, D *et al.* A meta-analysis of plant physiological and growth responses to temperature and elevated CO_2. **Oecologia**, v. 169, n.1, p. 1-13. 2012.

WAND, S. J *et al.* Responses of wild C_4 and C_3 grass (Poaceae) species to elevated atmospheric CO_2 concentration: a meta-analytic test of current theories and perceptions. **Global Change Biology**, v. 5, n. 6, p. 723-741. 1999. doi:10.1046/j.1365-2486.1999.00265.x

WATLING, J. R.; PRESS, M. C.; QUICK, P W. Elevated CO_2 induces biochemical and ultrastructural changes in leaves of the C_4 cereal sorgo. **Plant Physiology** , v.123, n. 3, p. 1143–1152. 2000. doi:10.1104/pp.123.3.1143

YAMORI, W. Improving photosynthesis to increase food and fuel production by biotechnological strategies in crops. **Journal of Plant Biochemistry & Physiology,** v. 1, n. 3. 2013. doi: 10.4172/2329-9029.1000113

YIN, X *et al.* Using a biochemical C_4 photosynthesis model and combined gas exchange and chlorophyll fluorescence measurements to estimate bundle-sheath conductance of maize leaves differing in age and nitrogen content. **Plant, cell & environment**, v. 34, n. 12, p. 2183-2199. 2011.

ZHANG, J *et al.* The effects of elevated CO_2, elevated O_3, elevated temperature, and drought on plant leaf gas exchanges: a global meta-analysis of experimental studies. **Environmental Science and Pollution Research**, v. 28, n. 12, p.15274-15289. 2021

SECOND SECTION: MANUSCRIPT

SUGARCANE PHOTOSYNTHESIS AND ASSOCIATED LEAF TRAITS IN RESPONSE TO HIGH [CO_2] AND DROUGHT

ABSTRACT

In theory, C_4 plants do not respond to the increase in atmospheric CO_2 concentration, since they already have a concentration mechanism in the vascular bundle sheath cells. However, it was observed that sugarcane plants (*Saccharum* spp.) may present higher photosynthesis and higher biomass production under conditions of high CO_2, suggesting that C_4 metabolism in these plants does not have maximum efficiency in the current CO_2 condition. The limitation can occur in the carboxylation or decarboxylation phases, associated with the process of photorespiration and CO_2 leakage.Therefore, the aim of this study was to identify the key processes of C_4 metabolism involved with the limitations in the mechanism of CO_2 concentration in sugarcane plants. The study was conducted in open-top chambers using two sugarcane cultivars (RB867515 and RB855536). Plants with 30 days after sprouting were grown under two CO_2 environments (400 and 680 µmol CO_2 mol^{-1} of air, aCO_2 and eCO_2, respectively) for 70 days. 60 DAS, plants were exposed to water restriction in order to enhance the flaws in photosynthetic metabolism. Biometric assessments, chlorophyll relative content, tolerance index (TI), biomass partitioning, response curves of CO_2 assimilation (A) to increasing intercelullar CO_2 concentration (A/Ci) and light intensities (A/Q), under 21% and 2% O_2 were performed. Leaf anatomical traits were also analised. It was found that plants of both varieties, when submitted to water deficit, presented reduced growth, leaf area and biomass accumulation when compared to well-watered plants. On the other hand, under elevated [CO_2], it was observed significant reduction of relative content of chlorophylls, but an increase in TI and a different biomass accumulation. 50 Days after sprouting (DAS), an increase in A was also detected. At 74 DAS, physiological and anatomical changes associated with acclimation were observed. High [CO_2] increased the water use-efficiency in the plants, leading to a better photosynthetic performance than plants grown under normal [CO_2]. eCO_2 led to decreases in the carboxylation rate of the PEPC enzyme, while the carboxylation rate of Rubisco was sustained. There was an increase in the quantum efficiency of carboxylation under eCO_2 without affecting A, which could be an indicator of the decrease in CO_2 leakage under this condition. During A/Q and A/C_i measurements at 2% O_2 and 400 µmol mol^{-1}, an increase in Rubisco activity was noticed, suggesting that A is limited by photorespiration. In this regard, we conclude that in the current CO_2 conditions, the photosynthetic process in sugarcane plants may be limited by the higher carboxylation rate of PEPC in relation to Rubisco, which leads to leakage of CO_2 from the bundle sheath cells to the mesophyll cells, resulting in lower quantum carboxylation efficiency. At the same time, our results indicate that plants were able to modulate their metabolism to the benefit of water conservation at the leaf level, since in this study they had overlapped the water use-efficiency over the gain in C by eCO_2.

Key words: Photosynthetic metabolism. C_4 photosynthesis. Climate change. *Saccharum* spp.

INTRODUCTION

The concentration of CO_2 ([CO_2]) in the atmosphere has been increasing in the last centuries. Climate prediction models suggest that the observed increase will continue for the next decades and it will be associated with changes in the rainfall regime (EPA, 2021), directly influencing the development of plants and ecosystems (PALIT et al., 2020). For this reason, studies have been carried out with the aim of increasing the understanding of how plants detect, respond, acclimate and adapt to environmental variations (WANG et al., 2014; SALES et al., 2021). However, in addition to the responses to high [CO_2] being species-dependent, they vary depending on the presence and intensity of stressors (DOMICIANO et al., 2020), since suboptimal conditions can reduce the internal availability of CO_2 in the plant (MARCHIORI et al., 2017).

The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a bifunctional enzyme that, in addition to carboxylase activity, has oxygenase activity in the C_3 photosynthetic cycle (DAI et al., 1993). C_4 photosynthetic metabolism is characterized by anatomical and biochemical changes in leaves that allow the maintenance of high CO_2 concentrations around the catalytic site of Rubisco, minimizing the occurrence of photorespiration. For C_4 photosynthesis to occur, the coordination between the activity of carboxylation and decarboxylation enzymes is needed (SAGE, 2004). Studies that describe the individual and combined effect of drought and high [CO_2] on carbon (C) metabolism have been performed mainly for C_3 species, with some approaches in C_4. Some of the relevant results showed that, contrary to what was expected for C_4 plants, the increase in [CO_2] had positive effects on A and productivity in sugarcane (*Saccharum* spp.), corn (*Zea mays* L.) and some C_4 forages (HABERMANN et al., 2020). The positive response was mainly associated with the presence of C_3 metabolism of developing leaves and/or increasing in water use efficiency (WUE) (WEI et al., 2022). Studies with sugarcane indicate that the negative effect on photosynthesis by drought was alleviated in plants cultivated under high [CO_2]. The observed improvements in A were mainly evidenced during leaf development and that thus being a factor that delays the adverse effects of drought (ZHANG et al., 2021). To date, there is no consensus in the scientific community about the effects of elevated [CO_2] on photosynthesis and growth of C_4 plants. Even sugarcane being a C_4 species, increases in photosynthesis were detected in response to the instantaneous increase in [CO_2] (A/C_i curve) when cultivated in the field or in pots (MACHADO et al., 2013; MARCHIORI et al., 2014; SALES et al., 2021).

Discrimination of carbon isotopes has been a tool to investigate and evaluate the photosynthetic performance of plants. In C_4 plants the discrimination against ^{13}C is lower when compared to C_3 plants, which means that C_4 plants have a higher amount of this heavy isotope than C_3 plants (EGGELS et al., 2021). Furthermore, it is known that genotypic variations and environmental conditions such as water deficit, intensity of solar radiation, and changes in temperatures influence fractionation during diffusion and carboxylation and, therefore, modify the values of $\delta^{13}C$ in the plant (CERNUSAK et al., 2013; EGGELS et al., 2021).

Given the worldwide importance of sugarcane as a bioenergetic source, any change in the carbon balance due to environmental variables may be well understood for correct assessments and predictions of the productive potential of these species under the climate change scenario (KANT et al., 2012). The vast majority of research trying to understand plant responses to climate change is carried out with C_3 species, since it is widely accepted that C_4 species do not respond to the increase in $[CO_2]$, as explained earlier (LEAKEY and LAU, 2012). The responses of C_4 plants to the increase in $[CO_2]$ are controversial and even when increased photosynthesis due to the increase in ambient $[CO_2]$ is observed, there is no clear justification for this response (MACHADO et al., 2013; MARCHIORI et al., 2014).

In view of these results, it is suggested that sugarcane responds to the increase in $[CO_2]$ and that this increase may be related to the inefficiency of C_4 photosynthetic metabolism. In addition to photorespiration, the reduction in photosynthetic efficiency can be caused by other factors. Although the mesophyll and vascular bundle sheath (BS) cells are separated by a suberized cell wall, which reduces CO_2 leakage (YIN et al., 2011), imbalances in metabolite flux between C_3 and C_4 cycle can increase the CO_2 leakage (ϕ) from the BS cells (HENDERSON et al., 1992; KROMDIJK et al., 2008). As C_4 photosynthetic metabolism has an additional energy cost of two mol ATP per mol fixed CO_2 with respect to C_3 plants, increasing ϕ will require more energy to regenerate phosphoenolpyruvate from pyruvate (HENDERSON et al., 1992; TAZOE et al., 2008).

Given this situation, the hypothesis that sugarcane plants respond positively to the increase in $[CO_2]$ due to inefficiencies in the mechanism of CO_2 concentration, specifically in the carboxylation process and CO_2 diffusion by increasing the water use efficiency, was tested. This information shows scientific and economic relevance, as it generates basic knowledge about the physiology of sugarcane in a climate change scenario.

MATERIAL AND METHODS

Plant material and experimental conditions

The experiment was conducted in four 1,8 m height x 1.06-m-diameter (1.30 m^3) *open top chambers* (OTCs) adapted from Drake et al. (1989) kept inside a greenhouse in Lavras, MG – Brazil (21º13'40''S and 44º57'50''W GRW, altitude 960 m). Two varieties of *Saccharum* spp., RB867515 and RB855536, were evaluated. RB855536 shows high agroindustrial productivity and susceptibility to drought, whereas RB867515 is more rustic, and it can be cultivated under low fertility and water restrictions (RIDESA, 2017). The plants were obtained from the active germplasm bank of the Experimental Station of the Federal University of Lavras - UFLA, located in the Center of Scientific and Technological Development in Agriculture – Muquém Farm in Lavras, Minas Gerais - Brazil. Just after the harvest, the stems, containing one bud were sectioned in five-centimeter cuttings and planted in pots (5 dm^3) filled with a mix of substrate soil and sand in a 2:1 ratio, respectively. Nutritional correction was performed based on chemical analysis of the mixed substrate (Table 1) according to van Raij et al. (1997). At 20 days after sprouting (DAS), seedlings of both varieties were left for 10 days to acclimatize inside the OTCs. Each OTC had 5 plants of each variety.

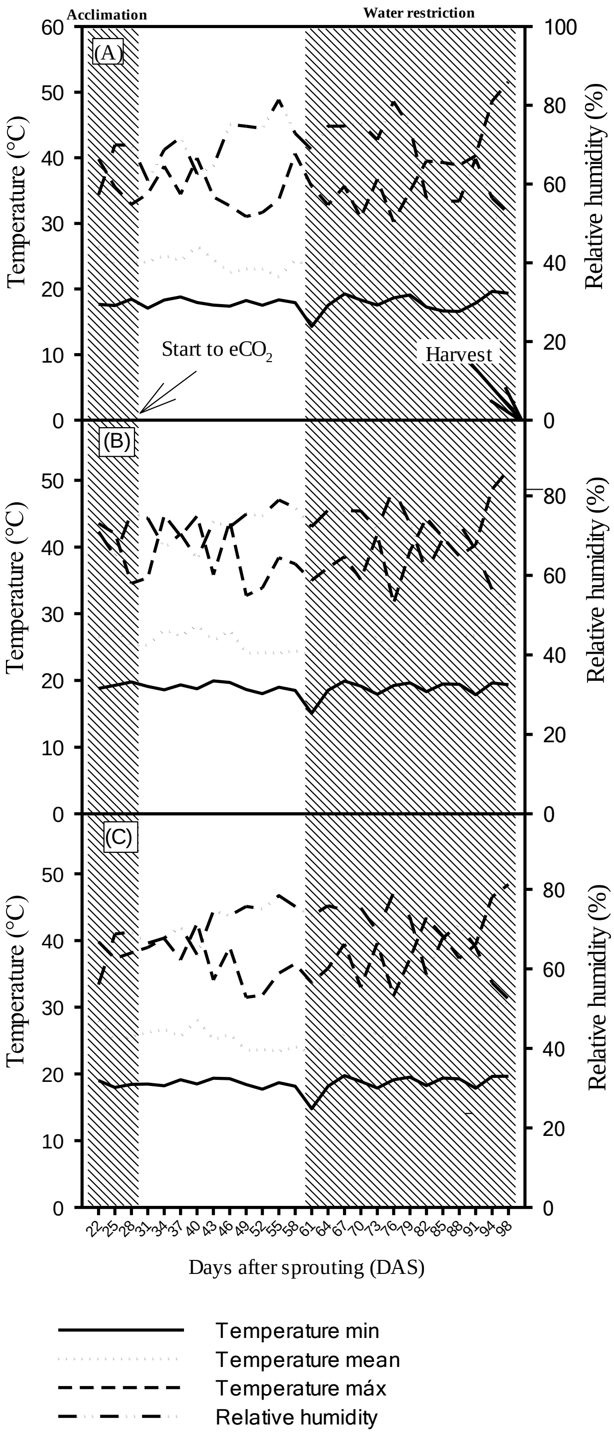
Table 1- Initial chemical analysis of the mix substrate used for plant cultivation

pH (KCl)	K	P	Na	Ca	Mg	Al	H^+ + Al	Zn	Fe	Mn	Cu	B	S
	--------mg dm^3-------			-----------cmol dm^3---------				---------------------mg dm^3-------------------					
6.0	51.74	20.57	11.20	2.26	0.28	0.06	2.01	3.4	32.4	21.9	6.9	0.2	22.9

Source: From the author (2022).

During the experimental period, the air temperature and relative humidity of the greenhouse and inside the OTCs was monitored every three hours using an RHT10 thermohygrometer (Extech Instruments, Nashua, NH, USA). The mean temperature and the mean relative humidity inside the greenhouse during the experimental period were 25.1 ± 1.0 ºC and 70.2 ± 1.3% (Figure 1A). Inside the OTC's with ambient [CO_2] it were 26.4 ± 0.2 and 71.0 ± 0.5% (Figure 1B) and inside in the OTC's with elevated [CO_2] these parameters were 25.9 ± 0.2 and 69.7 ± 0.5% (Figure 1C). There were no significant differences ($P<0.05$) for these two parameters among the three environments.

Figure 1- Temperature and Relative humidity recorded inside greenhouse (in A), Control chambers (± 400 µmol mol^{-1}, in B) and Elevated CO_2 chambers (± 680 µmol mol^{-1}, in C).



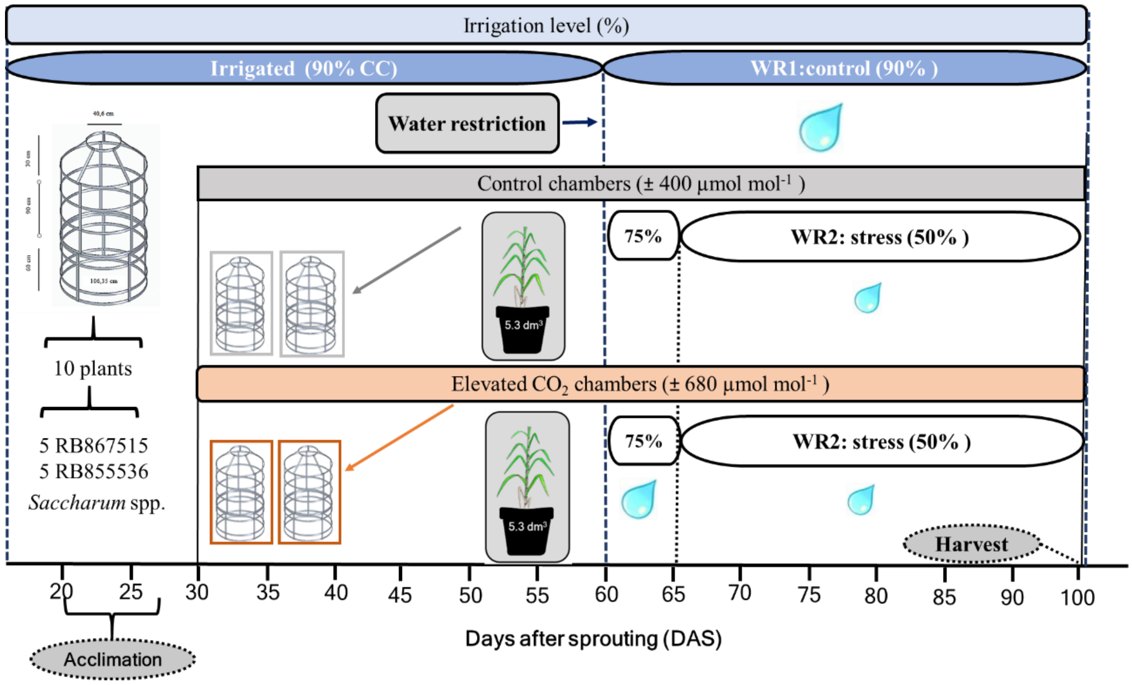
Source: From the author (2022).

Treatment imposition

The plants were subjected to two factors during the experiment: two CO_2 concentrations and two water regimes. The carbon dioxide enrichment treatments started when seedlings reached 30 DAS, where they were exposed to ambient CO_2 (±400 µmol mol^{-1}) or enriched ambient CO_2 (±680 µmol mol^{-1}); two OTCs were kept under elevated [CO_2] (eCO_2 chambers) while the other two chambers were under current ambient [CO_2] (cCO_2 chambers). The [CO_2] inside the OTCs was monitored every two hours using the infrared gas analyzer model SBA-5 (PP Systems, USA) and manually adjusting the flow rate of the CO_2 cylinders. The exposure time to elevated CO_2 levels occurred from 8:00h to 17:00h. The exposure time matched the day length during which the process of photosynthesis took place. Sugarcane plants were exposed to these [CO_2] levels for 70 days. All plants of both varieties were maintained at 90% soil capacity for 60 DAS.

At 60 DAS, the water regimes (WR) were imposed: (i) WR1 - plants watered regarding ~90% of the evapotranspiration from the previous day, which means that plants were maintained at 80-90% of the soil water capacity (Control); (ii) WR2 - plants watered regarding ~50% of the evapotranspiration from the previous day. The imposition of WR2 occurred in a controlled and gradual manner, with the amount of water being reduced for 75% and four days after 50%. WR2 was maintained until the end of the experiment. The drought effects on the plants were also verified by the visual registration of the rolling of leaf +1. For the control of irrigation, a humidity sensor (ThetaProbe, type ML2x, Delta T, UK) was used, adding the necessary amount of water based on the soil moisture calibration curve.

Figure 2 -Experiment Diagram of sugarcane plants grown under two CO_2 concentrations and two water conditions. OTCs - Open top chambers; WR - water regimes.



Source: From the author (2022).

Growth measurements

Growth measurements were carried out weekly after 30 DAS: The plant height, from the stem base to apex; stem height, from the root base to the insertion of leaf +1; diameter of the stem at 5 cm above the ground; and leaf area were evaluated. At the end of the experimental period (100 DAS), the plants were harvested and separated into leaves, stems and roots. The different plant sections were dried in an oven with air forced circulation at 70 ºC, until constant weight, for determination of dry mass. Root to shoot ratio (R:S ratio) was calculated from the above-mentioned variables.

The dry mass (DM) was used to calculate the stress tolerance index (TI, %), considering the ratio between the stress treatment data and the control, based on the following equation (FERNANDEZ, 1992):

$$\mathrm{TI}(\%) = \frac{\text{DM in stress treatment}}{\text{DM control treatment}} * 100 \qquad (1)$$

Whole plant gas exchange and leaf relative chlorophyll content

Net CO_2 assimilation (*A*), and transpiration (*E*) rates measurements were performed considering the entire canopy, 50 DAS. For *A* measurements, a 3.7 dm^3 volume chamber was used, in a air closed system. The chamber was sealed with crystal plastic to allow light to pass through, measurements were performed with ambient CO_2 (~400 µmol mol^{-1}). A hose was attached to the side of the chamber and connect to an infrared gas analyzer (model SBA-5, PP Systems, EUA). The gas-exchange system was connected to a computer to record CO_2 concentration inside the chamber. Plants remained in the chamber for 3 minutes and the decay of the CO_2 concentration was recorded according to its consumption and the increase of water vapour was associated to transpiration, by the process of gas exchange between the plant and the air. For the calculation, the CO_2 and water vapour values of the first minute was disregarded to avoid interference from the initial adjustment when placing the plant in the chamber.

The parameters considered for the calculation of net photosynthesis (*A*) and transpiration (*E*) are described in equations 3 and 4 (MITCHEL, 1992).

$$A = \frac{(C1 - C2)}{(T2 - T1) * V\frac{1}{L}} \tag{2}$$

$$E = \frac{(W2 - W1)}{(T2 - T1) * V\frac{1}{L}} \tag{3}$$

where, *A*: net photosynthesis (µmol m^{-2} s^{-1}); *C*1: inicial [CO_2] (µmol CO_2 mol^{-1} air); *C*2: final [CO_2] (µmol CO_2 mol^{-1} air); *T*1: initial time (s); *T*2: final time (s); *V*: chamber volume (m^3); L: leaf area (m^2) and *E*: transpiration (mmol m^{-2} s^{-1}); *W*1:inicial [H_2O] (mmol H_2O mol^{-1} air) e *W*2: final [H_2O] (mmol H_2O mol^{-1} air).

The correction of *A* and E values were performed considering the local atmospheric pressure (P), in MPa, and temperature (T) in Kelvin, described in:

$$A_c = A * P * T \tag{4}$$

$$E_c = E * P * T \tag{5}$$

The relative chlorophyll index was estimated 30 DAS every week on +1 leaves according to the Kuijper system of leaf classification (DILLEWIJN et al., 1952) using an atLEAF + chlorophyll meter (FT Green LLC, USA)

Photosynthetic responses to CO_2 and light

Leaf gas exchange was evaluated at 74 DAS (44 days after started the carbon dioxide enrichment treatments and six days after plants reached WR2), with a portable infrared gas analyzer (Li-6800, Licor, USA), which was warmed up daily prior to the measurements. It is very important to note that theses measurements were performed in leaves that were totally developed inside the OTCs. The response of *A* to the increase in the intercellular CO_2 partial pressure (C_i) was carried out with a photosynthetic photon flux (*Q*) of 2000 µmol m^{-2} s^{-1} and the reference air [CO_2] (*Ca*) inside the leaf chamber was varied between 50 and 1800 µmol mol^{-1} in 10 steps, for plants maintained at 400 µmol mol^{-1} 400, 250, 150, 50, 400, 600, 800, 1000, 1500 e 1800 µmol mol^{-1} and for CO_2 eleveted treatment plants: 680, 480, 280, 150, 50, 680, 800, 1000, 1500 e 1800 µmol mol^{-1}. The start of the points of the curves was the same in which the plants were being grown (ambient CO_2 - 400 µmol mol^{-1} or elevated CO_2 - 680 µmol mol^{-1}), aiming to maintain the growth conditions of the last 44 days and start from the stable state of *A*.

The response curves of CO_2 an light were done with 21 and 2% O_2. These concentration were obtained through the use of cylinder containing a mix of gases (98% N_2 and 2% 0_2) before mix with the CO_2 concentrations.

Two biochemical parameters were calculated from the analytical solutions of the equations proposed by Collatz et al. (1992):

$$\theta M^2 - M(V_T + \alpha Q) + V_T \alpha Q = 0 \tag{6}$$

$$\beta A_g^2 - A_g \left(M + kT * \frac{Ci}{p}\right) + M * kT * \frac{Ci}{P} = 0 \tag{7}$$

Where θ means *A/Q* curve convexity (described below), *M* is the flux determined by Rubisco and light limited capacities, V_T is the temperature-dependent substrate-saturated Rubisco capacity, α means apparent quantum efficiency of *A/Q* curve, β means A/C_i curve convexity, A_g means gross leaf CO_2 assimilation, k_T is temperature-dependent PEPC efficiency related to C_i and *P* is atmospheric pressure. A_g was calculated as $A_g = A + R_T$, where R_T is temperature-dependent leaf respiration.

In order to standardize the biochemical parameters, the initial slope of the C_4 photosynthetic response (*k*), related to the PEPC carboxylation efficiency and the C_4 maximum Rubisco capacity ($V_{\max}$) were temperature-corrected to 25°C. The temperature dependencies were calculated as follows (COLLATZ et al., 1992):

$$V_T = \frac{V_{max} Q_{10}^{\frac{TI-25}{10}}}{(1 + e^{0.3(I3-TI)})(1 + e^{0.3(TI-36)})} \tag{8}$$

$$kT = k Q_{10}^{\frac{TI-25}{10}} \tag{9}$$

$$R_T = \frac{R_D Q_{10}^{\frac{TI-25}{10}}}{1 + e^{1.\,3(TI-55)}} \tag{10}$$

where Tl means leaf temperature, R_D [μmol m^{-2} s^{-1}] means dark respiration and Q_{10} is the proportional increase in a parameter value for 10 °C increase in leaf temperature (considered two for all the parameters).

To estimate curve convexity (*β*), we fitted the *A/Ci* curves following the equation proposed by Prioul and Chartier (1977):

$$A = \left(\frac{\phi_{I0} * I + Pg_{max} - ((\phi_{I0} * I + Pg_{max})^2 \; - 4\theta * \; \phi_{I0} * I * Pg_{max})0.5}{2\theta}\right) - R_D \tag{11}$$

where A means net photosynthesis rate [μmol m^{-2} s^{-1}], ϕ_{Io} = quantum yield at I = 0 [μmol μmol^{-1}], I = photosynthetic photon flux density [2000 μmol m^{-2} s^{-1}], Pg_{max} = maximum gross photosynthesis rate [μmol m^{-2} s^{-1}], θ = convexity (dimensionless).

The response of A to Q was evaluated after leaf tissue was briefly acclimated under a photosynthetic photon flux density (Q) of 2000 μmol m^{-2} s^{-1} for 5 min. *Ca* was maintained at 400 μmol mol^{-1} and 680 μmol mol^{-1} considering the concentration of CO_2 in which the plants were exposed, After reaching steady-state, leaves were illuminated as follows: 2000, 1500, 500, 200, 100, 80, 60, 40, 20 and 0 μmol m^{-2} s^{-1}. The response curves of A to Q were fitted with the equation proposed by Prioul and Chartier (1977), previously described.

Water status

Leaf discs with 6 mm² area were collected and weighed immediately, followed by soaking in water at room temperature for 24 h to record their turgid weight. The same leaf discs were subjected to 70 °C for 3 days to determine their dry weight. The relative water content (RWC) of leaves was derived using the following model (BARRS and WEATHERLEY, 1962):

$$RWC = \left[\frac{FW - DW}{TW - DW}\right] * 100 \tag{12}$$

where, FW, DW and TW represent the fresh weight, dry weight and turgid weight of leaves, respectively.

Leaf anatomy

At the end of the experimental period, 105 DAS, a mid-region samples of one fully expanded leaf blade developed under the CO_2 treatment of each plant was collected and fixed in 70% formaldehyde, glacial acetic acid, and ethanol (FAA) for 72 h and then transferred to 70% ethanol (JOHANSEN, 1940). Paradermal sections, from abaxial (ABE) and adaxial epidermis (ADE), were taken from the median region of the leaf using a double-edged razor. The sections were clarified with 50% sodium hypochlorite, stained with 1% Safranin and placed in slides with 50% glycerin. For cross sections, samples of 2 cm^2 were taken from the middle section of the leaf. The material was sectioned with a microtome and stained using Safrablau (0.1% Astra Blue and 1% Safranin) and placed in slides with 50% glycerin (LIMA et al., 2013).

The slides were photographed using a light microscope, Zeiss Scope, model AX10 (ZEISS, Germany) coupled with a digital camera Canon Powershot, model G10 (Canon Inc., Huntington, NY, USA) and the images were analyzed using the ImageJ® software.

For each anatomical characteristic, five replicates were composed by five sections of each slide, totalling 25 sections per treatment. The anatomical characteristics evaluated were: adaxial and abaxial epidermis stomatal density (stomata mm^{-2}), polar (PD, µm) and equatorial diameter (ED, µm) of the stomata, and PD:ED ratio, ratio ADE/ABE and thickness of bundle sheath cells (µm).

Determination of carbon and organic nitrogen and isotopic ratios

At the end of the experiment, the whole leaf +2 was collected. Subsequently, leaves were oven-dried and ground to a fine powder for analysis of carbon isotopic compositions.The leaf samples were dried in an oven at 50 ºC for 48h and homogenized in a cryogenic mill (Geno / Grinder 2010 - SPEX SamplePrep, USA) using liquid nitrogen. An aliquot of 7.0 mg of each sample was weighed in a tin capsule using a scale with a resolution of 1 µg (XP6 - Mettler Toledo, Switzerland). The homogenization of the samples increases the representativeness of the small aliquot of the sample. The capsules were analyzed in a continuous flow isotopic ratio mass spectrometry system (CF-IRMS) using an IRMS (Delta V, Thermo Scientific, Germany) coupled to an Elemental Analyzer (EA, Flash 2000, Thermo Scientific, Germany) through a gas interface (ConFlo IV, Thermo Scientific, Germany). The EA determined the levels of Total Organic Nitrogen (TON) and Total Organic Carbon (TOC) expressed in percentage of dry mass. The IRMS determined the isotopic ratios of carbon $R(^{13}C/^{12}C)$ expressed as a relative difference in the isotopic ratio ($\delta^{13}C$) in ‰, from the V-PDB and $\text{Air}_{\text{atmospheric}}$ patterns according to the equation (COPLEN, 2011).

$$\delta^{13}C = \frac{R\left({}^{13}_{\delta}C/{}^{12}_{\delta}C\right)_{sample}}{R\left({}^{13}_{\delta}C/{}^{12}_{\delta}C\right)_{V-PDB}} - 1 \qquad (13)$$

The results were normalized using the NBS-22 and IAEA-N1 standards. The standard uncertainty of the IRMS for or $\delta^{13}C$ is ±0.15‰.

Also, the gas samples of the cylinders that contained CO_2 were used to enriche the OTC's were collected and stored in 12 mL glass tubes with septum and were analyzed directly in an IRMS (ABCA, Secon, UK). The results of R ($^{13}C/^{12}C$) were also expressed in $\delta^{13}C$ according to equation. The standard uncertainty for $\delta^{13}C$ was ± 0.10 ‰ for CO_2 samples.

From these results, the fractionation of C (*Δ*) was calculated according to the equation:

$$\Delta_{sample/reference} = \frac{\delta^{13}{}_{sample}}{\delta^{13}_{Ar-cylinder}} \tag{14}$$

Experimental design

The overall experimental design was completely randomized. A three o-level factorial scheme (2 x 2 x 2), with two CO_2 concentration (400 µmol mol^{-1} and 680 µmol mol^{-1}), two varieties (RB867515 and RB855536) and two water regimes (WR1 and WR2) was used. Each experimental unit was composed of one plant per pot, with five replicates per treatment (n=5).

Statistical analyses

The employed net photosynthetic light-and CO_2 response curves (A/C_i and A/Q curves) were fitted by the Solver function of Microsoft Excel. Statistical analyzes were conducted using the R Studio statistical software (Version 1.2.5033 © 2009-2019 RStudio, Inc.). The significance (F-tests) of main effects and interactions between treatment factors was assessed by analysis of variance (ANOVA). Assessment of residuals showed that the data satisfied the assumptions of the analysis on the raw scale, so it was not necessary to transform. Pairs of means were assessed and separated by the standard error of the difference between means (SED) and the Fisher's protected least significant difference (LSD) test at P<0.05.

RESULTS

The biomass allocation is modulated by [CO_2] and water deficit

Overall, regarding both sugarcane varieties, the accumulation of biomass was affected by the water deficit, with reductions of 27%, 33%, 15% and 25% for leaf, stem, root, and total biomass, respectively (Table 2). It was noticed that the material RB867515 showed greater biomass accumulation in all organs, except in the roots when compared to RB855536 regardless both water condition and [CO_2] ($P \leq 0.05$). On the other hand, the elevated [CO_2] promoted a variation in biomass allocation through different organs, as the accumulation of total biomass was the same in both conditions of CO_2, however organs like a stem and root showed greater accumulation under elevated of CO_2 (Table 2).

The water stress condition was also a key factor for biomass distribution pattern in the plants. Plants exposed to water stress condition increased the root formation, inducing an increase in the dry mass ratio of root and shoots, regardless the variety.

Table 2- Allocation of dry matter of leaves, stems, roots and the root/shoot ratio from two varieties of *Saccharum* spp. grown under two CO_2 concentrations and two water conditions.

Factor	**Leaf dry matter (g)**	**Stem dry matter (g)**	**Root dry matter (g)**	**Total biomass (g)**	**Root:Shoot ratio**
[CO_2]					
680 µmol mol^{-1}	6.62±0.39	8.29±0.52	7.36±0.31	22.29±1.20	0.49±0.01
400 µmol mol^{-1}	7.05±0.46	7.13±0.58	6.42±0.34	20.96±1.24	0.46±0.02
SED	---	0.76	0.45	--	--
$LSD_{0.05}$	--	0.93	0.82	--	--
Variety (V)					
RB867515	7.48±0.39	8.53±0.57	7.27±0.29	23.39±0.99	0.46±0.02
RB855536	6.16±0.41	6.9±0.50	6.51±0.37	19.98±1.29	0.48±0.01
SED	0.55	0.73	--	1.59	--
$LSD_{0.05}$	0.53	0.93	--	2.67	--
Water regimes (WR)					
90%	7.95±0.33	9.28±0.44	7.45±0.28	24.81±0.83	0.44±0.01
50%	5.8±0.33	6.19±0.4	6.34±0.34	18.63±1.10	0.51±0.01
SED	0.45	0.57	0.44	1.35	0.02
$LSD_{0.05}$	0.75	0.93	0.82	2.67	0.05

Note. [CO_2], CO_2 concentration in µmol mol^{-1}; (V), variety; (WR), water regimes. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 30 for all variables. Values are reported as means ± standard error (n=20).

Source: From the author (2022).

The growth of plants was impaired, with plants under water deficit having lower heights than well-irrigated plants ($P < 0.05$). The green leaf area, did not show differences between varieties or between CO_2 treatments, just only the occurrence of drought promoted a decrease of 66% in this parameter ($P < 0.05$). The variety RB867515, described as drought tolerant, was the one with the highest plant and stem height with 150 ± 2.6 cm and 35 ± 0.7, respectively, regardless water condition and [CO_2] (Table 3).

Table 3 - Plant and stem height, stem diameter and leaf area of two varieties of *Saccharum* spp. grown under two CO_2 concentrations and two water conditions.

Factor	Plant height (cm)	Stem height (cm)	Stem diameter (mm)	Leaf area (m^2)
[CO_2]				
680 µmol mol^{-1}	143.34±1.81	32.24±0.72	11.38±0.31	0.34±0.04
400 µmol mol^{-1}	146.62±3.91	32±1.05	11.01±0.34	0.31±0.04
SED	--	--	--	--
$LSD_{0.05}$	--	--	--	--
Variety (V)				
RB867515	150.31±2.67	34.58±0.71	11.39±0.25	0.32±0.04
RB855536	139.47±2.60	29.67±0.67	11±0.38	0.33±0.04
SED	3.63	0.95	--	--
$LSD_{0.05}$	5.92	1.78	--	--
Water regimes (WR)				
90%	149.56±3.16	32.93±0.93	11.94±0.25	0.5±0.02
50%	140.22±2.20	31.32±0.83	10.4±0.28	0.17±0.009
SED	3.75	--	--	0.02
$LSD_{0.05}$	5.92	--	--	0.05

Note. .[CO_2], CO_2 concentration in µmol mol^{-1}; (V), variety; (WR), water regimes. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 30 for all variables. Values are reported as means ± standard error (n=20).

Source: From the author (2022).

Physiological responses under elevated [CO_2] and water deficit

Reductions of 13% in the relative chlorophyll index values were detected for plants grown with 680 µmol mol^{-1}, but the drought conditions did not changed the relative chloropyll index. The water deficit treatment affected the plant water status as the RWC dropped from 84% in well-irrigated plants to 78% in plants under water deficit, but was not observed differences caused by CO_2 treatments, or varieties (Table 4).

Table 4- Relative chlorophyll index (SPAD) and Relative water count (RWC) of two varieties of *Saccharum* spp grown under two CO_2 concentrations and two water conditions. (Continue)

Factor	SPAD	RWC (%)
[CO_2]		
680 µmol mol^{-1}	28.79±2.09	82.85±1.51
400 µmol mol^{-1}	33.09±1.92	79.39±1.73

SED	2.09	--
LSD$_{0.05}$	2.71	--
Variety (V)		
RB867515	27.95±1.01	82.9±1.18
RB855536	33.93±1.74	79.35±1.94
SED	--	--
LSD$_{0.05}$	--	--
Water regimes (WR)		
90%	30.97±1.62	83.87±1.25
50%	30.91±1.63	77.79±1.80
SED	--	2.13
LSD$_{0.05}$	--	3.94

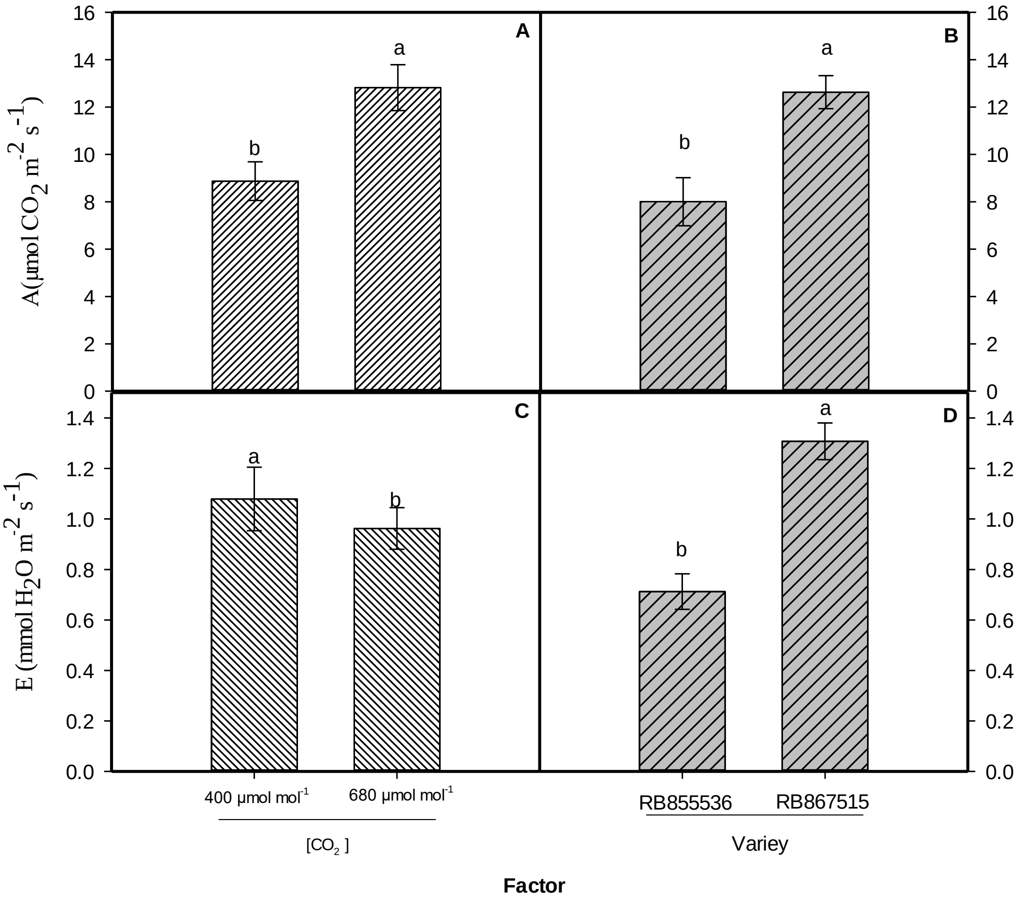
Note. .[CO_2], CO_2 concentration in μmol mol^{-1}; (V), variety; (WR), water regimes. SED, standard error of the difference between two treatment means LSD$_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 24 for SPAD and 29 for RWC. Values are reported as means ± standard error (n=16 for SPAD and 19 for RWC). (End)

Source: From the author (2022).

Photosynthesis and transpiration under elevated [CO_2]

Regarding the CO_2 assimilation (*A*), it was observed that the tolerant variety (RB867515) showed higher values than the susceptible one (RB855536). Likewise, the [CO_2] elevated condition favored a significant increase in *A* ($P \leq 0.001$) for both varieties (Figure 3A and B). Transpiration was higher at RB867515, but the exposure to elevated [CO_2] resulted in a decrease in this process (Figura 3C and D). There were no significant differences for the interaction between factors.

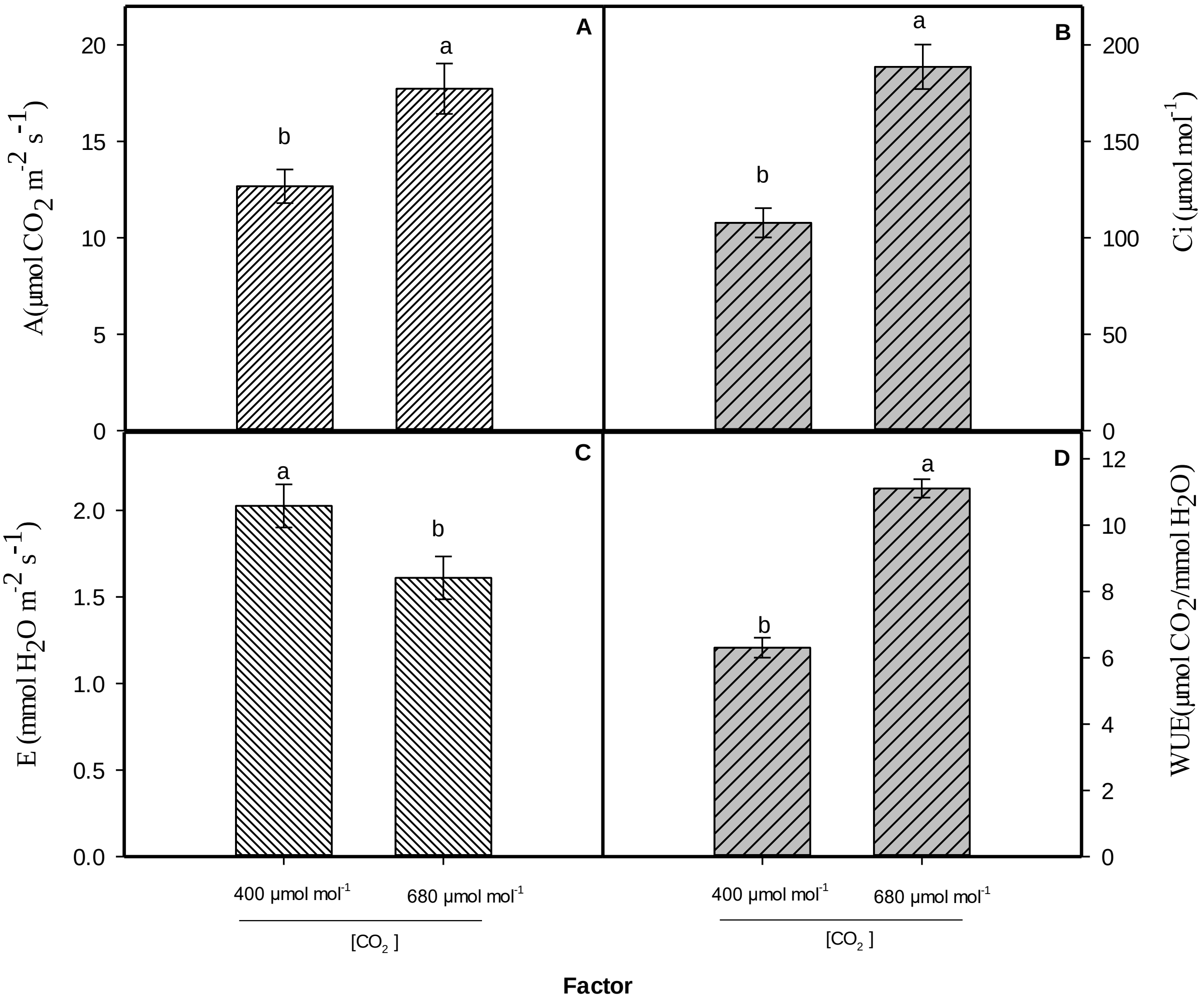
Figure 3- Photosynthesis (*A*, in A and B) and transpiration (*E*, in C and D) of two sugarcane varieties (RB867515 and RB855536) grown under ambient (400 µmol mol^{-1}) or elevated (680 µmol mol^{-1}) CO_2, at 50 day after sprouting (DAS), in a closed system considering all the leaves of the plants.



Note. Each histogram represents the mean value (n = 20) ± standard error ($LSD_{0.05}$, $P < 0.05$).
Source: From the author (2022).

Regarding to gas exchanges responses of sugarcane at 74 DAS. Were observed that [CO_2] elevated condition favored a significant increase in *A* ($P \leq 0.001$), C_i and WUE in both varieties (Figure 4A, B and D), whereas the transpiration decreased (Figure 4C).

Figure 4- Photosynthesis (*A*, in A), intercellular CO_2 (C_i, in B), transpiration (*E*, in C) and water use effciciency (WUE, in D) in leaves of sugarcane growing with ambient (400 µmol mol^{-1}) or elevated (680 µmol mol^{-1}) CO_2 at 74 days after sprouting (DAS).



Note. Each histogram represents the mean value (n = 20) ± standard error. $LSD_{0.05}$, $P < 0.05$.
Source: From the author (2022).

Stress tolerance under elevated [CO_2]

The stress tolerance index was estimated considering the total biomass, and showed a positive effect of elevated [CO_2], since plants submitted to high CO_2 showed higher values for this index than plants grown under environmental CO_2conditions ($P \leq 0.05$) (Table 5).

Table 5- Tolerance index of *Saccharum* spp plants grown under two CO_2 concentrations and two water conditions. (Continue)

Factor	Tolerance index (%)
[CO_2]	

680 µmol mol^{-1}	80.14±10.02
400 µmol mol^{-1}	66.94±7.21
SED	4.52
LSD$_{0.05}$	8.98

Note. [CO$_2$], CO$_2$ concentration in µmol mol^{-1}. SED, standard error of the difference between two treatment means; LSD$_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 12. Values are reported as means ± standard error (n=8). (End)

Source: From the author (2022).

Anatomical responses

For the anatomical traits, the stomatal density (SD) was significantly influenced by the variety and the water regimes. RB867515 variety showed higher SD in the adaxial surface than RB855536 (60 and 52 stomata mm^{-2}, respectively) and with the reduced water availability in WR2, there was an increase in the stomatal density (ADA) ($P < 0.05$) (Table 6). The SD was significantly influenced ($P \leq 0.05$) by the interactions between variety and CO$_2$ concentration (V x CO$_2$) variety and water regime (V x WR) and by the interaction between CO$_2$ concentration and water regime (CO$_2$ x WR). Plants of the RB855536 variety grown under 680 µmol mol^{-1} had the lowest SD (46 stomata mm^{-2}), whilst plants of the RB867515 variety under water deficit had the highest SD (67 stomata mm^{-2}) while plants grown with elevated CO$_2$ that maintained adequate irrigation had the lowest SE (46 stomata mm^{-2}) (Table 7).

On the abaxial surface, stomatal density was reduced for plants subjected to high CO$_2$ from 118 under 400 µmol mol^{-1} to 102 under high CO$_2$ concentration, regardless water regime and variety (Table 6). However, a higher SD was observed for plants grown under the environment with 400 µmol mol^{-1} and adequate irrigation ($P \leq 0.05$) (Table 7). Regarding the ratio of the number of stomata on adaxial/abaxial sides, it was observed that factors such as variety and water condition were significant ($P \leq 0.05$). Plants of RB855536 variety showed a higher ratio than RB867515 variety (0.57 and 0.45, respectively), and that the stress condition increased this variable from 0.48 in well-irrigated plants to 0.54 in plants under water deficit.

Table 6- Anatomical traits of leaves of two varieties of *Saccharum* spp. grown under two CO$_2$ concentrations and two water conditions.(Continue)

Factor	**Stomatal density (stomata mm^{-2})**		**PD:ED ratio**		**Ratio**	**Cell wall thickness (µm)**
	ADE	**ABE**	**ADE**	**ABE**	**ADE/ABE**	**-**
[CO$_2$]						
680 µmol mol^{-1}	53±3.34	102±4.84	3.52±0.122	3.61±0.11	0.52±0.03	2.29±0.2

400 μmol mol^{-1}	58±1.66	118±4.96	3.45±0.06	3.57±0.07	0.5±0.02	2.79±0.11
SED	--	6.75	--	--	--	0.22
LSD$_{0.05}$	--	12.43	--	--	--	0.42
Variety (V)						
RB867515	60±2.46	105±3.95	3.35±0.065	3.59±0.06	0.45±0.02	2.31±0.13
RB855536	52±2.59	115±6.07	3.62±0.112	3.59±0.11	0.57±0.02	2.76±0.18
SED	3.49	--	0.12	--	0.03	0.22
LSD$_{0.05}$	5.46	--	0.24	--	0.06	0.42
Water regimes (WR)						
90%	52±2.19	110±4.77	3.43±0.07	3.54±0.04	0.48±0.02	2.67±0.16
50%	59±2.92	110±5.69	3.54±0.11	3.64±0.12	0.54±0.03	2.4±0.16
SED	3.56	--	--	--	0.03	--
LSD$_{0.05}$	5.46	--	--	--	0.06	--

Note. [CO$_2$], CO$_2$ concentration in μmol mol^{-1}; (V), variety; (WR), water regimes; PD/ED ratio, ratio between the polar diameter and the equatorial diameter of the stomata; ratio, ratio between number of adaxial stomata (ADE) and number of abaxial stomata (ABE), cell wall thickness, thickness of bundle sheath cells; SED, standard error of the difference between two treatment means; LSD$_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 30 for all variables. Values are reported as means ± standard error (n=20). (End)

Source: From the author (2022).

Regarding the stomatal functionality (PD:ED ratio), on the abaxial surface there was no significant ($P > 0.05$) for any of the factors evaluated or for the interaction between them. On the adaxial side, the RB855536 variety presented higher values for this variable (Table 6). It was observed that plants of variety RB867515 when grown under 680 μmol mol^{-1} have lower PD:ED ratio, however the variety RB855536 under the same condition of CO$_2$ showed the highest values (3.26 and 3.78, respectively) (Table 7).

The cell wall thickness was higher in plants grown under high CO$_2$ and regardless of the water regime and the CO$_2$ condition, the RB855536 variety showed greater cell wall thickness of the sheath.

Table 7- Anatomical traits of leaves of two varieties of *Saccharum* spp. grown under two CO$_2$ concentrations and two water conditions. (Continue)

	Stomatal density (stomata mm^{-2})		**PD:ED ratio**
Interaction	**ADE**	**ABE**	**ADE**
(V) x [CO$_2$]			
RB867515 x 400	59±2.02	109±4.76	3.44±0.08
RB867515 x 680	61±4.77	102±6.61	3.26±0.1
RB855536 x 400	58±2.85	127±8.12	3.45±0.1

RB855536 x 680	46±3.57	102±7.8	3.78±0.2
SED	6.55	--	0.24
LSD$_{0.05}$	7.72	--	0.35
(V) x (WR)			
RB867515 x 90%	53±2.13	101±5.47	3.36±0.1
RB867515 x 50%	67±3.22	109±5.97	3.34±0.09
RB855536 x 90%	52±4.11	118±7.27	3.49±0.1
RB855536 x 50%	51±3.61	111±10.4	3.74±0.2
SED	6.35	--	--
LSD$_{0.05}$	7.72	--	--
[CO$_2$] x (WR)			
400-90%	59±2.32	126±4.13	3.4±0.08
400-50%	58±2.62	109±8.65	3.49±0.09
680-90%	46±2.47	93±4.01	3.45±0.12
680-50%	60±5.54	111±8.32	3.59±0.22
SED	6.65	12.63	--
LSD$_{0.05}$	7.72	12.43	--

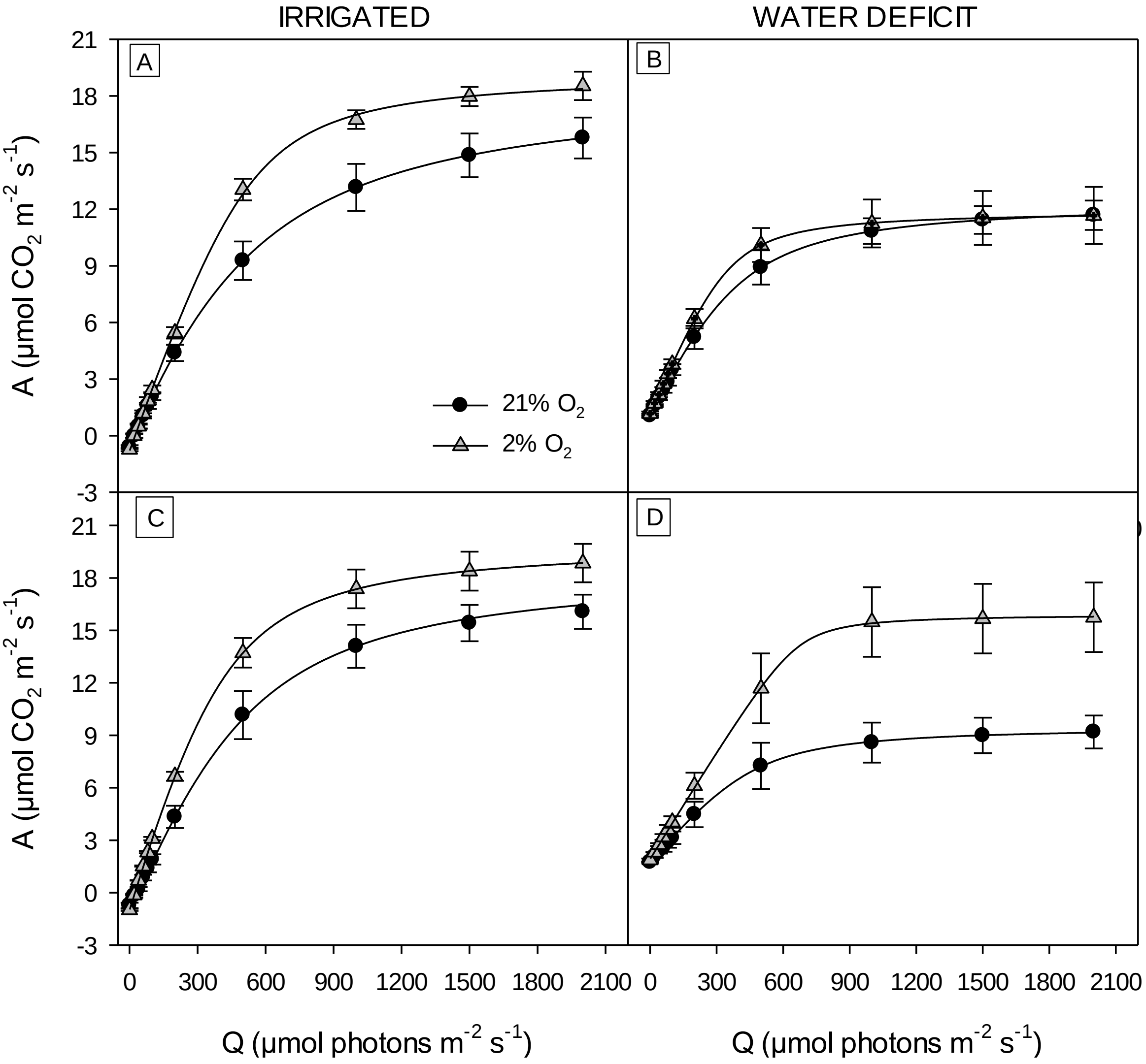
Note. (V), variety; [CO$_2$], CO$_2$ concentration in μmol mol^{-1}; (WR), water regimes; PD/ED ratio, ratio between the polar diameter and the equatorial diameter of the stomata; SED, standard error of the difference between four treatment means; LSD$_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 32 for all variables. Values are reported as means ± standard error (n=10). (End)

Source: From the author (2022).

Photosynthesis responses to increased photosynthetically active radiation.

The rate of net photosynthesis (*A*) in response to light from both varieties was affected only by the water deficit ($P \leq 0.05$) (Figures 5 and 6), with a reduction in the maximum CO$_2$ assimilation of 33% in the RB867515 variety and 26% in RB85553. The assimilation in the RB85536 variety was also influenced by the interaction between [CO$_2$] and water regime (WR), considering that plants grown under ambient CO$_2$ and that underwent stress presented lower photosynthetic rates when compared to the other treatments (Figures 5 and 6).

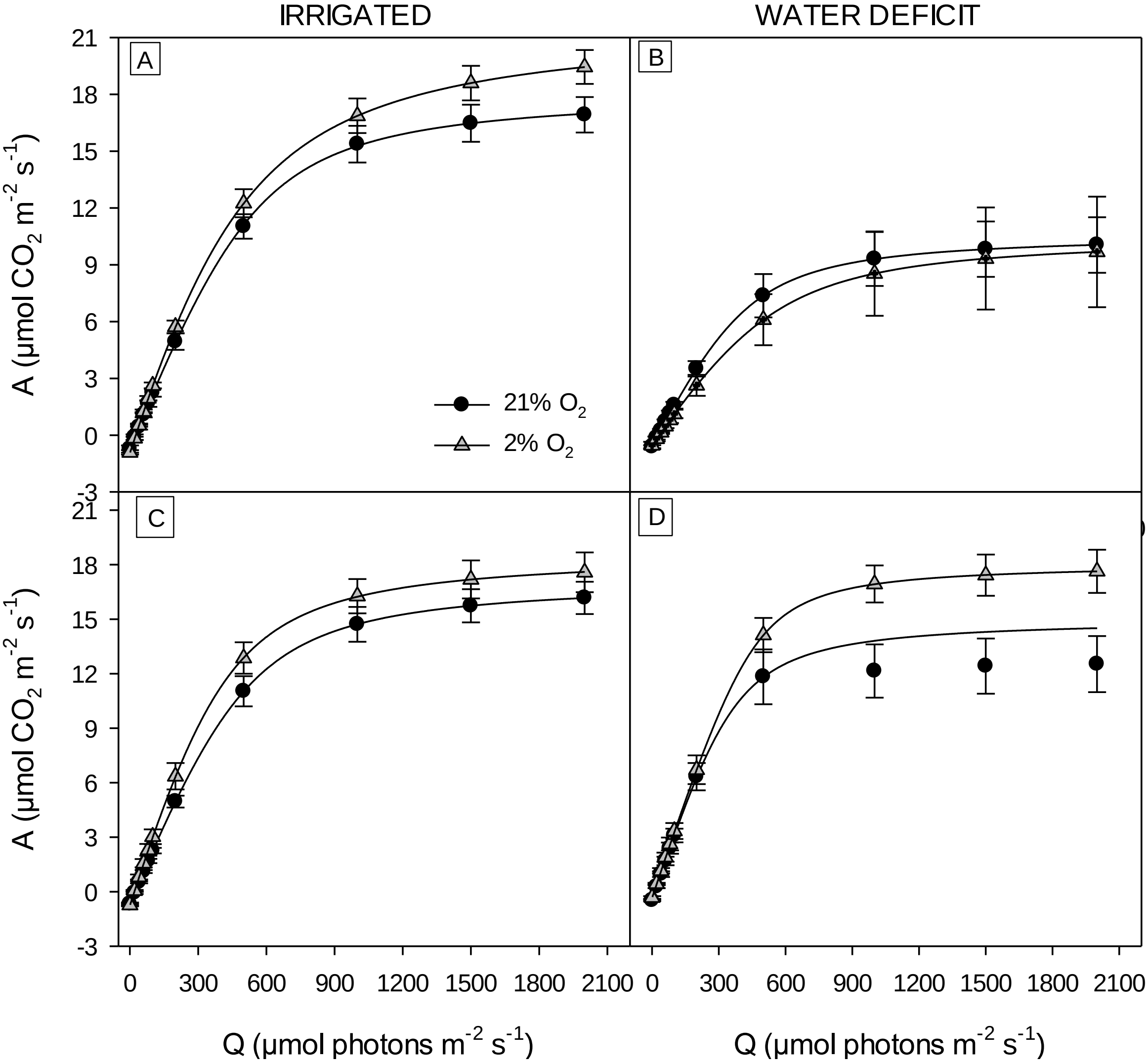
Figure 5- Responses of CO_2 assimilation (*A*) to the increase in photosynthetically active radiation (*Q*) of the RB867515 sugarcane variety grown under 400 µmol mol^{-1} (in A and B) and 680 µmol mol^{-1} (in C and D) under irrigated conditions and water deficit, on 21% and 2% O_2.



Note. Each symbol represents the mean value (n = 5) ± standard error.

Source: From the author (2022).

Figure 6- Responses of CO_2 assimilation (*A*) to the increase in photosynthetically active radiation (*Q*) of the RB855536 sugarcane variety grown with 400 µmol mol^{-1} (in A and B) and 680 µmol mol^{-1} (in C and D) under conditions irrigated and water deficit, on 21% and 2% O_2.



Note. Each symbol represents the mean value (n = 5) ± standard error.

Source: From the author (2022).

The light compensation point (ΓQ) for the RB867515 variety was influenced only by the oxygen condition ($P \leq 0.05$), as measurements with 2% of the gas were lower when compared with measurements of 21% O_2 (20.4 ± 1.8 and 26.1 ± 2.1 µmol photons m^{-2} s^{-1}, respectively) (Table 8). In the RB855536 variety, the factor that causes a decrease in the values of this parameter was the elevated [CO_2], since plants grown on 400 µmol mol^{-1} had an average value of 25.4 ± 2.1 µmol m^{-2} s^{-1} while plants grown on 680 µmol mol^{-1} showed an average value of 18.0 ± 1.7 µmol m^{-2} s^{-1}.

Regarding the apparent quantum efficiency Φ, in the RB867515 variety, only the occurrence of stress caused decrease in this parameter ($P \leq 0.05$) (Table 8). For the RB855536 variety, the water deficit affected this variable as an isolated factor and the CO_2 concentration promoted an increase in Φ from 0.028 ± 0.003 in plants on ambient CO_2 to 0.036 ± 0.002 in plants on eCO_2, regardless of the water regime and the oxygen condition (Table 10).

There was significance for the interactions between oxygen and the water regimes (O_2 x WR) and for interaction between CO_2 and the water regimes (CO_2 x WR). Since curves performed on well-irrigated plants with 2% O_2 showed the highest quantum yield when compared to the rest of the treatments. In the CO_2 x WR interaction, the lowest efficiency was registered for stressed plants that grew on 400 µmol mol^{-1} (Table 11).

Table 8- Maximum CO_2 assimilation (*A*), light compensation point (ΓQ), apparent quantum yield (Φ) and convexity factor (θ) of the RB867515 variety grown under two water conditions and evaluated on two oxygen conditions.

Factor	***A*** **(µmol CO_2 m^{-2} s^{-1})**	**ΓQ (µmol photons m^{-2} s^{-1})**	**Φ**	**Θ**
Oxygen (O_2)				
21%	13.54±1.15	26.14±2.1	0.03±0.002	0.73±0.05
2%	14.83±0.99	20.38±1.86	0.03±0.002	0.87±0.03
SED	--	2.73	--	0.06
$LSD_{0.05}$	--	5.40	--	0.11
Water regimes (WR)				
90%	16.79±0.53	22.68±3.03	0.03±0.002	0.74±0.05
50%	11.3±1.14	23.91±2.95	0.02±0.002	0.87±0.03
SED	1.23	--	0.003	0.06
$LSD_{0.05}$	2.55	--	0.006	0.11

Note. (O_2) O_2 concentration ; (WR), water regimes. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 30 for all variables. Values are reported as means ± standard error (n=20).

Source: From the author (2022).

Dark respiration (Rd) of the RB867515 variety was affected by the interaction between the water condition and the CO_2 concentration. Thus, plants in drought stress grown in the eCO_2 treatment maintained the lowest ($P < 0.05$) Rd when compared to the other of the treatments (0.43 µmol m^{-2} s^{-1}) (Table 9).

Table 9- Dark respiration (Rd) of the RB867515 variety grown under two CO_2 concentration and two water conditions.

Interaction	RB867515
$[CO_2]$ x (WR)	Rd (μmol CO_2 m^{-2} s^{-1})
400 - 90%	0.67±0.07
400 - 50%	0.72±0.08
680 - 90%	0.8±0.09
680 - 50%	0.43±0.10
SED	0.34
$LSD_{0.05}$	0.25

Note. $[CO_2]$, CO_2 concentration in µmol mol^{-1}; (WR), water regimes. SED, standard error of the difference between four treatment means; $LSD_{0.05}$, least significant difference at $P<0.05$; residual degrees of freedom = 30 for all variables. Values are reported as means ± standard error (n=10).

Source: From the author (2022).

In the RB855536 variety, only water restriction caused a 28% decrease in respiration values (Table 10). The convexity factor for the RB867515 variety was influenced by the concentration of oxygen and the water regime as isolated factors, as plants evaluated with 2% oxygen and stressed plants showing the highest values for this variable (Table 8). On the other hand, the RB855536 variety under elevated CO_2 concentration showed the highest convexity factor (0.87 ± 0.02) (Table 10).

Table 10- Maximum CO_2 assimilation (*A*), light compensation point (ΓQ), dark respiration (Rd), apparent quantum yield (Φ) and convexity factor (θ) of the RB855536 variety grown under two CO_2 concentrations and two water conditions. (Continue)

Factor	A (μmol CO_2 m^{-2} s^{-1})	ΓQ (μmol photons m^{-2} s^{-1})	Rd (μmol CO_2 m^{-2} s^{-1})	Φ (μmol of CO_2 μmol^{-1} photons)	θ
$[CO_2]$					
400 µmol mol^{-1}	14.68±1.24	25.44±2.08	0.7±0.083	0.028±0.003	0.75±0.042
680 µmol mol^{-1}	16.48±1.00	17.99±1.69	0.63±0.062	0.036±0.002	0.87±0.024
SED	--	2.60	--	0.003	0.047
$LSD_{0.05}$	--	5.09	--	4.00E-03	0.096
Water regimes (WR)					
90%	17.71±0.62	21.82±1.61	0.76±0.068	0.035±0.002	0.8±0.025
50%	13.11±1.34	21.36±2.64	0.55±0.068	0.028±0.003	0.82±0.049

SED	1.43	--	0.093	0.003	0.053
$\mathbf{LSD_{0.05}}$	2.50	--	0.191	4.00E-03	--

Note . [CO_2], CO_2 concentration ; (WR), water condition. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 27 for all variables. Values are reported as means ± standard error (n=20). (End)

Source: From the author (2022).

Plants of the variety RB855536 when evaluated under 2% oxygen showed the highest photosynthetic rates and a higher apparent quantum yield (18.54 µmol m^{-2} s^{-1} and 0.04 respectively). In the drought condition together with ambient CO_2 this variety was affected by the lowest rate of A with a value of 9.99 µmol m^{-2} s^{-1} (Table 11).

Table 11- Maximum CO_2 assimilation (*A*) and apparent quantum yield (Φ) of the RB855536 variety grown under two water conditions and evaluated in two O_2 concentration.

Interaction	**RB855536**	
Oxygen(O_2) x Water regimes(WR)	**A**	**Φ**
21%-90%	16.97±1.00	0.03±0.002
21%-50%	11.64±1.66	0.03±0.003
2%-90%	18.54±0.74	0.04±0.002
2%-50%	14.58±2.19	0.03±0.005
SED	--	0.067
$\mathbf{LSD_{0.05}}$	--	0.06
[CO_2] x (WR)		
400 -90%	17.97±0.81	0.03±0.002
400 -50%	9.99±1.52	0.02±0.003
680 - 90%	17.42±1.07	0.04±0.003
680 -50%	15.53±1.77	0.04±0.002
SED	2.53	0.046
$\mathbf{LSD_{0.05}}$	3.35	0.06

(O_2), O_2 concentration; [CO_2] CO_2 concentration in µmol mol^{-1} ; (WR), water condition. SED, standard error of the difference between four treatment means; $LSD_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 27 for all variables. Values are reported as means ± standard error (n=10)

Source: From the author (2022).

Photosynthesis responses to increased intercellular CO_2 concentration.

In the photosynthesis response curve to the increase in internal CO_2 concentration (A/C_i), the varieties RB867515 and RB855536 showed a decrease in the values of the maximum rate of carboxylation by Rubisco (V_{max}) due to the drought. However, there was a response positive for V_{max} in plants of the RB855536 variety evaluated under 2% O_2, since they went from 17.16 μmol m^{-2} s^{-1}under conditions of 21% O_2 to 20.55 under 2% O_2 (Table 12). Maximum carboxylation rate of PEPC (V_{pmax}) in both varieties was influenced by the concentration of CO_2 independent of water condition and oxygen concentration ($P \leq 0.05$), with high CO_2 promoting a decrease in the values for this variable (Table 12).

Table 12- Rate of carboxylation by Rubisco (V_{max}) and maximum carboxylation rate of PEPC (V_{pmax}) of two varieties of *Saccharum* spp. grown under two CO_2 concentrations, two water conditions and two oxygen conditions.

Factor	RB867515		Factor	RB855536	
	V_{max}	V_{pmax}		V_{max}	V_{pmax}
Oxygen- (O_2)	(μmol CO_2 m^{-2} s^{-1})	(mol CO_2 m^{-2} s^{-1})	**Oxygen- (O_2)**	μmol CO_2 m^{-2} s^{-1}	(mol CO_2 m^{-2} s^{-1})
21%	17.91±1.72	0.28±0.02	21%	17.16±1.12	0.21±0.02
2%	16.73±1.35	0.26±0.02	2%	20.55±1.41	0.24±0.03
SED	--	--	**SED**	1.74	--
$LSD_{0.05}$	--	--	**$LSD_{0.05}$**	2.39	--
[CO_2]			**[CO_2]**		
400 μmol mol^{-1}	17.29±1.53	0.33±0.02	400 μmol mol^{-1}	20.04±1.62	0.28±0.02
680 μmol mol^{-1}	17.39±1.61	0.19±0.01	680 μmol mol^{-1}	17.89±1.00	0.16±0.02
SED	--	0.03	**SED**	--	0.03
$LSD_{0.05}$	--	0.06	**$LSD_{0.05}$**	--	0.06
Water regimes (WR)			**Water regimes (WR)**		
90%	22.07±0.75	0.3±0.02	100%	21.98±0.78	0.24±0.02
50%	11.66±0.81	0.24±0.03	50%	15.02±1.28	0.2±0.03
SED	1.08	--	**SED**	1.44	--
$LSD_{0.05}$	1.95	--	**$LSD_{0.05}$**	2.39E+00	--

(O_2), O_2 percentage; (CO_2), CO_2 concentration ; (WR), water condition. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at P< 0.05; residual degrees of freedom = 27 for all variables. Values are reported as means ± standard error (n=20).

Source: From the author (2022).

There was a significant interaction for V_{max} between O_2 and CO_2 (O_2 x CO_2) in the RB867515 variety and between CO_2 and water regimes (CO_2 x WR) in RB855536. Being that evaluations with 2% of oxygen in plants grown at 400 μmol mol^{-1} of RB867515 variety, presented the lowest value for this variable (15.26 ± 2.03) (Table 13) while plants of variety RB855536, grown on 400 μmol mol^{-1} and well-irrigated showed the maximum value with 23.85 ± 0.97 (Table 14).

Table 13- Rate of carboxylation by Rubisco (V_{max}) of RB867515 Variety grown under two CO_2 concentrations and two oxygen conditions.

Interaction	**RB867515**
(O_2) x [CO_2]	**Vmax** (µmol CO_2 m^{-2} s^{-1})
21%: 400	19.1±2.29
21%:680	16.58±2.84
2%:400	15.26±2.03
2%:680	18.21±1.89
SED	4.30
$LSD_{0.05}$	2.68

Note. (O_2), O_2 percentage; [CO_2], CO_2 concentration in µmol mol^{-1}. SED, standard error of the difference between two treatment means; $LSD_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 25 for the V_{max}. Values are reported as means ± standard error (n=10).

Source: From the author (2022).

Regarding V_{pmax}, there was significant interaction ($P \leq 0.05$) only for the RB55536 variety, as increase in plants grown in ambient CO_2 and evaluated with 2% O_2 (Table 14). Likewise, increases were recorded for this variable in plants under adequate irrigation that grew in ambient CO_2 and were evaluated under 2% O_2.

Table 14- Rate of carboxylation by Rubisco (V_{max}) and maximum carboxylation rate of PEPC (V_{pmax}) of RB855536 variety grown under two CO_2 concentrations, two water conditions and two oxygen conditions.(Continue)

Interaction	**RB855536**
[CO_2] x (WR)	***V_{max}*** (µmol CO_2 m^{-2} s^{-1})
400:90%	23.85±0.97
400:50%	14.32±2.27
680:90%	19.88±0.76
680:50%	15.61±1.69
SED	2.84
$LSD_{0.05}$	3.27
(O_2) x [CO_2]	***V_{pmax}*** (mol CO_2 m^{-2} s^{-1})
21%: 400	0.22±0.04
21%:680	0.19±0.03
2%:400	0.34±0.03

2%:680	0.13±0.03
SED	0.07
LSD$_{0.05}$	0.08

(O_2) x [CO_2]x (WR)	***V*$_{\mathbf{pmax}}$** (mol CO_2 m^{-2} s^{-1})
21:400 90	0.28±0.05
21:400:50	0.15±0.04
21:680:90	0.13±0.01
21:680:50	0.26±0.06
2:400:90	0.37±0.07
2:400:50	0.32±0.04
2:680:90	0.19±0.04
2:680:50	0.05±0.02
SED	0.08
LSD$_{0.05}$	0.12

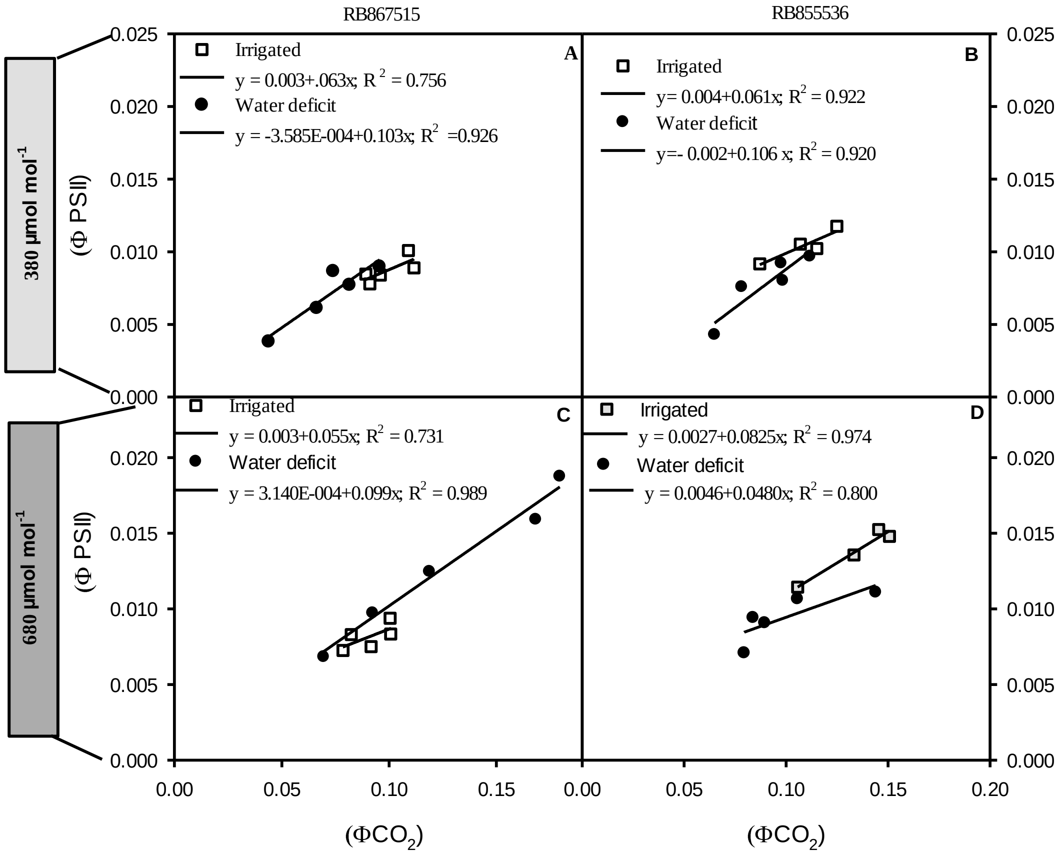
Note.(O_2), O_2 percentage; (CO_2), CO_2 concentration in µmol mol^{-1}; (WR), water regimes in %. SED, standard error of the difference between two treatment means; LSD$_{0.05}$, least significant difference at $P < 0.05$; residual degrees of freedom = 27 for the all variables. Values are reported as means ± standard error (n=10) for interactions (CO_2 x WR) and (O_2 x CO_2) (n=5) and for interaction (O_2 x CO_2 x WR).

(End)

Source: From the author (2022).

The relationship between ΦPSII and ΦCO_2 was linear for all treatments. However, for well watered plants for the tolerant variety (RB867515), high [CO_2] caused a decrease in the slope value, from 0.63 to 0.055. For RB855536, plants grown under water deficit and high CO_2 levels had the least steep slope (0.0480) for the correlation relationship between ΦPSII and ΦCO_2 (Figure 7).

Figure 7- The relationship between ΦPSII and ΦCO_2 of two varieties of *Saccharum* spp. grown with 400 µmol mol^{-1} (A and B) and 680 µmol mol^{-1} (C and D) under conditions irrigated and water deficit.



Note. On the left side, the equations that represent the correlation between the variables quantum yield of PSII (ΦPSII) and quantum yield of CO_2 fixation (ΦCO_2), with the value of m in the linear equation Y = mx + b, corresponding to the value of ($\Phi PSII / \Phi CO_2$) of each material in each water condition.
Source: From the author (2022).

Carbon and Nitrogen Responses

There was an increase in the percentage of total organic carbon for plants subjected to elevated CO_2 regardless of variety and water regime ($P \leq 0.05$), however, water restriction as an isolated factor caused decreases in this variable. On the isotopic composition, it was observed that concentrations of 400 µmol mol^{-1} promoted a decrease in **$\Delta^{13}C$** (Table 15).

Table 15- Percentage of total organic nitrogen (PTON), percentage of total organic carbon (PTOC), and carbon discrimination ($\Delta^{13}C$) of two varieties of *Saccharum* spp. grown under two CO_2 concentrations and two water conditions. (Continue)

Factor	**PNOT (%)**	**PTOC (%)**	**$\Delta^{13}C$ (‰)**
Variety (V)			
RB867515	0.56±0.02	40.13±0.19	-4.73±0.31

RB855536	0.62±0.03	40±0.09	-4.66±0.33
SED	--	--	--
LSD$_{0.05}$	--	--	--
[CO$_2$]			
400 µmol mol^{-1}	0.63±0.03	39.87±0.17	-5.88±0.03
680 µmol mol^{-1}	0.56±0.02	40.26±0.10	-3.51±0.07
SED	--	0.19	0.071
LSD$_{0.05}$	--	0.31	0.15
Water regimes (WR)			
90%	0.57±0.01	39.8±0.14	-4.7±0.32
50%	0.62±0.04	40.33±0.12	-4.69±0.32
SED	--	0.18	--
LSD$_{0.05}$	--	0.31	--

.*Note*.[CO$_2$], CO$_2$ concentration in µmol mol^{-1}; (V), variety; (WR), water regimes. SED, standard error of the difference between two treatment means; LSD$_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 24 for all variables. Values are reported as means ± standard error (n=16). (End)
Source: From the author (2022).

The total percentage of organic nitrogen was influenced by the interaction between CO$_2$ concentration and water regime ($P \leq 0.05$), and the 400 µmol mol^{-1} environment together with water restriction promoted an increase for this variable. Concerning PTOC, the highest value for PTOC was registered for plants of the RB867515 variety grown on 680 µmol mol^{-1} and for plants in the same condition but which had adequate irrigation (Table 16).

Table 16- Percentage of total organic nitrogen (PTON), percentage of total organic carbon (PTOC) of two varieties of *Saccharum* spp. grown under two CO$_2$ concentrations and two water conditions.(Continue)

Interaction	**PTON**
[CO$_2$] x (WR)	**%**
400: 90%	0.54±0.02
400:50%	0.71±0.05
680:90%	0.60±0.01
680:50%	0.52±0.03
SED	0.06
LSD$_{0.05}$	0.09
(V) x **[CO$_2$]**	**PTOC %**
RB855536:400	39.97±0.09
RB855536:680	40.04±0.16
RB867515:400	39.78±0.35
RB867515:680	40.48±0.1

SED	0.38
$LSD_{0.05}$	0.44

Variety (V) x [CO_2] x (WR)	**PTOC %**
RB855536:400:90%	39.87±0.12
RB855536:400:50%	40.06±0.13
RB855536:680: 90%	39.77±0.15
RB855536:680:50%	40.31±0.26
RB867515:400: 90%	39.14±0.33
RB867515:400:50%	40.43±0.44
RB867515:680: 90%	40.43±0.15
RB867515:680:50%	40.54±0.16
SED	0.60
$LSD_{0.05}$	0.62

Note. (V), variety; [CO_2], CO_2 concentration, in µmol mol^{-1}; (WR), water regimes ; SED, standard error of the difference between two treatment means. $LSD_{0.05}$, least significant difference at $P< 0.05$; residual degrees of freedom = 24 for the all variables. Values are reported as means ± standard error (n=8) for interactions (CO_2 x WR) and (V x CO_2) (n=4) and for interaction (V x CO_2 x WR). (End)

Source: From the author (2022).

DISCUSSION

Responses to eCO_2 have been correlated with photosynthetic limitations as Rubisco is fundamentally inefficient, due to its relatively low affinity for CO_2 and low catalytic rate of the carboxylation reaction. Thus, with the increase in CO_2 supply, the photosynthetic process is optimized and increases in biomass may be observed (LEAKEY and LAU, 2012). This explains why C_3 plants respond more strongly than C_4 plants, once the last already show mechanism for CO_2 concentration, being more efficient and therefore less responsive to variations in CO_2 concentration in the environment. However, our results clearly show the sugarcane varieties, even being a C_4 plant, respond to the increase in CO_2, since the increase in A, decrease in E and differentiated biomass allocation were observed in well-watered plants. Considering that under high CO_2 plants improve WUE so during a drought event, this effect could alleviate stress, without implying that C_4 plants are exclusively responsive under these conditions.

Water deficit is one of the abiotic factors that most affects C and N metabolism, since it affects the hydraulic conductivity and decreases stomatal conductance (g_s), which reduces transpiration rate (E). Consequently, the absorption and transport of nitrogen compounds via transpiration stream is compromised. There is also limitation of photosynthesis, inducing increases in photorespiration and changes in the redox state of the cell (BUCHANAN and JONES, 2015). Limitation in photosynthesis is mainly a result of a decrease in the concentration of internal carbon (C_i), due to the reduction in stomatal conductance, and photoinhibition, due to the excess of reducing power formed in the photochemical phase of photosynthesis (DING et al., 2018).

In this sense, in the present work the intensity and duration of the water restriction was enough to cause changes in the process of CO_2 diffusion and use of light by the two varieties (Figures 5 and 6). Considering the same varieties, other study showed the predawn water potentials (Ψpd) of -0.5 MPa (moderate stress) and -1.0 MPa (severe stress) lead to molecular and physiological changes in the two materials, since genes involved in photosystem I (PSI) were repressed (Vital et al., 2017). Proteins involved in photosynthesis are the main target for oxidation and there is post-translational modulation of Rubisco, which leads to the loss of enzyme function, limiting photosynthesis and consequently the availability of carbohydrates for the biosynthesis of sucrose and starch. In relation to PEPC activity, there was no change caused by water restriction. Lobo et al. (2016) indicated that during drought, biochemical limitations of photosyntesis are often found, and in C_4 plants during the stress period, there is a preferential inhibition of Rubisco rather than PEPC.

These limitations in plant metabolism lead to changes in growth and biomass accumulation as has been reported in several studies (VITAL et al., 2017; DING et al., 2018; ENDRES et al., 2019). Specifically in sugarcane crop, it is known that characteristics such as plant height, stem diameter, among other morphological parameters, strongly depend on the plant genetic potential. However, the interaction with the environment determines the morphological and growth responses of this species under stress (LIMA et al., 2016). This was observed in our study, since water stress resulted in a decrease in the biomass and in the allocation patterns of the varieties evaluated here (Tables 2 and 3). However, it is noteworthy that the genotype RB867515 was less affected by the imposed stress, as it presented higher biomass production than RB855536.

Here, the sugarcane varieties showed some of the typical responses of plants exposed to high CO_2 during long term. Among these responses, the pattern of biomass allocation changed, with greater accumulation of biomass in stems and roots than in leaves (Table 2). Approbato (2015) attribuited this effect to the interaction between eCO_2 and high temperatures. However, in this experiment, the temperature variation between OTCs with high CO_2 and ambient CO_2 was only ± 1.02 ºC ($P \leq 0.05$). Therefore, the differential allocation of biomass was attributed only to the effect of CO_2. Because the increase in dry matter in sink organs (stem and root) depends mainly on assimilation and transport of carbon from source organs (leaves) (LARCHER, 2000), the increase in A under high CO_2 (Figure 3B) associated to the higher amount of total organic carbon (TOC) found in plant leaves under eCO_2 (Table 15) may have positively influenced the transport of photoassimilates, by increasing the difference between source and sink, stimulating the accumulation of biomass in these organs. Also we observed decrease in transpiration (Figure 3B), which favors a higher water use efficiency (WUE) (DE SOUZA et al., 2008).

The relative chlorophyll index is correlated with chlorophyll and/or N content present in the leaves (YANG et al., 2015). The values recorded under high CO_2 (Table 4) could be related to four main causes: *i.* due to the effect of dilution by growth, where plants grown under high CO_2 tend to increase their size due to the gain of C (Table 15), however, the amount of N supplied is not incremented (FERRI, 2014); *ii.* associated with negative regulation of the enzymes nitrite reductase (NiR), nitrate reductase (NR) and chloroplast glutamine synthase (GS_2) (DING, 2018). The reason is that during the increase in *A* of plants under eCO_2, there is a decrease in the amount of reduced ferredoxin (Fd red) available to N assimilate in leaves (HOANG, 2017; DOMICIANO et al., 2020); *iii.* by the reduction in stomatal conductance under eCO_2, which consequently affects the water mass flow to transport nitrogen to the leaves (DOMICIANO et al., 2020). However, the foliar organic nitrogen content showed no differences between the [CO_2] treatments (Table 15). Therefore, in our case, the differences do not refer to the content of N. *iv.* Finally, the morphophysiological acclimation may be a cause of the decrease in pigment levels in plants exposed to eCO_2 (WALTER et al., 2015). CO_2 acclimation responses are related to decreases the amount of enzymes following the least cost theory, where, the relative investment in photosynthetic capacity and water transport is optimized, so the enough photosynthetic rate is reached at the lowest cost (SMITH and KEENAN, 2020). Given this, decreases in the amount of enzymes require mechanisms allowing to avoid possible excess in formation of reducing power and ATP, from photochemical phase. Accordingly, one of the strategies would be to decrease the amount of pigments, aiming to reduce light interception and consequently a possible photoinhibition.

The high [CO_2] also altered the responses of plants under water deficit, and promoted an increase in the drought tolerance index (Table 5). In this regard, and as previously mentioned, it has been widely reported that one of the main responses of plants to eCO_2 is decreases in stomatal conductance (g_s) which in turn, leads to a reduction in transpiration, improving the water use efficiency. Thus, the high CO_2 may mitigate some of the deleterious effects caused by drought (Vu and Allen Jr, 2009).

Our results also showed that sugarcane plants show variations in anatomical patterns due to exposure to water deficit and CO_2 enrichment, because there was changes in stomatal density (number of stomata per area), stomatal functionality (relationship between polar and equatorial stomatal diameter) and cell wall thickness of the vascular bundle sheath cells (Table 6 and 7), considering the leaves that were totally grown and developed inside the OTCs. In this sense, it has been reported that anatomical traits are strongly influenced by genotype, but also by environment (LIMA, 2013) and that the occurrence of changes in leaf anatomy reflects plant plasticity to climatic variations, being associated to physiological performance under a specific condition (TARATIMA et al., 2019).

On general, it was observed that plants of the RB867515 variety showed higher stomatal density on the adaxial surface, regardless of the water condition and [CO_2] when compared to the RB855536 variety (Table 6). Water deficit as an isolated factor also promoted an increase for this variable (Table 6). Variation in SD is one of the indicators to determine the diffusive efficiency of the leaves in exchange gases. In a regular way, an increase in number of stomata leads to a greater functionality of these, as a consequence of the decrease in their size, allowing a faster regulation to the environment (stomatal aperture and closure). Thus, when a leaf is developed under stressfull conditions, the plant may adjust the number of stomata on the adaxial surface, limiting the excess transpiration (CASTRO et al., 2009). Therefore, considering the drought tolerant characteristic of the RB867515 variety, it can be expected that it presents higher stomatal density when facing a drought event (Table 7).

Most of anatomical alterations caused by eCO_2 were described in C_3 plants, but anatomical responses of C_4 species have been little addressed. Despite this, Habermann et al. (2020) reported that a C_4 grass (*Panicum maximum* Jacq.) also show this type of response. Thereby, in this work, we observed when the plants were grown under eCO_2 (disregarding wáter treatment or varieties) a decrease in the number of stomata on the abaxial surface were detected (Table 6). Among the varieties, RB855536 showed the greatest reduction in SD under high CO_2. It is known the control of the number of stomata is a protective mechanism that increases the use of water resources, and this response is considered a long-term acclimation mechanism that occurs only when the leaves are fully mature developed under a CO_2-enriched atmosphere. Regarding the functionality of the stomata, it was seen that the high CO_2 increased the values for this variable in the RB855536 variety (Table 7). This response would represent an advantage for this material in future climate change scenarios, since drought events will be more frequent as well as an increase in CO_2 concentration, once in leaves with smaller stomata, lower transpiration rates are observed, and the difference in the size of the stomatal aperture is more significant for water diffusion than for CO_2 diffusion (Rocha, 2005; Castro; Pereira and Paiva, 2009). Consequently, increase the stomatal functionality favors higher WUE by obtaining carbon dioxide even with smaller stomatal aperture.

In general is observed three distinct types of responses to high CO_2: *i.* increase photosynthesis and biomass by optimizing the diffusion and biochemical phases (WALIA et al., 2022); *ii*. Acclimation, that leads to greater water use efficiency (LEAKY and LAU, 2012); and *iii.* maintenance of metabolism indifferent to gas enrichment (WALIA et al., 2022). In this case, an acclimation response was recorded since plants grown under high [CO_2] showed a better water use efficiency. Considering this resource is probably the factor that most limits the growth and development of plants, this type of response may be based on trade-offs, since increases in photosynthesis at that moment would not have a greater effect on their biological cycle than would have to save water.

Untill now it is known how the two varieties of sugarcane, even being a C_4 plant, show typical responses of C_3 plants exposed to high atmospheric CO_2. However, the answer to the questions of why C_4 plants respond to this environment and whether this response is conditioned by photosynthetic limitations specifically in the biochemical phase have not yet been approached. In this way, through the A/C_i and A/Q curves, it will be analyzed what probably happened. First, it is worth mentioning that exposing plants to high CO_2 theoretically would favor two of the three phases of photosynthesis: the diffusive phase, as it increases the difference in the concentration gradient among atmosphere and leaf mesophyll; and biochemical phase, by supplying a greater amount of CO_2 to the enzymes responsible for carboxylation, their activity would be stimulated, which would consequently result in increases in photosynthetic rates.

In this work, from the diffusion phase it can be mentioned that at a first moment (20 days after treatment with eCO_2, transpiration was reduced by the high CO_2 (Figure 2-B), due to a decrease in stomatal conductance. Likewise, it was observed that anatomical characteristics such as stomatal density and functionality were altered as a result of this factor (after 44 days of exposure to CO_2) (Table 6). However, these changes seem to have more relation to an acclimation response, and WUE was favored rather than the CO_2 diffusion process. It is important to highlight that despite these plants having lower C_i, the A values were not affected when compared with plants in ambient CO_2, probably by the least cost theory (SMITH and KEENAN, 2020).

Another parameter reinforcing the diffusion of CO_2 was altered is supported by the discrimination of C isotopes, since plants under CO_2-enriched atmospheres show lower discrimination against carbon 13 compared to plants under 400 umol mol^{-1} (Table 15). The discrimination of carbon isotopes in C_4 species reflects the biochemical fractionation of Rubisco and PEPC, as well as their interconnectivity. It is known that under conditions that promote higher g_s, the CO_2 diffusion rate into the leaf is maximum. Thus, inside the stomatal cavity, it will be enriched with ^{12}C compared to the air, due to the fact that the heavy isotope ($^{13}CO_2$) has a lower diffusion rate than the lighter one ($^{12}CO_2$). In addition, the Rubisco enzyme present in the vascular bundle sheath cells (BS) also discriminates against the heavy isotope (^{13}C). On the other hand, when the plant presents conditions that lead to a lower g_s, where the flow of CO_2 is limited, the enzymes start to take advantage of all the substrate provided at the time. So, Rubisco, in this case, would use a higher proportion of ^{13}C that is being provided by PEPC, since it has a lower discrimination against ^{13}C (BRÜGGEMANN et al., 2011).

Regarding the biochemical phase, although the activity of the carboxylation enzymes was not evaluated at a first moment (22 days under eCO_2), increases in *A* were observed due to the high CO_2 (Figure 3-A). After, when performing A/C_i curves to evaluate the activity of carboxylation enzymes, there was no change in Rubisco activity, however PEPC activity was reduced without affecting the photosynthetic rates of both varieties (Table 12). Studies indicate that the increase in *A* is not linear, and under prolonged exposure to high [CO_2], after the initial increase mentioned, decreases in *A* are produced by negative feedback (regulation) or by acclimation (DOMICIANO et al., 2020).

It is known that plants acclimated to high [CO_2] can maintain their photosynthetic rates even with diffusive limitations or they can limit photosynthesis in order to conserve resources at the leaf level (SMITH e KEENAN, 2020). So, the plants are able to prioritize metabolic processes to adjust to a specific condition, which represents an advantage in the biological context (MARCHIORI et al., 2014).

In this case, at first, the plants exposed to eCO_2 responded positively to the treatment, increasing photosynthesis which was converted in more biomass. But, as the time under eCO_2 passed, the plants showed few acclimation responses, since *A* was no longer stimulated and there was changes in anatomical and physiological characteristics in order to improve the water use, without this imply in reductions of *A* or biomass accumulation.

Five important facts that were observed in our plants grown under eCO_2 condition were: *i.* Rubisco's activity has not changed (Table 12); *ii.* PEPC activity was decreased (Table 12); *iii.* the light compensation point was lower, while the apparent quantum efficiency was higher (variety RB8555536) (Table 10); *iv.* the amount of leaf TOC was higher (Table 15); and *v.* conditions that inhibit photorespiration (2% O_2) promoted increases in $V_{\max}$ (Table 14). It has been reported that under current CO_2 condition, there is a higher rate of carboxylation by PEPC in the mesophyll in relation to carboxylation by Rubisco in the BFV cells, which may favour the increase of CO_2 leakage (WATLING, 2000). CO_2 leakage (ϕ) is defined as the fraction of

CO_2 fixed by PEPC that escapes from the BSC (FARQUHAR, 1983). As C_4 photosynthetic metabolism has an additional energy cost of two mols of ATP per mol of fixed CO_2 compared to C_3 plants, increasing ϕ will require more energy for PEPC to refix CO_2 (KROMDIJK et al., 2010). Thus, the apparent quantum efficiency (ϕ) that expresses the efficiency in which light is absorbed for CO_2 fixation (µmol of fixed CO_2 µmol^{-1} of absorbed photons) can be used to determine if there is an inadequate energy expenditure due to leakage.

In this context, the maintenance of photosynthetic rates of plants exposed to eCO_2, lower PEPC activity and no change in Rubisco activity, can be explained by the fact that with lower amount of available C_i, PEPC carboxylates lower amount of CO_2 than in the current CO_2 condition (*i.e.* low enzyme activity). Due to ambient CO_2 conditions, the enzymes have different carboxylation rates with PEPC being higher than that of Rubisco, but under eCO_2 condition, even if PEPC decreases its activity, V_{max} was not change and therefore A is not affected either.

As the PEPC carboxylation rate in mesophyll cells is considered to be similar to the decarboxylation rate in BSC, since under eCO_2, V_{pmax} decreased, probably ϕ was smaller. This can be correlated with the decrease in light compensation point and the increase in apparent quantum efficiency for CO_2 fixation in RB855536 (Table 10). Leakage generates higher energy expenditure due to the amount of ATP that has to be produced to regenerate phosphoenol pyruvate. Thus, reductions in the amount of CO_2 that has to be refixed allowed to increase the efficiency in which light is used to carboxylate. Furthermore, it is considered that the measurements of A are based on a difference between the amount of CO_2 that enters the leaf mesophyll and the amount of CO_2 that leaves it. However, the value of A is not an indication that the plant is incorporating all the CO_2 into organic molecules at that moment, since the CO_2 that is leaking and remains inside the mesopphyl cells is considered for leakiness calculation. These data can be related to the higher values of leaf TOC found in plants under CO_2 enrichment (Table 15), which is an indicator that under high CO_2 plants are able to incorporate a greater amount of C into organic compounds, probably due to the decrease in leakage.

The linear response in the relationship of ΦPSII and φCO_2 indicates that in sugarcane plants, A is the main sink for linear electron transport (Figure 5). However, in the RB867515 genotype, the relation was reduced by high [CO_2]. On the other hand, at 400 µmol mol^{-1}, the ratio of ΦPSII : ΦCO_2 was larger, probably due to the extensive overcycling of the C_4 pump due to CO_2 leakage. Under these conditions, the ratio would exceed the theoretical minimum energy requirement, as more energy would be required for re-fixation of CO_2. Under eCO_2, the relationship of ΦPSII : ΦCO_2 decreased, probably caused by the more energy produced by non-cyclic electron transfer which was used by the Calvin cycle. The lower requirement for pseudocyclic photophosphorylation may also increase the efficiency in utilizing linear electron transfer for CO_2 assimilation (COUSINS et al., 2001).

C_4 plants are recognized to balance the C_3 and C_4 cycles by reducing ϕ under limiting conditions. In sugarcane, for example, low nitrogen contents increase ϕ, which leads to a lower efficiency in carboxylation and, therefore, in the CO_2 concentration mechanism. However, it was observed that a low conductance in the sugarcane vascular bundle sheath (g_{bs}) would balance the low PEPC activity and thus maintain high CO_2 concentration at Rubisco sites under low nitrogen (TOFANELLO et al., 2021). This can be explains few of our results, because the nitrogen fertilization was based on nutritional recommendations (made for actual environmental conditions), but it was not estimated to increase the dose of N due to the effect of dilution by growth. However, in plants grown with eCO_2, even with reduced PEPC activity, photosynthetic rates remained unchanged. At the same time, was observed that the variety RB855536, plants under water deficit had the lowest slope (0.0480) (Figure 7 D) for the correlation under high levels of CO_2.

In relation to measurements performed with 2% O_2, in the *A/Q* curves, it was observed that although there were no changes in *A* in both varieties, the light compensation point in the RB867515 variety decreased (Table 8), while the apparent quantum efficiency of CO_2 fixation (ϕ) and the V_{max} in RB855536 increased with photorespiration inhibition (Table 11). It was expected that high concentrations of CO_2 would improve the assimilatory flux of CO_2 due to the higher concentration of this gas around the catalytic site of the Rubisco enzyme. This would result in greater carboxylation reaction rather than the oxygenation reaction, thus decreasing photorespiration. However, the plants showed acclimation, and this response was not observed, since V_{max} was not altered under eCO_2. But, the data found under 2% O_2 show that in the current CO_2 condition, photorespiration is a factor that affects the carboxylation activity of Rubisco and that the light curve parameters were benefited because by inhibiting photorespiration, there was probably less leakage of CO_2 and consequently the photochemical phase of photosynthesis was more efficient.

Finally, we emphasize that due to the responses presented by the plants evaluated here, the imposition of drought to potentiate the failures in the photosynthetic mechanism was not observed.

CONCLUSION

Despite the acclimation process observed in plants, the results suggest that in the current CO_2 condition there are failures in the C_4 photosynthetic process, due to CO_2 leakage, as a result of different carboxylation rate for the two enzymes (PEPC and Rubisco) and an inhibition at V_{max} by photorespiration. The eCO_2 caused anatomical changes that led to a better WUE, low C_i and reduced g_s, consequently there was lower PEPC activity and unchanged V_{max}. The apparent CO_2 carboxylation efficiency was improved, indicating that under eCO_2 condition, the leakage was reduced and the efficiency in the use of photons to fix CO_2 was improved by not presenting unnecessary energy expenditure in the re-fixation of CO_2. At the same time, we indicate that plants are able to modulate their metabolism to the benefit of water conservation at the leaf level, since in this study they had overlapped the efficient use of water over the gain in C by eCO_2.

REFERENCES

APPROBATO, A. U. Análises fisiológicas e bioquímicas da forrageira tropical *Panicum maximum* Jacq.(Poaceae) cultivada em elevado CO_2 atmosférico e aquecimento. Tese (Doutorado em Biologia comparada)-Universidade de São Paulo, Ribeirão Preto. 2015.

BAKER, N. R. Chlorophyll fluorescence: a probe of photosynthesis in vivo. **Annual. Reviews of Plant Biology,** v. 59, p. 89-113. 2008
.
BERTOLLI, S.C.; RAPCHAN, G.; SOUZA, G.M. Photosynthetic limitations caused by different rates of water-deficit induction in *Glycine max* and Vigna unguiculata . **Photosynthetica**, v. 50, p. 329–336. 2012. doi:10.1007/s11099-012-0036-4

BARRS, H.; WEATHERLEY, P. A re-examination of the relative turgidity technique for estimating water deficits in leaves. **Australian Journal of Biological Sciences**,v. 15, n. 3, p.413-428. 1962.

BUCHANAN B, G. W.; JONES R. Biochemestry & molecular biology of plants (L. John Wiley & Sons Ed. Wiley-blackwell. ed.). UK. 2015.

CERNUSAK, L.A *et al.* Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. **New Phytologist,** v. 200, n. 4, p. 950-956. 2013. doi: 10.1111/nph.12423

COLLATZ, G. J.; RIBAS-CARBO, M.; BERRY, J. A. Coupled photosynthesis-stomatal conductance model for leaves of C_4 plants. **Functional Plant Biology,**.v. 19, n. 5, p. 519-538. 1992.

COPLEN, T. B. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. **Rapid Communications in Mass Spectrometry**, v. 25, n. 17, p. 2538-2560. 2011.

DAI, Z.; KU, M. S.; EDWARDS, G. E. C_4 photosynthesis (the CO_2-concentrating mechanism and photorespiration). **Plant Physiology**, v. 103, n. 1, p. 83-90. 1993.

DE SOUZA, A. P *et al.* Elevated CO_2 increases photosynthesis, biomass and productivity, and modifies gene expression in sugarcane. **Plant, Cell & Environment**, v.31, n. 8, p.1116-1127. 2008.

DING, L *et al.*. Is nitrogen a key determinant of water transport and photosynthesis in higher plants upon drought stress? **Frontiers in Plant Science**. v. 9. 2018. doi: 10.3389/fpls.2018.01143

DOMICIANO, D *et al.* Nitrogen sources and CO_2 concentration synergistically affect the growth and metabolism of tobacco plants. **Photosynthesis research**, v. 144. n.3, p. 327-339.2020.

DRAKE, B. G *et al.* An open top chamber for field studies of elevated atmospheric CO_2 concentration on saltmarsh vegetation. **Functional Ecology**, v.3, n. 3, p. 363-371. 1989.doi: doi.org/10.2307/2389377

EGGELS, S.; BLANKENAGEL, S.; SCHÖN, CC. . The carbon isotopic signature of C_4 crops and its applicability in breeding for climate resilience. **Theory Applied Genetic** v.134, p. 1663–1675. 2021.doi:10.1007/s00122-020-03761-3

FARQUHAR GD. On the nature of carbon isotope discrimination in C_4 species. **Australian Journal of Plant Physiology**, v.10, n. 2, p. 205–226. 1983.

FERNANDEZ, G.C.J. Effective selection criteria for assessing plant stress tolerance. p. 257-270. In Adaptation of food crops to temperature and water stress: Proceedings of an International Symposium, Taiwan, China. 13-18 August. Publication nr 93-410. 531. 1992. **Vegetable Research and Development Center**, Taipei, China

FERRI, C. Gramíneas forrajeras perennes de crecimiento estival (C_4) para la región Pampeana semiárida, en el contexto de la intensificación ganadera y del cambio climático. **Resultados finales. Proyectos de investigación científica y tecnológica orientados al desarrollo productivo provincial. Universidad Nacional de La Pampa, EdUNLPam, Santa Rosa**, v. 1 n. 1, p. 92-145. 2014.

HABERMANN E *et al.*. A increasing atmospheric CO_2 and canopy temperature induces anatomical and physiological changes in leaves of the C_4 forage species *Panicum maximum*. **Plos uno,** v.15, n. 8. 2020. doi:10.1371/journal.pone.0238275

HENDERSON, S.A.; VON CAEMMERER, S.; FARQUHAR, G.D. Short-term measurements of carbon isotope discrimination in several C_4 species. **Australian Journal of Plant Physiology**, v.19,n. 3, p. 263 – 285. 1992.

HOANG, T. D *et al.* Photosynthetic response and nitrogen use efficiency of sugarcane under drought stress conditions with different nitrogen application levels. **Plant Production Science**, v. 20, n. 4, p. 412-422. 2017.

KANT, S *et al.* Improving yield potential in crops under elevated CO_2: integrating the photosynthetic and nitrogen utilization efficiencies. **Frontiers in Plant Science,** v. 19. 2012.

KROMDIJK, J.; GRIFFITHS, H.; SCHEPERS, H.E. Can the progressive increase of C_4 bundle sheath leakiness at low PFD be explained by incomplete suppression of photorespiration?. **Plant, Cell & Environment**, v. 33, p. 1935–1948. 2010.

KROMDIJK, J *et al.* Bundle sheath leakiness and light limitation during C_4. **Plant Physiology**,v. 148, n. 4, p.2144–2155. 2008. doi: 10.1104/pp.108.1298902008

LAWLOR, D. W. Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. **Annals of Botany**, v. 89, n. 7, p. 871-885. 2002.

LEAKEY, A. D. Rising atmospheric carbon dioxide concentration and the future of C_4 crops for food and fuel. **Proceedings of the Royal Society B: Biological Sciences**,v. 276, n. 1666, p. 2333-2343. 2009.

LEAKEY, A. D.; LAU, J. A. Evolutionary context for understanding and manipulating plant responses to past, present and future atmospheric [CO_2]. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v. 367, n.1588, p. 613-629. 2012.

LONG, S. P *et al.*Rising atmospheric carbon dioxide: plants FACE the future. **Annual Review of Plant Biology**, v.55, p. 591-628. 2004. doi: 10.1146/annurev.arplant.55.031903.141610

LUDWIG, M. Evolution of the C_4 photosynthetic pathway: events at the cellular and molecular levels. **Photosynthesis research**, v.117, n. 1 p.147-161. 2013.

MACHADO, D. F. S *et al.* Baixa temperatura noturna e deficiência hídrica na fotossíntese de cana-de-açúcar. **Pesquisa Agropecuária Brasileira**, v.48, n. 5, p. 487-495, 2013.doi:10.1590/S0100-204X201300050000

MARCHIORI, P. E.; MACHADO, E. C.; RIBEIRO, R. V. Photosynthetic limitations imposed by self-shading in field-grown sugarcane varieties. **Field Crops Research**, v.155, p. 30-37. 2014.doi: 10.1016/j.fcr.2013.09.025

MARCHIORI P. E. R *et al.* Physiological plasticity is important for maintaining sugarcane growth under water deficit. **Frontiers in Plant Science**, v.8. 2017. doi:10.3389/fpls.2017.02148.

MITCHELC.A .Measurement of photosynthetic gas exchange in controlled environments. **Hortscience**, v. 27, n. 7. 1992.

MORONEY, J. V *et al.* Photorespiration and carbon concentrating mechanisms: two adaptations to high O_2, low CO_2 conditions. **Photosynthesis research**, v. 117, n. 1, p. 121-131. 2013.

PALIT, P *et al.* An integrated research framework combining genomics, systems biology, physiology, modelling and breeding for legume improvement in response to elevated CO_2 under climate change scenario. **Current Plant Biology**, 22100149. 2020.

PRIOUL, J.; CHARTIER, P. Partitioning of transfer and carboxylation components of intracellular resistance to photosynthetic CO_2 fixation: a critical analysis of the methods used. **Annals of Botany**, v.41, n. 4, p.789-800. 1977.

RAIJ, B *et al.* Recomendações de adubação e calagem para o estado de São Paulo.2.ed. Campinas: IAC (Boletim técnico, 100). 285p.1996.

SAGE, R. F. The evolution of C_4 photosynthesis. **New phytologist**,v. 161, n. 2, p. 341-370. 2004.

SALES, C. R *et al.* Flexibility of C_4 decarboxylation and photosynthetic plasticity in sugarcane plants under shading. **Environmental and Experimental Botany**,v., n.149, p. 34-42. 2018.

SALES, C. R *et al.* Improving C_4 photosynthesis to increase productivity under optimal and suboptimal conditions. **Journal of Experimental Botany**, v. 72. n. 17, p. 5942-5960. 2021. doi: 10.1093/jxb/erab327.

SMITH, N. G.; KEENAN, T. F. Mechanisms underlying leaf photosynthetic acclimation to warming and elevated CO_2 as inferred from least-cost optimality theory. **Global Change Biology**,v 26, n. 9, p. 5202-5216. 2020.

STOKES, J. C *et al.* Measuring and modelling CO_2 effects on sugarcane. **Environmental Modelling & Software**, v.78, p. 68 -78. 2016.

TARATIMA, W. *et al.* Leaf anatomical responses to drought stress condition in hybrid sugarcane leaf (*Saccharum officinarum* 'KK3').**Malaysian Applied Biology**, *v.48, n.* 3, p. 181-188. 2019.

TAZOE, Y *et al.* Relationships between quantum yield for CO_2 assimilation, activity of key enzymes and CO_2 lea- kiness in *Amaranthus cruentus* a C_4 dicot, grown in high or low light. **Plant Cell Physiology,** v. 49, n. 1, p. 19-29. doi: 10.1093/pcp/pcm160. 2008.

TOFANELLO, V. R *et al.* Role of bundle sheath conductance in sustaining photosynthesis competence in sugarcane plants under nitrogen deficiency. **Photosynthesis Research**,v. 149, n. 3, p. 1-13. 2021. doi: 10.1007/s11120-021-00848-w.

VITAL, C. E *et al.* An integrative overview of the molecular and physiological responses of sugarcane under drought conditions. **Plant Molecular Biology**, v. 94, n. 6, p. 577-594. 2017.

VON CAEMMERER, S.; FURBANK, R. T. The C_4 pathway: an efficient CO_2 pump. **Photosynthesis Research**, v. 77, n. 2, p. 191-207. 2003.

VU, J. C.; ALLEN JR, L. H. Growth at elevated CO_2 delays the adverse effects of drought stress on leaf photosynthesis of the C_4 sugarcane. **Journal of Plant Physiology**, v. 166, n. 2, p. 107-116. 2009.

WEI, Z *et al.* Elevated CO_2 effect on the response of stomatal control and water use efficiency in amaranth and maize plants to progressive drought stress.**Agricultural Water Management**, p.266, 107609. 2022.

WALIA, S.; RATHORE, S.; KUMAR, R. Elucidating the mechanisms, responses and future prospects of medicinal and aromatic plants to elevated CO_2 and elevated temperature. **Journal of Applied Research on Medicinal and Aromatic Plants**, v. 261. 2022.

WALTER, L.C.; TELLES, H.R E.; STRECK, N. A.Mecanismos de aclimatação das plantas à elevada concentração de CO_2 . **Ciência Rural** , v. 45, n. 9 , p. 1564-1571. 2015. doi: 10.1590/0103-8478cr20140527.

WATLING, J.R.; PRESS, M.C.; QUICK, W. P. Elevated CO_2 Induces Biochemical and Ultrastructural Changes in Leaves of the C_4 Cereal Sorghum. **Plant Physiology**, v. 123, n. 3, p. 1143–1152. 2000. doi: 10.1104/pp.123.3.1143

WANG, D *et al.* A meta-analysis of plant physiological and growth responses to temperature and elevated CO_2. **Oecologia**, v. 169, n.1, p. 1-13. 2012.

WEATHERLEY, P. Studies in the water relations of the cotton plant: I. The field measurement of water deficits in leaves. **New Phytologist**,v. 49, n. 1, p. 81-97. 1950.

YANG, H *et al.* SPAD Values and Nitrogen Nutrition Index for the Evaluation of Rice Nitrogen Status, **Plant Production Science**, v. 17, n.1, p. 81-92. 2015. doi: 10.1626/pps.17.81

YIN, X *et al.* Using a biochemical C_4 photosynthesis model and combined gas exchange and chlorophyll fluorescence measurements to estimate bundle-sheath conductance of maize leaves differing in age and nitrogen content. **Plant, Cell & Environment**, v. 34, n. 12, p. 2183-2199. 2011.

ZHANG, J *et al.* The effects of elevated CO_2, elevated O_3, elevated temperature, and drought on plant leaf gas exchanges: a global meta-analysis of experimental studies. **Environmental Science and Pollution Research**, v. 28, n. 12, p.15274-15289. 2021.