UNIVERSIDADE ESTADUAL PAULISTA JÚLIO DE MESQUITA FILHO

CÂMPUS DE JABOTICABAL

EFEITO COMBINADO DA ELEVADA [CO_2] ATMOSFÉRICA E DÉFICIT HÍDRICO EM GENÓTIPOS DE CANA-DE-AÇÚCAR

Renan Gonçalves da Silva

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Agronomia (Genética e Melhoramento de Plantas).

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TÍTULO DA TESE: EFEITO COMBINADO DA ELEVADA [CO_2] ATMOSFÉRICA E DÉFICIT HÍDRICO EM GENÓTIPOS DE CANA-DE-AÇÚCAR

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“Um homem não pode fazer o certo numa área da vida, enquanto está ocupado em fazer o errado em outra. A vida é um todo indivisível”.

(Mahatma Gandhi)

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EFEITO COMBINADO DA ELEVADA [CO_2] ATMOSFÉRICA E DÉFICIT HÍDRICO EM GENÓTIPOS DE CANA-DE-AÇÚCAR

RESUMO - As projeções atuais indicam um aumento na concentração atmosférica de dióxido de carbono [CO_2] e, consequentemente, a intensificação da escassez dos recursos hídricos. A deficiência hídrica no solo, assim como outros fatores associados às alterações climáticas, são fatores ambientais que afetam significativamente a produtividade das culturas. Como a vegetação terrestre poderá atenuar os efeitos das mudanças climáticas? Como reagirão as plantas aos ambientes estressantes previstos para os próximos anos? Com base nos estudos que consideraram os efeitos da elevada [CO_2] e que foram realizados com plantas de metabolismo C_4, verificamos que se faz necessário investir no entendimento das respostas fisiológicas e genéticas que são reguladas frente aos múltiplos estresses (Capítulo 2). Desse modo, o objetivo dessa pesquisa foi compreender as principais respostas fisiológicas, bioquímicas e moleculares de genótipos de cana-de-açúcar, em decorrência da deficiência hídrica no solo associada ao aumento na [CO_2]. Dois genótipos de cana-de-açúcar (IACSP94-2094 e IACSP94-2101), contrastantes em relação a diversas características agronômicas (por exemplo, recomendação de plantio e rusticidade), foram cultivados em casa de vegetação e, após 60 dias, submetidos ao bioensaio de elevada [CO_2] e déficit hídrico. O experimento foi realizado em delineamento inteiramente casualizado. Os tratamentos consistiram de plantas em condições ideais de irrigação e [CO_2] de 410 ppm (controle), e plantas submetidas à elevada [CO_2] (800 ppm) e déficit hídrico (estresse), por 30 dias. As análises compreenderam parâmetros biométricos, como o crescimento da parte aérea e do sistema radicular; parâmetros fisiológicos, como a medida da eficiência quântica do fotossistema II (F_vF_m), conteúdo de pigmentos fotossintéticos, entre outros; parâmetros bioquímicos e moleculares, relacionando a atividade de enzimas antioxidantes, conteúdo de glutationa (GSH), flavonoides, e a identificação por RNA-seq do cabedal gênico diferencialmente expresso. Em relação aos parâmetros de crescimento, as análises estatísticas mostraram o “agrupamento” das plantas avaliadas nas condições impostas (Capítulo 3). Os coeficientes de correlação entre os parâmetros biométricos foram positivamente significativos, ou

seja, o estresse hídrico foi um fator limitante para ambos os genótipos, promovendo de forma geral, uma redução significativa no desenvolvimento da parte aérea e radicular das plantas. No entanto, observa-se vias de respostas/defesa diferencialmente regulados nesses genótipos (Capítulo 4). Um total de 2.081 e 4.266 genes foram diferencialmente expressos (DEGs) nos genótipos IACSP94-2094 e IACSP94-2101, respectivamente. O genótipo IACSP94-2101, mais responsivo ao estresse abiótico, ativa um conjunto mais expressivo de genes e induz um número considerável de peroxidases. Por outro lado, no genótipo IACSP94-2094, possivelmente ocorre um acúmulo transiente de peróxido de hidrogênio (H_2O_2) e malondialdeído (MDA), sinalizando para a ativação do sistema antioxidante e danos menos severos nos fotossistemas (PSI e PSII). DEGs relacionados à sinalização mediada por ácido abscísico, fotossíntese e metabolismo secundário foram identificados em ambos os genótipos. Ressalta-se que o presente trabalho realizou uma abordagem global do transcriptoma. Genes potencialmente de interesse podem ser caracterizados funcionalmente e desse modo, pode-se reconsiderar os efeitos dos estresses abióticos e definir estratégias biotecnológicas que visam o melhoramento genético de cana-de-açúcar.

Palavras-chave: expressão gênica, mudanças climáticas, *Saccharum* spp., sistema antioxidante.

COMBINED EFFECT OF ELEVATED [CO_2] ATMOSPHERIC AND WATER DEFICIT ON SUGARCANE GENOTYPES

ABSTRACT - Current projections indicate an increase in the atmospheric concentration of carbon dioxide [CO_2] and consequently an intensification of the scarcity of water resources. Water deficiency in the soil, as well as other factors associated with climate change, are environmental factors that significantly affect crop productivity. How can terrestrial vegetation mitigate the effects of climate change? How will plants react to the stressful environments expected in the coming years? Based on studies that considered the effects of elevated [CO_2] and that were carried out with plants with C_4 metabolism, we found that it is necessary to invest in the understanding of the physiological and genetic responses that are regulated under multiple stresses (Chapter 2). Thus, the objective of this research was to understand the main physiological, biochemical and molecular responses of sugarcane genotypes, due to water deficiency in the soil associated with increased [CO_2]. Two sugarcane genotypes (IACSP94-2094 and IACSP94-2101), contrasting in relation to several agronomic characteristics (e.g., recommendation for planting and rusticity), were grown in a greenhouse and after 60 days, submitted to elevated [CO_2] and water deficit experiment. The experiment was carried out in a completely randomized design. The treatments consisted of plants under ideal irrigation conditions and 410 ppm [CO_2] (control), and plants subjected to elevated [CO_2] (800 ppm) and water deficit (stress), for 30 days. The analyzes included biometric parameters such as the growth of the aerial part and the root system; physiological parameters, such as the quantum efficiency of photosystem II measurement (F_vF_m), photosynthetic pigments content, among others; biochemical, and molecular parameters, relating the antioxidant enzymes activity, glutathione (GSH) content, flavonoids, and RNA-seq for differentially expressed gene identification. Regarding the growth parameters, the statistical analyzes showed the “grouping” of the plants evaluated under the imposed conditions (Chapter 3). The correlation coefficients between the biometric parameters were positively significant, that is, water stress was a limiting factor for both genotypes, promoting, in general, a significant reduction in the development of the aerial and root

parts of plants. However, we observed differently regulated response/defense pathways in these genotypes (Chapter 4). A total of 2,081 and 4,266 genes were differentially expressed (DEGs) in IACSP94-2094 and IACSP94-2101 genotypes, respectively. The IACSP94-2101 genotype, more responsive to abiotic stress, activates a more expressive set of genes and induces a considerable number of peroxidases. Otherwise, in the IACSP94-2094 genotype, there is possibly a hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) transient accumulation, signaling of the antioxidant system activation and less severe damage to photosystems (PSI and PSII). DEGs related to abscisic acid-mediated signaling, photosynthesis, and secondary metabolism were identified in both genotypes. It is noteworthy that the present work carried out a global approach to the transcriptome. Potentially interesting genes can be functionally characterized and in this way, we can reconsider the abiotic stresses effects and define biotechnological strategies aimed at the genetic improvement of sugarcane.

Keywords: gene expression, climate change, *Saccharum* spp., antioxidant system.

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

A cana-de-açúcar (*Saccharum* spp.), considerada uma gramínea apropriada para produção de biomassa é conhecida pela capacidade de armazenamento de sacarose nos colmos e tolerante a temperaturas mais elevadas. Entretanto, o rendimento da cultura de cana-de-açúcar é consideravelmente influenciado por fatores ambientais, como a deficiência hídrica no solo e oscilações na temperatura.

Sabe-se que as modificações no regime hídrico e na temperatura média global são, em parte, consequências das mudanças climáticas devido ao aumento da emissão de gases de efeito estufa. A concentração atmosférica de dióxido de carbono ([CO_2]) tem aumentado expressivamente nos últimos anos e isso pode, diretamente ou indiretamente, afetar os processos biológicos em diferentes níveis de organização das plantas. No entanto, na literatura são os poucos estudos realizados com plantas de metabolismo C_4 (por exemplo, cana-de-açúcar e milho) que objetivaram investigar os efeitos da elevada [CO_2] no crescimento, fisiologia e principalmente na modulação das respostas genéticas.

De modo geral, a deficiência de água no solo é um dos principais fatores limitantes do crescimento e da produção da cana-de-açúcar, por ocasionar mudanças na captação de radiação, temperatura da folha, redução das taxas de assimilação de CO_2 e transpiração, bem como, redução significativa no crescimento das plantas. Mas quais seriam os efeitos no crescimento das plantas considerando-se os cenários em que mais de um estresse ambiental (abiótico ou biótico) estão associados? Especialmente, como seria o desempenho da cana-de-açúcar em ambientes de elevada [CO_2] e déficit hídrico?

As alterações relatadas, principalmente na fotossíntese, são indutoras do estresse oxidativo nas plantas. Esse estresse advém da produção de espécies reativas de oxigênio (ERO) e é potencializado por estresses, como a seca. A eliminação de ERO pode ocorrer por diferentes mecanismos antioxidantes, mediados por sistemas enzimáticos ou não.

O entendimento destes e de outros mecanismos em resposta às alterações climáticas, está intimamente relacionado com o perfil de genes expressos e, consequentemente, como os processos biológicos são regulados. Neste contexto, torna-se possível identificar vias de respostas importantes para o desenvolvimento de estratégias de melhoramento, bem como os cultivares (genótipos) de cana-de-açúcar que apresentam características de tolerância às diferentes condições ambientais limitantes.

2. OBJETIVOS

2.1 Objetivo geral

Tendo em vista as previsões de aumento na concentração atmosférica de CO_2 e demais efeitos associados, como a deficiência hídrica no solo imposta à cultura da cana-de-açúcar, a pesquisa consistiu na investigação de alguns mecanismos de respostas modulados em dois genótipos contrastantes de cana-de-açúcar (IACSP94-2094 e IACSP94-2101), submetidos à elevada [CO_2] e deficiência hídrica.

2.2 Objetivos específicos

i) Através de uma sucinta revisão de literatura, abordar os principais efeitos da alta [CO_2] no metabolismo fotossintético C_4, com especial atenção aos dados de expressão gênica diferencial;

ii) Comparar os dois genótipos de cana-de-açúcar, por meio de uma abordagem baseada na análise estatística das múltiplas características do crescimento vegetal;

iii) Identificar as principais vias de respostas e compreender como o sistema antioxidante é modulado nos dois genótipos de cana-de-açúcar;

3. REVISÃO DE LITERATURA

3.1 Cana-de-açúcar

A cana-de-açúcar (*Saccharum* spp.), espécie pertencente à família Poaceae, é uma gramínea de origem asiática conhecida pela capacidade de acúmulo de sacarose nos colmos (Moore e Maretzki, 1996; Zuurbier e Vooren, 2008), considerada robusta e eficiente na produção de biomassa (Belloti, 2012). A cultura de cana-de-açúcar tem boa adaptação em ambientes de temperaturas mais elevadas, mas não tolera baixa disponibilidade de água, sendo, assim, uma cultura típica de regiões tropicais e subtropicais, onde o clima quente e úmido favorece o seu cultivo (Tejera et al., 2007).

É considerada uma das culturas de metabolismo C_4 mais importantes do mundo, assim como o milho (*Zea mays*) e o sorgo (*Sorghum bicolor*) (Silva et al., 2020), possuindo considerável importância ecológica e econômica (Vu et al., 2006).

No Brasil, a cana-de-açúcar chegou logo após o descobrimento do país (Canabrava, 2005), e, atualmente, é considerado o maior produtor mundial, seguido pela Índia e China (FAOSTAT, 2018) (Figura 1). A produção acumulada no ano de 2020 é de 590 milhões de toneladas (UDOP, 2020), com uma estimativa de produção total para a safra 2020/21 de 642,1 milhões de toneladas, o que representa uma redução de 0,1% em relação ao período anterior. A redução também é esperada para a produção de açúcar e etanol (safra 2020/21) em relação a safras anteriores (CONAB, 2020).

De acordo com Gascho e Shih (1983), a cana-de-açúcar é caracterizada por quatro diferentes fases de desenvolvimento, que envolvem a emergência dos brotos (I), o perfilhamento e estabelecimento da cultura (II), o período de grande crescimento (início do acúmulo de sacarose) (III) e a maturação (IV). As duas fases iniciais estão intimamente relacionadas ao bom estabelecimento da cultura e fornecimento de colmos adequados para uma boa produtividade (Câmara, 1993).

No entanto, o crescimento é significativamente dependente de fatores climáticos (Silva et al., 2010) e, portanto, o rendimento dessa cultura é influenciado negativamente por um grande número de fatores externos (abióticos e bióticos), com

destaque para os efeitos advindos da restrição hídrica no solo, salinidade e temperatura (Lawlor e Cornic, 2002).

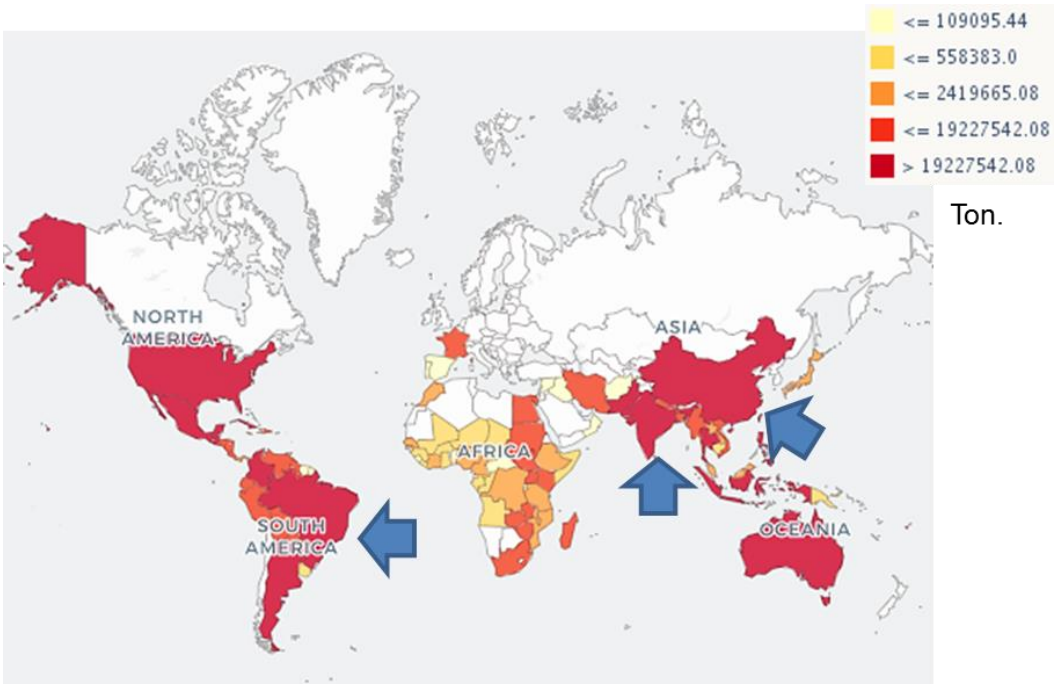

Figura 1. Produção de cana-de-açúcar por país. Fonte: adaptado de FAOSTAT, 2018.

3.2 Mudanças climáticas e déficit hídrico

As modificações no regime hídrico e na temperatura global são, em parte, consequências das mudanças climáticas devido ao aumento da emissão de gases de efeito estufa (GEE) (IPCC, 2007; Carvalho et al., 2010). Tem sido crescente a preocupação mundial em relação às mudanças no clima, principalmente, em razão do aumento das emissões de gases como o dióxido de carbono (CO_2) e, consequentemente, dos efeitos ocasionados pelo aquecimento global do planeta (NASA, 2020). Desse modo, a busca por estratégias para mitigar essa emissão, como por exemplo, a maximização do sequestro de carbono no solo e na vegetação tem se tornado essencial (Carvalho et al., 2010).

A [CO_2] tem aumentado consideravelmente desde a revolução industrial. Ao longo de 50 anos, essa concentração aumentou cerca de 100 ppmv (partes por milhão de volume) e, atualmente, o acréscimo anual foi de 2,7 ppmv. A [CO_2] em outubro de 2020 foi de 411,2 ppmv (NOAA, 2020). Alguns estudos fazem previsões para 2100 de

níveis em 900 ppmv, compreendendo cerca de 0,1% da nossa atmosfera (NASA, 2015).

Sabe-se que, em teoria, plantas com metabolismo fotossintético C_4 não são capazes de apresentar respostas tão expressivas ao incremento de CO_2, mas o que se observa em diversos experimentos (Anderson et al., 2001; Souza et al., 2008; Souza et al., 2015) é que muitas respostas carecem de melhores entendimentos. Estudos realizados com cana-de-açúcar (Vu et al., 2006; Souza et al., 2008) e sorgo (Vara Prasad et al., 2009) indicam o aumento nas taxas fotossintéticas. No entanto, Martins (2012), verificou a redução da taxa fotossintética em cana-de-açúcar submetida à elevada concentração de CO_2 e baixo suprimento de nitrogênio no solo. Outras alterações como mudanças na taxa de transpiração, no metabolismo de carboidratos e no acúmulo de proteínas também foram observadas por Prins e colaboradores (2010), estudando plantas de milho em ambientes com níveis aumentados desse gás.

As respostas fisiológicas das plantas podem ser distintas frente ao aumento da concentração de CO_2 atmosférico, principalmente quando está associado a estresses bióticos e abióticos (por exemplo, déficit hídrico) e problemas nutricionais no solo (Martins, 2012; Souza et al., 2015).

O aumento da taxa fotossintética, bem como na biomassa e no conteúdo de sacarose, é indicativo de que a principal regulação da fotossíntese em cana-de-açúcar sob o aumento de CO_2 ocorre por meio do sistema de captura de luz, devido ao aumento na taxa de elétrons destas plantas e da expressão de genes relacionados à captura de luz e transporte de elétrons (Souza, 2011). Além disso, diversos genes relacionados ao acúmulo e mobilização de reservas, ciclo celular e crescimento têm se mostrado sensíveis a modificações nas concentrações de açúcares (Rolland et al., 2006), principalmente a sacarose (Hanson e Smeekens, 2009), com destaque para os genes codificadores das proteínas hexoquinases e trealose 6-fosfato, que estão envolvidas nos mecanismos de sinalização e percepção de açúcares em cana-de-açúcar (McCormick et al., 2008; Smeekens et al., 2010).

Associado a esse cenário ambiental, sabe-se que a deficiência de água no solo é um dos principais fatores limitantes do crescimento da cana-de-açúcar, por ocasionar simultaneamente mudanças morfológicas e fisiológicas (Silva e Costa,

2004), como: alterações na captação de radiação, temperatura da folha, condutância estomática, transpiração e respiração (Qing et al., 2001; Silva et al., 2007), redução das taxas de assimilação de CO_2 e crescimento das plantas (Solari et al., 2006). Todas essas alterações afetam de maneira direta o desenvolvimento dos perfilhos e a altura final dos colmos e, consequentemente, a produção e acúmulo de açúcar (Gascho e Shih, 1983; Sugiharto, 2004).

Embora sejam conhecidos os principais danos decorrentes da seca no crescimento das plantas, os efeitos primários do déficit hídrico em níveis bioquímicos e moleculares ainda não estão bem elucidados (Zhu, 2002; Chaves e Oliveira, 2004). Em cana-de-açúcar submetida à deficiência hídrica foram identificados mais de 1.600 genes diferencialmente expressos e envolvidos, por exemplo, com a regulação da fotossíntese e mecanismos de resposta (defesa) ao estresse (Rodrigues et al., 2011).

As plantas C_4 podem apresentar variações bioquímicas em relação ao tipo de enzima de descarboxilação (Kanai e Edwards, 1999). No caso da cana-de-açúcar, as enzimas desse metabolismo podem ser a PEPCK (Phosphoenolpyruvate carboxykinase) (Calsa Jr. e Figueira, 2007) e NADP-ME (NAD+ - malic enzyme) (Bowyer e Leegood, 1997). Segundo Bowyer e Leegood (1997), admite-se que o mecanismo de transporte de compostos de quatro carbonos presente nessa espécie seja referente à NADP-ME. Entretanto, trabalhando com cultivares contrastantes de cana-de-açúcar em relação ao déficit hídrico, Belloti (2012), verificou que o maior acúmulo de transcritos e maior atividade de PEPCK observado na cultivar tolerante (IACSP94-2094) sob o estresse hídrico, indicam que a via de descarboxilação do tipo PEPCK aparentemente poderia ser ativada em condições de seca.

Portanto, muitas são as vias de respostas que precisam ser investigadas para que assim haja uma melhor compreensão de muitos processos biológicos, relacionados, por exemplo, com a fotossíntese, sistemas de defesa, ativação de mecanismos antioxidantes, entre outros. Ressalta-se que, nesse contexto, seja de extrema importância considerar não somente os estresses ambientais isoladamente, mas principalmente os efeitos dos estresses associados.

3.3 Sistema antioxidante

Os efeitos de muitos estresses abióticos podem ser extremamente prejudiciais às plantas. No caso da deficiência hídrica no solo, um dos efeitos imediatos no metabolismo vegetal é o estresse oxidativo, relacionado às alterações bioquímicas que ocorrem nos tecidos vegetais e, consequentemente, com o aumento na formação de espécies reativas de oxigênio (ERO) (Scandalios; Acevedo e Ruzsa, 2000; Chaki; Begara-Morales e Barroso, 2020), como os radicais superóxidos ($O_2^{\bullet -}$), hidroxila ($OH^{\bullet}$) e produtos como o peróxido de hidrogênio (H_2O_2) e oxigênio singleto ($_1O_2$) (Demidchik, 2015). O estresse oxidativo leva a um sério desequilíbrio celular, causando efeitos diretos na fotossíntese e no crescimento das plantas (Alves, 2019).

As plantas desenvolveram diferentes estratégias naturais para tolerar ou evitar condições de estresse hídrico (Taiz e Zeiger, 2004). O mecanismo de fechamento estomático reduz a evaporação através das folhas, sendo esta uma das respostas primárias ao estresse (Finkelstein e Rock, 2002; Sirichandra et al., 2009). Os mecanismos de tolerância ao déficit hídrico podem estar associados com a tolerância ao estresse oxidativo, assim como, ao aumento da atividade enzimática e não enzimática do sistema antioxidante (Smirnoff, 1995; Chaki; Begara-Morales e Barroso, 2020).

Desse modo, a eliminação de ERO pode ocorrer por intermédio do sistema antioxidante, composto por algumas enzimas, como a superóxido dismutase (SOD – EC 1.15.1.1), responsável por catalisar a dismutação de $O_2^{\bullet -}$ para H_2O_2 e O_2 nos cloroplastos, mitocôndria e outros compartimentos (Gill et al., 2015). É possível encontrar nesses diferentes compartimentos subcelulares três isoformas da enzima SOD, a Mn-SOD, Cu/Zn-SOD e Fe-SOD (Corpas et al., 2006; Alves, 2019).

Além da SOD, que é a primeira enzima a atuar (Ahmad et al., 2010), a catalase (CAT – EC EC 1.11.1.6) e outras peroxidases participam de forma eficiente das reações convertendo o H_2O_2 em água (H_2O) e O_2 (Garg e Manchanda, 2009). Outras enzimas como a ascorbato peroxidase (APX – EC 1.11.1.11) e glutationa redutase (GR – EC 1.6.4.2) adquirem um papel importante na eliminação de ERO (ASADA, 1999). A desintoxicação do H_2O_2 pela APX ocorre por meio da oxidação do ascorbato (ASC) a monodehidroascorbato (MDHA). O ASC pode ser regenerado pela ação da

enzima monodehidroascorbato redutase (MDHAR) a partir do MDHA, através do pode redutor de NADPH (Mittler, 2002; Anjum et al. 2014).

Por outro lado, a GR é necessária para reduzir a glutationa oxidada (GSSG) ao seu estado reduzido (GSH) (Ghisla e Massey, 1989). Desta forma, o GSH formado pode ser usado pela glutationa peroxidase (GPX – EC 1.11.1.9). A enzima GPX por sua vez converte o H_2O_2 em H_2O (Gill et al., 2013).

A enzima glutationa S-transferase (GST - EC 2.5.1.18) também participa do sistema através de vias adjacentes auxiliares (Kumar e Trivedi, 2018). Desse modo, todas essas enzimas integrantes do ciclo da Ascorbato-Glutationa pertencem a um importante sistema de eliminação de ERO e minimização dos efeitos do estresse oxidativo.

Os compostos não enzimáticos mais conhecidos em plantas são os antioxidantes ASC e a GSH, muito importante na proteção das membranas celulares (Alscher, 1989). Mas também existem os flavonoides, alcaloides e outros compostos que atuam diretamente ou indiretamente na defesa antioxidante, ou seja, neutralizando os efeitos de ERO (Gratão et al., 2015). Os flavonoides são uma grande classe de metabólitos secundários que podem apresentar funções antioxidantes em plantas superiores (Winkel-shirley, 2002; Agati e Tattini, 2010). Os denominados “flavonoides antioxidantes” são encontrados no cloroplasto, o que sugere um papel como eliminadores de oxigênio singleto e estabilizadores da membrana externa do cloroplasto (Agati et al., 2012).

3.5 RNA-seq

O sequenciamento de última geração (NGS) veio para sustentar uma revolução contínua nas ciências. O NGS mudou o escopo dos estudos de expressão genica. As tecnologias de sequenciamento de RNA (RNA-seq), que aplicam os princípios de NGS aos DNAs complementares (cDNAs) derivados de populações transcritas, foram usadas pela primeira vez para estudar plantas há apenas alguns anos e fornecem acesso imediato às informações do transcriptoma de alta resolução (Martin et al., 2013).

Muitos estudos que retratam esse tipo de abordagem molecular são recentemente divulgados na comunidade científica. Pesquisas que envolvem RNA-seq e objetivam investigar os mecanismos regulados em cana-de-açúcar, compreendem uma diversidade de condições experimentais (Tabela 1).

A quantidade de sequências que são geradas pelo sequenciamento, bem como o número de genes que são analisados varia de estudo para estudo, mas de uma forma geral, contribuem para uma abordagem global ou direcionada dos processos/mecanismos biológicos que são modulados nas condições estudadas. No entanto, ressalta-se que ainda não existem estudos para algumas condições ambientais, como determinados estresses bióticos, deficiência nutricional, elevada concentração atmosférica de CO_2 (com exceção do presente trabalho), entre outros.

Tabela 1. Alguns estudos de RNA-seq realizados com cana-de-açúcar e diferentes condições experimentais.

Condição	Descrição	Referência
Não estressante	Conteúdo de lignina	Vicentini et al. (2015)
	Conteúdo de fibra	Kasirajan et al. (2018)
Abiótico	Baixa temperatura	Selvarajan et al. (2018)
	Deficiência hídrica	Nawae et al. (2020)
	Toxicidade por alumínio	Rosa-Santos et al. (2020)
Biótico	Interação com fungo	Que et al. (2014)
	Interação com bactéria	Malviya et al. (2020)

Ademais, sabe-se que a compreensão do transcriptoma da cana-de-açúcar é limitada (Grivet e Arruda, 2002). Não se sabe quantas isoformas de transcrição resultam do splicing alternativo neste transcriptoma potencialmente complexo. A cana-de-açúcar é um híbrido poliploide entre *Saccharum officinarum* e *S. spontaneum*, e cada híbrido de cana-de-açúcar tem o seu próprio conjunto cromossômico único (Hoang et al., 2017). Adicionalmente, segundo Hoang et al. (2017), a maioria dos estudos de cana-de-açúcar foi baseada em sequências genômicas/transcritos de sorgo, por possuir um alinhamento ortólogo com o genoma da cana-de-açúcar. Portanto, de acordo com esses autores, ainda existe uma necessidade da construção

das sequências de comprimento total de isoformas transcritas distintas, para facilitar a análise da expressão diferencial de isoformas e também para estender nossa compreensão do transcriptoma de cana-de-açúcar.

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CAPÍTULO 2 – INCREASED [CO_2] CAUSES CHANGES IN PHYSIOLOGICAL AND GENETIC RESPONSES IN C_4 CROPS: A BRIEF REVIEW[1]

Abstract: Climate change not only worries government representatives and organizations, but also attracts the attention of the scientific community in different contexts. In agriculture specifically, the cultivation and productivity of crops such as sugarcane, maize, and sorghum are influenced by several environmental factors. The effects of high atmospheric concentration of carbon dioxide ([CO_2]) have been the subject of research investigating the growth and development of C_4 plants. Therefore, this brief review presents some of the physiological and genetic changes in economically important C_4 plants following exposure periods of increased [CO_2] levels. In the short term, with high [CO_2], C_4 plants change photosynthetic metabolism and carbohydrate production. The photosynthetic apparatus is initially improved, and some responses, such as stomatal conductance and transpiration rate, are normally maintained throughout the exposure. Protein-encoding genes related to photosynthesis, such as the enzyme phosphoenolpyruvate carboxylase, to sucrose accumulation and to biomass growth and are differentially regulated by [CO_2] increase and can variably participate owing to the C_4 species and/or other internal and external factors interfering in plant development. Despite the consensus among some studies, mainly on physiological changes, further studies are still necessary to identify the molecular mechanisms modulated under this condition. In addition, considering future scenarios, the combined effects of high environmental and [CO_2] stresses need to be investigated so that the responses of maize, sugarcane, and sorghum are better understood.

Keywords: CO_2 enrichment; C_4 plants; gene expression; photosynthesis; productivity.

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1. Introduction

The increased atmospheric concentration of carbon dioxide ([CO_2]) is one of the factors responsible for global warming [1] and the consequent irreversible climate change for hundreds of years [2]. This increase has been significant since the industrial revolution, and over the last 50 years, [CO_2] levels have increased from 320 to 390 ppmv (parts per million volume) [3,4]. In 2018, [CO_2] levels reached 409.23 ppmv [5] and there are some predictions that for 2100, [CO_2] will reach 800 ppmv [4].

High [CO_2], as well as radiation, temperature, and water availability, influence crops [6]. Climate change affects plant growth and development, mainly owing to changes in photosynthetic carbon assimilation [7]. Plants absorb atmospheric CO_2 by chemically reducing carbon, and this reduction is actually much more, because in addition to storing chemical energy in the plant, photosynthesis also provides carbon skeletons for the organic molecules that comprise the vegetal structure [8]. Theoretically, plants can mitigate climate change through the photosynthetic conversion of atmospheric CO_2 into carbohydrates and other organic compounds [9]. However, the direct and indirect effects of these changes on plants need to be better evaluated, especially regarding to their impact on crop production and productivity [7]. Increased [CO_2] levels affect most physiological processes in plants; however, plants under this condition can present a mix of positive and negative responses [5].

C_3 and C_4 species respond differently to this increase [10] because [CO_2] levels affect biological processes at different levels of organization [11]. There are many studies on plant responses to increased [CO_2] with C_3 species. Less attention has been given to C_4 plants, owing to the assumption that the mechanism of CO_2 accumulation in C_4 plants makes them insensitive to high [CO_2] levels [7].

However, studies on plants such as *Saccharum officinarum* L. [12,13] and *Sorghum bicolor* L. [14] in high-level CO_2 environmental conditions indicate increased photosynthetic rates. Changes in the transpiration rate and metabolite production (e.g., glucose, mannose, and galactose) have been reported in *Zea mays* L. [9]. In addition, increased [CO_2] can also improve plant susceptibility to pests and diseases, mainly because of decreased phytochemical and phytohormone production, with these being part of the plant defensive system [15,16].

Several genes were identified in C_3 and C_4 plants that are directly involved in plant responses to environmental conditions [17]. In soybeans (*Glycine max* '93B15'), 327 genes were responsive to increased [CO_2], indicating that this environmental condition stimulates carbohydrate degradation, increasing energy and leaf expansion and growth precursors [18]. C_4 plants under increased [CO_2] presented changes in the expression of genes involved in the photosynthetic mechanism of *Zea mays* [19] and differential expression of dozens of genes also related to photosynthesis and development in sugarcane plants [13].

Environmental factors, such as water availability and temperature, are associated with a considerably increased atmospheric [CO_2]. In C_3 plants, the interactive effects of water deficiency and increased [CO_2] were studied by Medina et al. [20] in wheat genotypes (*Triticum turgidum* L.), finding that these effects depend on genotype and severity of water stress, especially for the expression of genes responsive to water conditions, such as catalase and superoxide dismutase, negatively regulated in three of the four evaluated genotypes. More than 1600 differentially expressed genes involved in photosynthetic processes and stress response mechanisms were identified in sugarcane under water deficiency [21]. Molecular studies using cDNA arrays identified several genes induced by biotic and abiotic stresses [22,23], revealing that drought-tolerant or sensitive genotypes respond differently to this stress at the gene expression level [23].

Therefore, expanding our knowledge on physiological and genetic responses in different crops can improve the development of cultivars that are better adapted to the new climatic conditions to which they will be subjected in the coming decades. Considering the interest in elucidating these responses in C_4 plants (sugarcane, maize, and sorghum), this brief review approaches the main effects of high [CO_2] through considering different exposure periods (time) and emphasizing mostly physiological aspects related to photosynthetic metabolism and differential gene expression, despite the lack of data in the literature.

2. C_4 Plants of Commercial Importance

Although C_4 plants represent a small portion of the world's plant species, they have substantial ecological and economic significance [12]. *Zea mays*, *Sorghum bicolor*, and

Saccharum spp. stand out among C_4 species and are considered the most important world crops in terms of production [24–27]. These plants are grasses belonging to the Poaceae family, generally growing in warmer climates with considerable light intensity [28,29].

Grains and cereals, such as *Zea mays* and *Sorghum bicolor*, are essential food group in the human diet, as they provide energy and essential nutrients. They are also a source of food for animals and of raw materials to produce biocomposites. Maize is currently the most cultivated crop in the world, being used as food and raw material for ethanol, starch, and oil production [30]. However, climatic instability can influence crop yield, as is the case in the south of Brazil. Climate changes have negatively influenced the productive potential of maize crops (0.8% reduction in average productivity levels) [31]. Sorghum has also become important in terms of cultivation, being used in animal feed or as a food base for millions of people, mainly in Africa and Asia [32]. A considerable portion of the global sorghum harvest areas are in Africa and Asia. The majority of sorghum production is in Africa (41%), the Americas (38%), and then Asia (18%) [33]. Recent reports show growth in cereal production in the 2019/2020 harvest in Brazil, with an increase of 24.7% in sorghum production compared to the previous harvest.

Saccharum spp. is a grass of Asian origin that is known for its sucrose storage capacity in the stalks [34,35], and is considered a robust crop with efficient biomass production and great potential for bioenergy production [36]. Brazil has the largest production in the world, estimated at 35.3 million tons of sugar and 29.3 billion liters of ethanol in the 2019/2020 harvest, followed by India, China, and other countries [37].

The productivity of these crop plants can be negatively or positively influenced, mainly owing to cultivation in different regions and the consequent effects of atmospheric [CO_2], global warming, and precipitation rate changes [38,39]. Recent projections show significantly decreased yields in major crops until the end of the 21st century, even with the effects of CO_2 fertilization on crops [40,41]. Understanding plant responses to increased [CO_2] is essential to predict net primary productivity and for ecosystem service provision, which can meet future food and fuel demands [41].

3. C_4 Photosynthesis Mechanism and CO_2

In plants, carbon is assimilated through the phosphate-reducing cycle (Calvin cycle) [25]. Once CO_2 has been absorbed into the aerial space of the leaf and mesophyll, it is converted into bicarbonate and pre-assimilated into the mesophyll cytoplasm by the enzyme phosphoenolpyruvate carboxylase (PEPC) [42,43]. Some plants have a C_4 biochemical mechanism, responsible for photosynthetic processes. Approximately 8100 plant species use C4 carbon fixation [44], and on the basis of the decarboxylation mechanism in the perivascular sheath, these plants can be classified into different subgroups [45]. Unlike the C_3 photosynthetic pathway, which has only one carboxylation step, the C_4 photosynthetic pathway has two carboxylation steps [46].

During photosynthesis, the incident light is absorbed by chlorophyll molecules, responsible for transferring the captured energy to photosystem I (PSI) and II (PSII) reactions, which direct the energy to the electron transport chain [47]. The CO_2 absorbed by C_4 species is diffused through the stomata and hydrated by the carbonic anhydrase enzyme [48], being subsequently assimilated by oxaloacetate using PEPC located in the cytosol as the substrate [49]. Upon receiving the electrons, oxaloacetate is reduced to malate or aspartate, or even both. It depends on the subtype of C_4 species, as they are divided by the type of enzyme that performs the decarboxylation: nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME), nicotinamide adenine dinucleotide-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK) [50–53].

The CO_2 is supplied to the active site of the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), located in the chloroplasts of the vascular sheath cells, and is used for carbohydrate production [49,54]. According to Jenkins et al. [55], in C_4 plants, [CO_2] around the vascular sheath Rubisco is 10 times higher than the [CO_2] present in the atmosphere, explaining the importance of this enzyme in the C_4 photosynthetic apparatus.

Plants with the C_4 mechanism use these biochemical processes to concentrate CO_2 in the leaves where the Rubisco enzyme acts by fixing CO_2 [8]. As this is minimally affected by increased [CO_2], this is probably directly related to the high [CO_2] in the sheath cells of C_4 plants. Increased [CO_2] often has little direct effect on the

photosynthetic rates of these plants; however, they reduce stomatal conductance, consequently improving photosynthesis [56,57].

The Rubisco enzyme changes the flow of atmospheric CO_2 in carbon assimilation [58]. At current CO_2 levels, this assimilation can be saturated in C_4 species, mainly because PEPC uses HCO_3 (bicarbonate system) as the substrate instead of CO_2 [59]. Although studies that consider the [CO_2] mechanism in C_4 plants under high [CO_2] conditions are less comprehensive, it is remarkable that global concerns regarding climate change and the real impacts on the productivity of several crops have led to studies that evaluated how these plants will respond to the predicted [CO_2] increase. What are the effects on plant development and gene response modulation? Are the physiological mechanisms changed? These questions have been investigated through experiments evaluating high [CO_2] application, experimental conditions, periods of stress exposure, and integrative stress effect (but little research involving genetic machinery), among other experimental aspects (Table 1).

Despite the limited number of published studies, they present a similar context, pointing to the same line of knowledge that, owing to their [CO_2] mechanisms, C_4 plants have an almost saturated photosynthetic capacity under the current atmospheric CO_2 conditions. In this context, many questions arise, e.g., how do C_4 plants really respond to high [CO_2] conditions? Understanding how these plants, economically important for the world, are influenced by increased [CO_2] levels is essential for the development of breeding programs aimed at improving or adapting plant growth and increasing productivity considering future climate changes [12].

Table 1. Experimental designs established in some research with C_4 plants (maize, sugarcane, and sorghum) and elevated atmospheric concentration of carbon dioxide ($[CO_2]$) environments.

Plant	$[CO_2]$ Applied [1]	Experimental Conditions [2]	Reference
Zea mays	AC: 370 µmol mol^{-1} EC: 750 µmol mol^{-1}	Soil–plant–atmosphere research chambers	[60]
	AC: 350 µL L^{-1} EC: 700 µL L^{-1}	CEC	[9]
	AC: 378 µL L^{-1} EC: 550 µL L^{-1}	FACE	[61]
	AC: 394 ppm EC: 566 ppm	OTCs	[19]
	AC: 400 ppm EC: 550 ppm	OTCs	[62]
Saccharum spp.	AC: 360 µmol mol^{-1} EC: 720 µmol mol^{-1}	TGG	[12]
	AC: 370 ppm EC: 720 ppm	OTCs	[13]
	AC: 360 µmol mol^{-1} EC: 720 µmol mol^{-1}	TGG	[63]
	AC: 400 ppm EC: 750−800 ppm	OTCs	[59]
Sorghum bicolor	AC: 370 µmol mol^{-1} EC: 570 µmol mol^{-1}	FACE	[64]
	AC: 370 µmol mol^{-1} EC: 570 µmol mol^{-1}	FACE	[65]
	AC: 400 µmol mol^{-1} EC: 800 µmol mol^{-1}	OTCs	[66]

[1] AC = ambient condition; EC = under elevated $[CO_2]$. [2] CEC = controlled environment cabinets; FACE = free air CO_2 enrichment; OTCs = open top chambers; TGG = temperature gradient greenhouses.

4. Physiological, Biochemical, and Gene Expression Changes

Increased [CO_2] levels affect morphological, physiological, and productive plant aspects [67]. Many growth parameters have been shown to be positively altered in this climatic condition. In maize at the vegetative stage, there is an increase in leaf area (over 50%) in 550 ppm [CO_2] [68], and in studies with sugarcane (30%) [12] and sorghum (25%) [69], in 720 µmol mol^{-1} and 900 µL CO_2 L^{-1}, respectively. Other measurements, such as plant height and biomass are increased under high [CO_2] (Table 2). According to Souza et al. [13] in sugarcane crops it was observed that productivity can increase in environments with high [CO_2], however, just as the authors report, it is necessary to consider that some factors influence these growth responses (e.g., the experiment was not performed under field conditions).

Photosynthetic metabolism, plant growth, and biomass accumulation are directly related to productivity. Sorghum and maize showed no increase in productivity of grain yield under high [CO_2] conditions [70,71], and in sugarcane, the productivity, evaluated by the results of direct allocation of sucrose to the stem, was satisfactory under high [CO_2] conditions [13,72]. However, experimental and environmental factors can improve different responses, as observed for some growth characteristics (leaf area and biomass) in maize, resulting in improved crop yield under high [CO_2] conditions associated with increased precipitation [73].

Table 2. Growth responses of C_4 plants (maize, sugarcane, and sorghum) under elevated [CO_2] conditions.

Plant	Growth Parameters	Elevated [CO_2]	Effects *
Zea mays	Leaf area	550 ppm	(↑) [1] (↑) [2]
	Plant height	550 ppm	(↑) [1] (↑) [2]
	Total masses	70 Pa–550 ppm	(↑) [3] (=) [4]
Saccharum spp.	Leaf area	720 µmol mol^{-1}	(↑)[5]
	Plant height	720 ppm	(↑)[6]
	Total masses	720 ppm	(↑)[6]
Sorghum bicolor	Leaf area	795–900 µ L^{-1}	(↑) [7] (↑) [8]
	Plant height	795 µ L^{-1}	(=) [7]
	Total masses	900 µ L^{-1}	(↑) [8]

* Reference: [1] Mina et al. [68]; [2] Adishesha et al. [74]; [3] Sicher and Barnaby [75]; [4] Huang et al. [62]; [5] Vu et al. [12]; [6] Souza et al. [13]; [7] Chaudhuri et al. [76]; [8] Khanboluki et al. [69]. The symbols mean an increase (↑) and no difference (=) in relation to control plants ([CO_2] ambient).

Some studies evaluated the physiological, biochemical, and molecular responses of C_4 plants under high [CO_2] conditions [12,13,19,62,63,64,75,77]. Figure 1 summarizes some of the main effects of high [CO_2] on C_4 plant photosynthesis, specifically maize, sugarcane, and sorghum. In this schematic representation, we emphasize physiological and genetic changes that occur owing to the duration of exposure of C_4 plants to excess CO_2.

Carbohydrates are the main products generated in the photosynthetic process, being processed in the stem and accumulated in the form of sugars in sugarcane [78]. Thus, increased photosynthetic rates and sucrose content indicate that the main regulation of sugarcane photosynthesis in high [CO_2] [5] may occur through the light capture system, being possibly related to the increased electron rate of these plants and the expression of genes related to light capture and electron transport [13]. Several genes related to reserve accumulation and mobilization, cell cycle, and growth have been shown to be sensitive to changes in sugar concentrations [79], mainly sucrose [80].

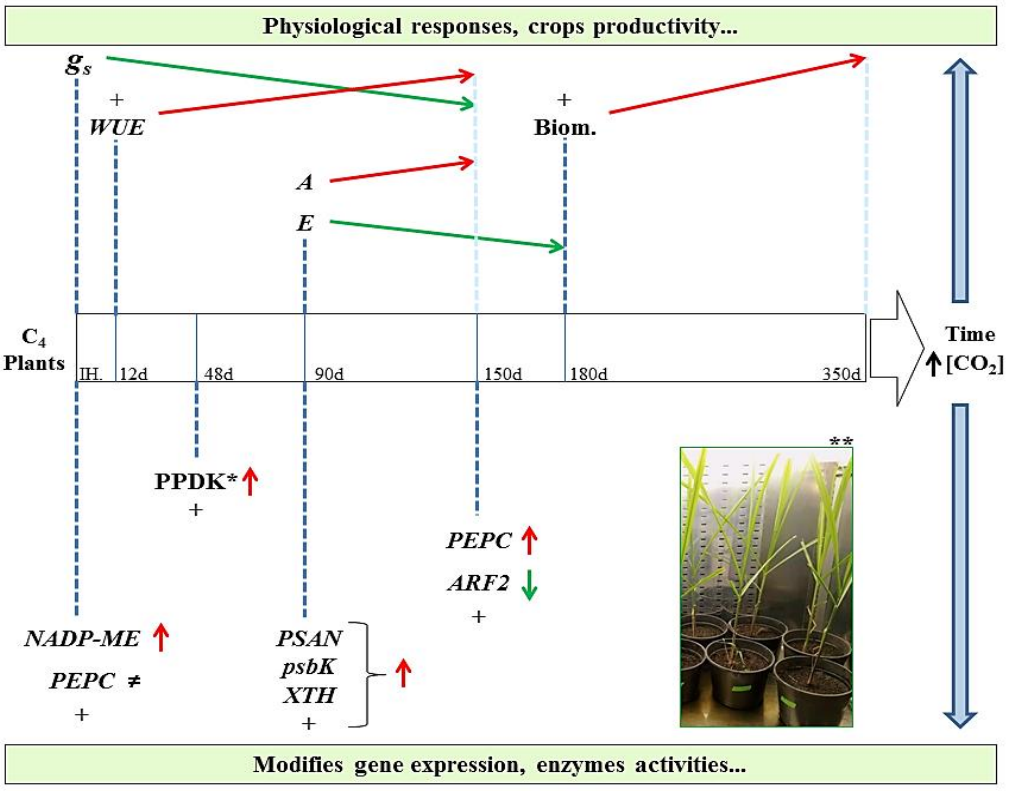

Figure 1. Schematic representation of some effects of elevated CO_2 on C_4 plants (maize, sugarcane, and sorghum). The green arrows indicate inhibition or decrease, while the red arrows indicate promotion or increase. The (+) sign indicates that other physiological

parameters or genes/enzymes have also been modulated, and the (≠) sign indicates differential expression in some cultivars (plants C_4) or experiments. * Represents pyruvate phosphate dikinase (PPDK) enzyme activity. ** Example of experiments under elevated CO_2 conditions: sugarcane plants evaluated in fitotron plant growth chamber.

Souza et al. [13] reported that among the main gene categories regulated in sugarcane after 150 days of exposure to elevated [CO_2] are those related to the cell cycle and plant development, along with a significant difference in height and biomass between control and treated plants. The increased biomass of C_4 plants under high [CO_2] was typically attributed to reduced stomatal opening and conductance, as well as to subsequent improvement in the leaf water status [64,81] (Figure 1).

Sugarcane also shows a positive regulation of a few specific genes involved in sugar metabolism, such as the induction of the xyloglucan endotransglucosylase/hydrolase (XTH) gene after 90 days under high [CO_2] [13]. XTH can hydrolyze xyloglucan backbones, consequently contributing to plant tissue expansion [82], thus suggesting a greater potential for leaf cells to expand (increased leaf area) and accumulate more sugars [13].

Genes related to PSI and PSII (photosystem I reaction center subunit N (PSAN) and photosystem II protein K (psbK)) were positively regulated in sugarcane after 90 days under high [CO_2], concomitantly with the reported increase in CO_2 assimilation rate [13]. In contrast, genes encoding Rubisco-related subunits were inhibited, as reported in maize by Huang et al. [62]. Therefore, despite the probable photosystem and electron transport chain functional normality in C_4 plants due to increased atmospheric CO_2, inhibited or lower CO_2 fixation by Rubisco in the Calvin cycle may be possible. In addition, C_4 plants invest more in the PEPC and pyruvate phosphate dikinase (PPDK) photosynthetic cycle and less in Rubisco [83].

Some factors, such as, the experimental conditions ([CO_2] levels and exposure time) can influence in C_4 plants responses. In general (but lacking consensus), it is understood that the photosynthetic apparatus may acclimatize in the initial periods of plant exposure to high [CO_2] [83]. In relation to control plants (ambient [CO_2]), it has been shown that CO_2 assimilation increases (A) [13], which was also observed in the increasing exchange rate of leaf CO_2 in sorghum, maize, and sugarcane, especially in younger leaves [12,84,85]. Leaf CO_2 exchange rate increase (20%) in sugarcane

plants (7 days after leaf emergence) grown at double-ambient (720 µmol mol^{-1}) CO_2 [12].

Photosynthesis of the sorghum second leaves of FACE-grown plants (6 days, under daytime values of 566 µL l^{-1} and 373 µL l^{-1} [CO_2] and the mean nighttime values of 607 µL l^{-1} and 433 µL l^{-1} [CO_2] for FACE and control, respectively) was 37% greater than that of control-grown plants [84], and in youngest fully expanded leaf of maize grown under 550 µmol mol^{-1} [CO_2], authors observed at times that A was up to 41% greater in leaves grown and measured under elevated [CO_2] compared with ambient [CO_2] [85]. Similar effects of increased [CO_2] to other physiological responses in relation ambient [CO_2] (control plants) have been shown in these three C_4 species. The stomatal conductance (gs) decreases [13,19,64]; the transpiration rate (E) decreases [64,77]; and the expression of PEPC, NADP-ME, and other genes/enzymes related to photosynthetic metabolism changes [13,19] (Figure 1).

Some responses remain unchanged (for example, gs) with the long-term exposure of sugarcane plants (70–120 days) to high [CO_2] (720 ppm), but other processes can be adjusted (regulated), such as the decreased photosynthetic capacity of plants due to carbohydrate accumulation in the leaf [13,62,86] and the modulation of the expression levels of genes related to growth, such as auxin response factor 2 (ARF2). Some studies showed the essential roles of ARFs in plant growth and development, as well as in adapting to stress [87,88]. However, the role of ARF2 becomes evident in the results reported by Lim et al. [89] as a true repressor of auxin signaling, that is, it directly interferes with the coordination of plant growth [90]. The repression of ARF2 in sugarcane after 150 days of exposure to 720 ppm [CO_2], the differential expression of carbohydrate biosynthesis-related genes in sugarcane, such as glucose-6-phosphate dehydrogenase and xyloglucan endo-transglycosylase/hydrolase [13], and in maize exposure to 550 ppm [CO_2], such as fructose-bisphosphate aldolase and glucose-1-phosphate adenylyltransferase [62], may explain the probable regulation of mechanisms related to growth control and the consequent biomass accumulation in C_4 plants.

More experimental evaluations under high [CO_2] are necessary to further understand the connection between photosynthetic capacity and the expression of many genes, as verified for the ribulose bisphosphate carboxylase small chain gene

(rbcS) by Huang et al. [62]. In maize grown under increased atmospheric CO_2, Rubisco, PEPC, NADP-MDH (NADP-malate dependent dehydrogenase), NADP-ME, and PPDK activities were negatively regulated [83], as also reported by Vu and Allen [63] in mature sugarcane (*Saccharum officinarum* L. 'CP72-2086') leaves. However, Rubisco, PPDK, and NADP-MDH enzymes were positively regulated in another sugarcane genotype under the same [CO_2] conditions (*Saccharum officinarum* L. 'CP73-1547') [12].

C_4 plants may show biochemical variations related to the type of decarboxylation enzyme [53] and probably in the role played by Rubisco in high atmospheric CO_2 conditions. In maize, the expression of the NADP-ME gene was positively regulated in the B106 genotype, and the PEPC and NADP-MDH genes were negatively regulated in the same genotype. Different responses (except for the NADP-ME gene) were obtained in the analysis of the B76 maize genotype [19]. Therefore, genetic variability considerably influences different physiological and gene expression responses to high [CO_2].

Little information is available about the molecular responses regulated in maize, sugarcane, and sorghum, and this approach shows the importance of C_4 plants under high [CO_2] conditions and the need of further studies involving this subject. The understanding needs to include aspects of physiology, metabolic mechanisms, and molecular mechanisms regulated in this condition, especially with regard to the photosynthetic enzyme efficiency, osmotic adjustment, antioxidant metabolism, and secondary metabolite production, among other processes that may directly or indirectly influence the adaptability responses to the climatic condition.

5. Perspectives

As previously reported, the elevated [CO_2] contributes to the increase of biomass in maize, sugarcane, and sorghum. However, high [CO_2] levels are closely related to water stress, one of the major factors of crop production and productivity losses [5]. How would a C_4 plant sensitive to water stress react in conditions of high atmospheric [CO_2]? Would it activate the same set of genes already identified under normal CO_2 conditions? What are the physiological mechanisms and gene and/or protein profiles regulated under these conditions?

Increased [CO_2] influences the photosynthetic and carbohydrate metabolisms. However, we know that the study efforts will be directed towards the investigation of the combined action of different environmental stresses on plants and high [CO_2] (Figure 2). What is expected for the coming years are studies that involve physiological responses, secondary metabolism pathways, hormones, and mainly gene and protein profiles modulated by the combined effect, aiming at C_4 plants more adapted to climatic diversities that are tolerant and productive.

In this sense, studies aiming to provide information on the molecular machinery through which C_4 plants respond to increased atmospheric CO_2 are fundamental to supply genetic data to the scientific community. Several gene groups (more than 1300 differentially expressed genes) were identified by Ge et al. [91] through analysis of the maize transcriptome under high [CO_2]. These data, associated with physiological aspects, can provide additional support to understand the effect of this increase and the interactive effect of stresses.

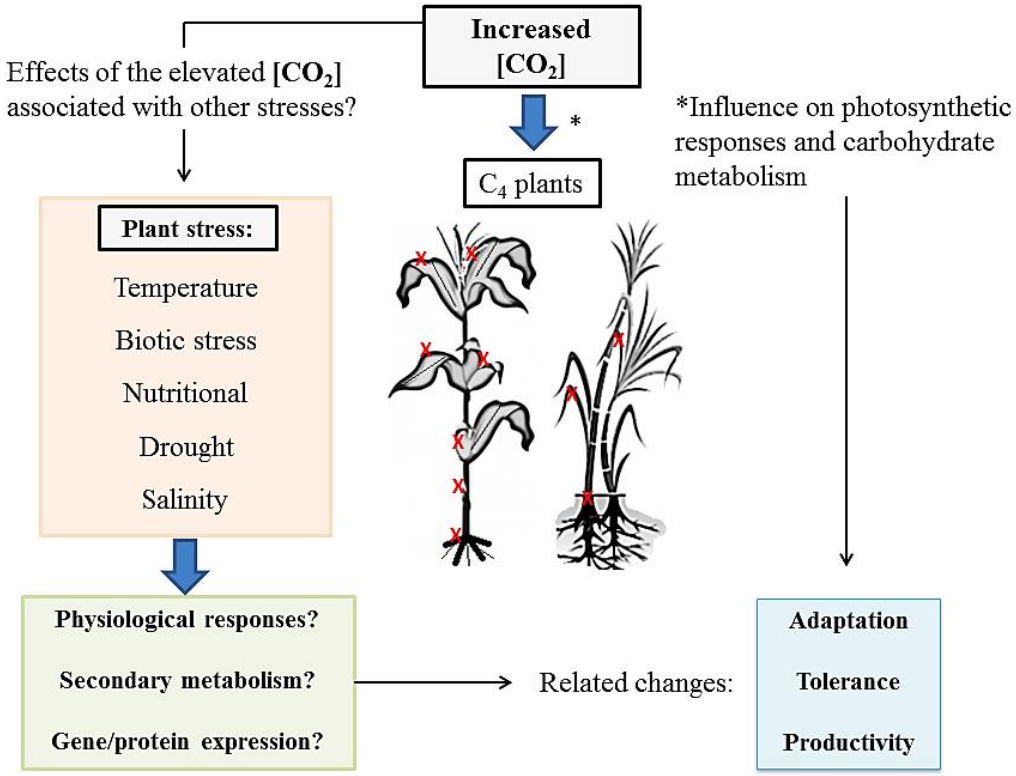

Figure 2. Environmental factors and increased [CO_2] influence on physiology and general metabolism of C_4 plants.

6. Conclusions

The physiological responses of C_4 plants to increased [CO_2], such as transpiration rate, stomatal conductance decreases, and photosynthetic enzymes are differentially expressed in maize, sugarcane, and sorghum. The gene regulation involved in carbohydrate metabolism and plant growth changes according to time of exposure to stress and experimental aspects.

C_4 plants can present advantages in high [CO_2] environments and, owing to climatic forecasts involving increased temperature and decreased precipitation, the understanding of adaptive physiological and genetic mechanisms is fundamental in order to guarantee the satisfactory growth of these plants.

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CAPÍTULO 3 – STATISTICAL TECHNIQUES APPROACH: TWO SUGARCANE GENOTYPES GROWTH UNDER ELEVATED [CO_2] ATMOSPHERIC AND DROUGHT[1]

Abstract

This article aims was to compare two sugarcane genotypes under elevated atmospheric carbon dioxide (CO_2) concentration and soil water deficit, through an approach based on statistical analysis of plant growth multiple characteristics. For that, Pearson's correlation, dissimilarity and principal components (PC) analysis were performed, of some parameters such as stem length (culm) and roots length, number of leaves, dry mass of plant tissues, among other characteristics. Biometric attributes have a positive and significant correlation with each other. However, the dry mass values have a negative correlation with the other parameters, meaning, therefore, there is a growth reduction as there is also a decrease in plants water conditions subjected to stress. Based on the dissimilarity and PC analysis, observed that genotypes genetically diverge and the imposed environmental condition influences growth responses. This preliminary study is an indication that this approach is essential for understanding the variations between the sugarcane genotypes responses. In addition, is evident (due to divergence) that analyses about the physiological and molecular responses of these genotypes in this environmental condition will contribute to the identification of regulated biological processes and possible tolerance mechanisms.

Keywords: Multivariate analysis; Growth; Climate changes; *Saccharum* spp.

[1]Este capítulo corresponde ao artigo científico publicado no periódico *Research, Society and Development*. Doi: 10.33448/rsd-v9i9.7645

1. Introduction

Sugarcane (*Saccharum* spp.), from to the Poaceae family, has great importance for the national economy, mainly due to its use as a raw material in the sugar, ethanol, and bioenergy production and also for animal feed (Tejera et al., 2007; Torquato & Ramos, 2013). Brazil is the world's largest sugarcane producer, with annual production above 700 million tons (FAOSTAT, 2018) and stands out as the second largest ethanol producer (USDA, 2019).

This dynamic scenario in the sugar and alcohol sector is directly related to the expansion of new planting areas and / or the development of strategies to improve sugarcane, aiming to increase productivity. Among the strategies, the search for genotypes (cultivars) more adapted to different edaphoclimatic conditions of the country is considered very important.

Adapted to tropical and subtropical climates, sugarcane needs a large volume of water in production system, especially when is in complete vegetative development (Tejera et al., 2007). However, changes in the water availability and global temperature negatively influence plant growth leading to significant reductions in productivity.

These changes are partly a consequence of climate change due to the increased greenhouse gases emission (IPCC, 2007). The CO_2 concentration ($[CO_2]$) has increased considerably since the industrial revolution. Currently, atmospheric $[CO_2]$ is in the range of 409 ppm (Misra et al., 2019), with forecasts for 2100 ranging from 700 to 900 ppm (NASA, 2015).

Increase of CO_2 in the atmosphere affects plants biological processes at different organization levels (Mooney et al., 1999). Researches indicate changes in photosynthetic rates, stomatal conductance, carbohydrate metabolism and other important physiological processes for the sugarcane growth (Vu et al., 2006; Souza et al., 2008).

However, compared to the studies carried out with C_3 metabolism plants, there are few studies with C_4 metabolism plants, (e.g. sugarcane and sorghum) that aimed to investigate the physiological, biochemical, molecular and growth responses under atmospheric conditions of $[CO_2]$ expected in the coming years.

The associated effects of increased $[CO_2]$ and water deficit can induce morphophysiological changes in sugarcane plants. Those changes may become

important clues to compare genotypes regarding to their tolerance levels to environmental conditions, and the correlation between the characteristics (biometric, physiological, among others) contributes to the definition of criteria that guide a more efficient selection.

The multivariate analysis techniques as a tool to find patterns and relationships between several variables simultaneously are important for the genetic analysis of the relationship between evaluated characteristics and genetic material (Queiroz et al., 2011). To estimate the genetic divergence biometric models are used and, using statistical methods, with multiple information, it is possible to measure the dissimilarity (Sudré et al., 2005).

Thus, considering that few studies have been done combining the action of different types of stress on plants, especially in sugarcane, in the present study, statistical analyses were carried out of some growth parameters from two sugarcane genotypes under the combined effect of elevated [CO_2] and soil water deficit.

2. Methodology

Sugarcane plants from IACSP94-2094 (genotype A), and IACSP94-2101 (genotype B), 50 days old, were supplied by the Agronomic Institute of Campinas (IAC), Ribeirão Preto - SP, Brazil. Genotypes are contrasting regarding to agronomic characteristics such as: production, planting recommendation, tolerance to unfavorable environments, and others. Genotype A is considered tolerant to water deficit (Machado et al., 2009). Plants were transferred to pots (10 L) containing sand and soil mixture (1:1, v / v). After a 7-day acclimatization period in growth chambers (Fitotron-Weiss Gallenkamp, UK), under controlled temperature (28°C) and humidity (60%) conditions, plants were subjected to the combined stress effects for a period total of 30 days.

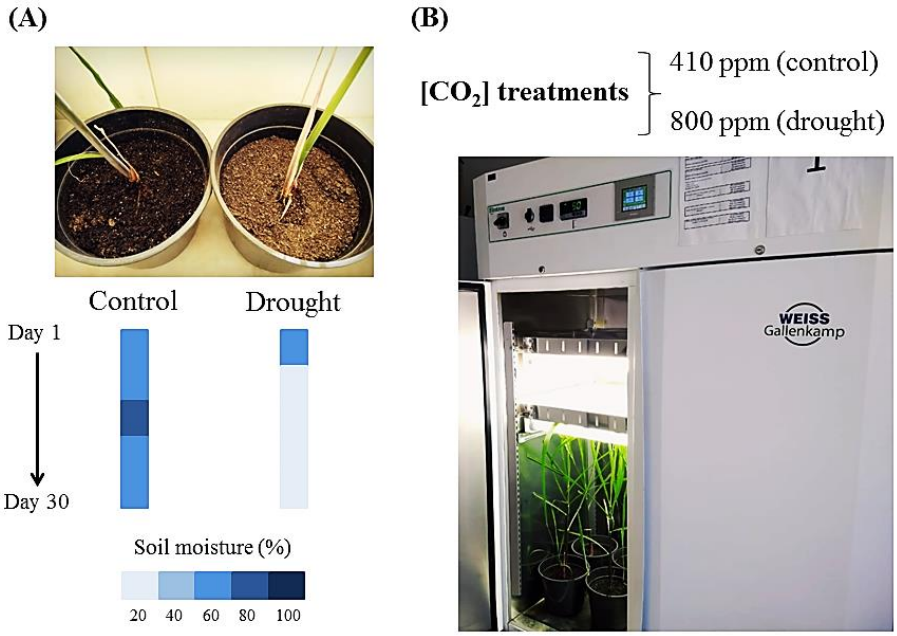
The methodology used is it of a quantitative nature (Pereira et al., 2018). Experimental design was completely randomized, two genotypes and two stress conditions, with three biological replicates. The established stress conditions consisted of plants in pots properly irrigated in a chamber with an atmospheric [CO_2] of 410 ppm (control); and plants subjected to water deficiency (irrigation suppression) in a chamber with [CO_2] of 800 ppm (Figure 1).

After 30 days plants were evaluated for growth and physiological parameters, including stem length (SL), stem diameter (SD), green leaves (GL or number of leaves), roots length (RL), roots area (RA), roots volume (RV), dry mass leaves (DML), dry mass stem (DMS), and dry mass roots (DMR). RA and RV measurements were determined in three plants per treatment, using SAFIRA software (Root and Fibers Analysis System) (Jorge & Rodrigues, 2008). DML, DMS, and DMR were measured using an analytical balance. For the dry mass (DM) determination, we obtained the dry mass of the samples (1 gram of leaves, stem, and roots) after drying at 65 °C for 24 h in an oven. DM calculation followed the formula DM (leaves, stem, and roots) = [(dry weight/ fresh weight)] × 100.

Data of the evaluated parameters were submitted to variance analysis (ANOVA) by the AgroEstat software - System for Statistical Analysis of Agronomic Tests, version 1.1.0 (Barbosa & Maldonado, 2014). Pearson's correlation, Dissimilarity, and Principal components analysis (PCA) were carried out using XLSTAT software package (Addinsoft, France).

Dissimilarity (Agglomerative hierarchical clustering – AHC) between treatments was measured by the Euclidean distance and the agglomeration method was performed by the Ward’s method, to determine the number of clusters previously. In the PCA, the eigenvalues were extracted from the covariance matrix that generated the eigenvectors called principal components.

Figura 1. Experimental Design. (A) Soil moisture monitored over the 30 stress days; (B) Control pot plants were subjected to treatment with 410 ppm [CO_2] and plants in drought pots were subjected to treatment with 800 ppm [CO_2].



Source: Authors.

3. Results and Discussion

The correlations analysis between the biometric (SL, SD, GL, RL, RA, and RV) and physiological attributes (DML, DMS, and DMR) showed significant values in the correlation matrix. Strong and positive correlation (≥0.730) between SL, SD, and GL with all biometric parameters; RL with SL, SD, and GL; RV with SL, SD, GL, and RA; RA with all parameters except RL, was observed (Figure 2A). These results indicate that plants of both genotypes under the imposed stress condition (elevated [CO_2] and drought) showed changes in the growth and development of the aerial part and the root system.

The stem growth and the number of leaves of both genotypes were affected by environmental condition imposed, and these changes are significantly related to the root length (e.g., correlation coefficient between SL and RL higher than 0.800) (Figure 2A). In contrast, there is a negative correlation between physiological measures (dry

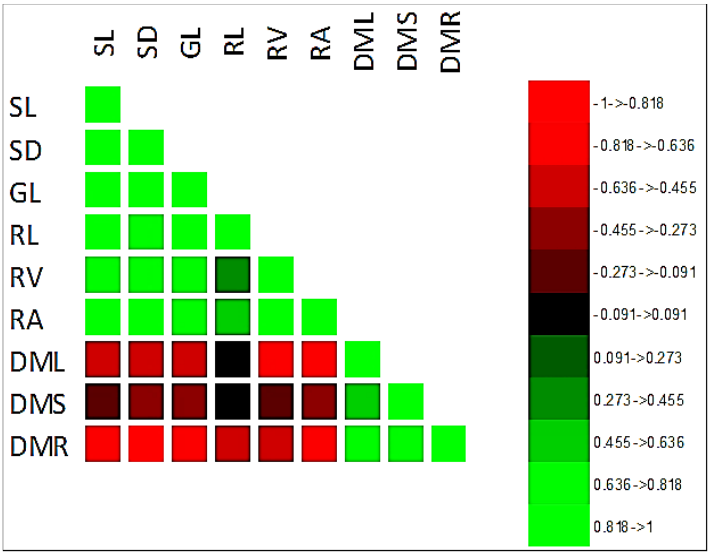
mass) and the biometric parameters evaluated (red correlation values) (Figure 2B). Dry mass analysis of 1 gram of samples is directly related to the water content available in plant tissues (water loss). Therefore, higher DML, DMS, and DMR values represent an increase water loss.

Figure 2. Pearson's correlation coefficients of biometric and physiological traits evaluated in two sugarcane genotypes under elevated [CO_2] and drought. (A) Correlation matrix (Pearson's) considering the data for the two genotypes. Values in bold are different from 0 with a significance level alpha=0.05; (B) Heatmap representing the negative (red) and positive (green) correlation values.

(A)

Variables	SL	SD	GL	RL	RV	RA	DML	DMS	DMR
SL	**1**	**0.962**	**0.945**	**0.828**	**0.803**	**0.879**	-0.498	-0.147	**-0.721**
SD		**1**	**0.954**	**0.816**	**0.730**	**0.834**	-0.548	-0.382	**-0.869**
GL			**1**	**0.830**	**0.769**	**0.816**	-0.514	-0.283	**-0.770**
RL				**1**	0.401	0.494	0.008	0.018	-0.553
RV					**1**	**0.938**	**-0.795**	-0.206	**-0.578**
RA						**1**	**-0.781**	-0.283	**-0.672**
DML							**1**	**0.628**	**0.662**
DMS								**1**	**0.762**
DMR									**1**

(B)



SL stem lenght
SD stem diameter
GL green leaves
RL roots lenght
RA roots area
RV roots volume
DML dry mass leaves
DMS dry mass stem
DMR dry mass roots

Source: Authors.

According to the correlation coefficients (Figure 2), the sugarcane genotypes under elevated [CO_2] and drought, have reduced measures of growth attributes and DM values increased, consequently due to water loss. As these characteristics can be associated with more expressive productivity, they are then considered valid during the breeding procedure, since they contribute to selection of sugarcane genotypes more tolerant to water deficit conditions (Silva et al., 2008).

Additionally, it appears that based on the characteristics evaluated, the four treatments (three biological replicates per treatment) are distributed in 3 groups according to dendrogram that shows the hierarchy groups (Figure 3) and distribution of the cluster centroids (average distance centroid ranging from 4531.166 to 2977.588) (Table 1).

Cluster 1 was formed by genotype A, control plants. The plants of genotype B (control) and one plant of genotype A (under stress) constitute the second cluster. Cluster 3 was formed by two plants of genotype A under stress and plants of genotype B under stress. Therefore, there is a clear difference between genotypes and an approximation of these taking into account all parameters evaluated and the interaction with the environment (genotype x environment) (Figure 3). That is, the genotypes under stress (high [CO_2] and drought) show some similar growth responses.

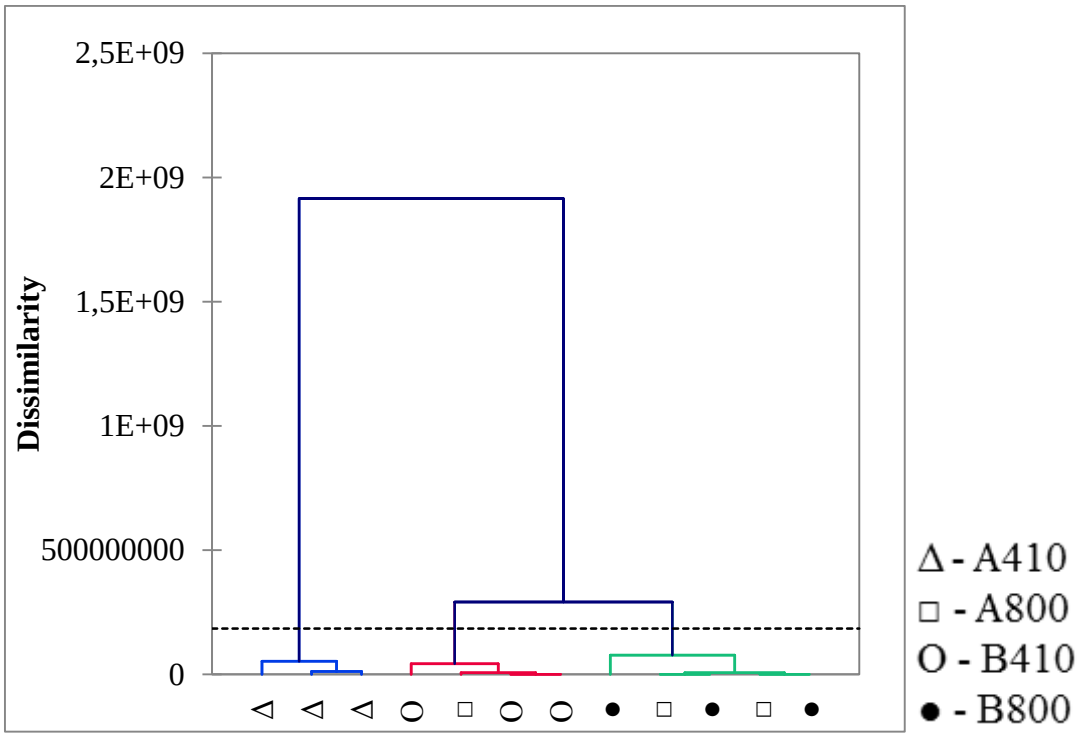
However, it is emphasized that the existing genetic variability is a crucial point to identify the studied genotypes as divergent (genetic divergence expressed as a measure of dissimilarity). Considering this genetic divergence between the evaluated genotypes, it becomes necessary to understand how other physiological responses (photosynthetic apparatus), biochemical and molecular mechanisms (carbohydrate metabolism, antioxidant defense, etc.) are regulated in the predicted climatic condition. Queiroz et al. (2011), evaluated two sugarcane cultivars (IAC91-2195 e IAC91-5155), and presented information’s on drought tolerance, based on the responses of biochemical and physiological indicators to water stress by assessing the levels of osmoprotectors and biometric variables of initial growth.

The variability can be explained by the analysis of the main components, resulted in two principal components (PC), which explained 87.316% of the variance (cumulative variability) (Figure 4A). The two-dimensional plane formed by the components F1 (69.34%) and F2 (17.97%) retained this original variance above 87%

(Figure 4B). The treatments are distributed on a coordinate factor plan considering the relationship between the active variables.

Variables related to the biometric parameters (SL, SD, GL, RL, RA, and RV) explain much of the variability between the genotypes. DM variables (DML, DMS, and DMR) differentiate genotypes under stress (A800 and B800) and contribute to the least variability (Figure 4B). However, plants of genotype B (B800) have higher DM values. This difference is an important factor that must be taken in consideration, as it is a good reference of water conditions in plants and other vital processes at the cellular level.

Figure 3. Dendrogram of dissimilarity indices based on 12 treatments plus GE (genotype x environment) interaction. A410 (genotype A control); A800 (genotype A under stress); B410 (genotype B control); and B800 (genotype B under stress).



Source: Authors.

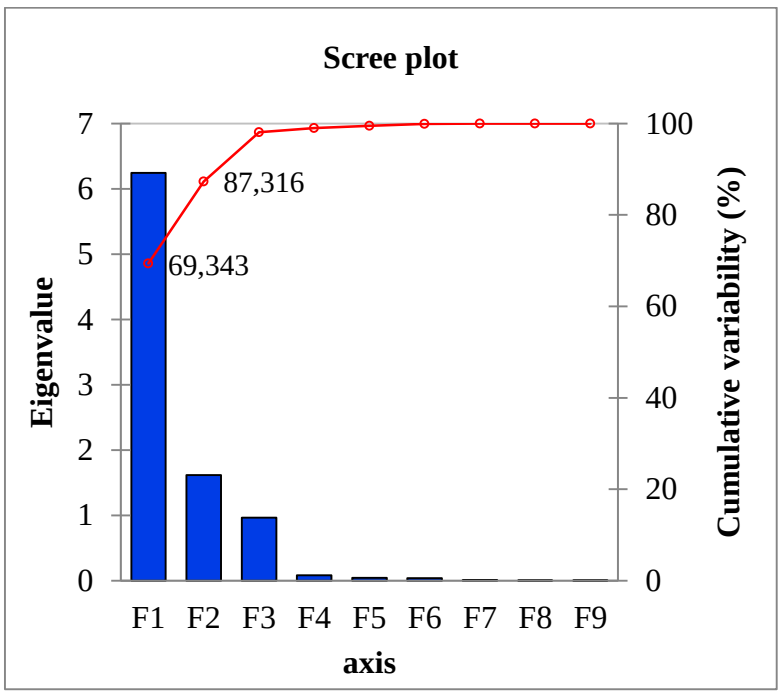
Table 1. Distribution of the cluster centroids.

Class	1*	2*	3*
Objects	3	5	4
Within-class variance	32362914.902	21026102.545	16562951.450
Average distance to centroid	4531.166	3411.634	2977.588
	Δ	□	□
	Δ	□	O
	Δ	●	O
		●	O
		●	

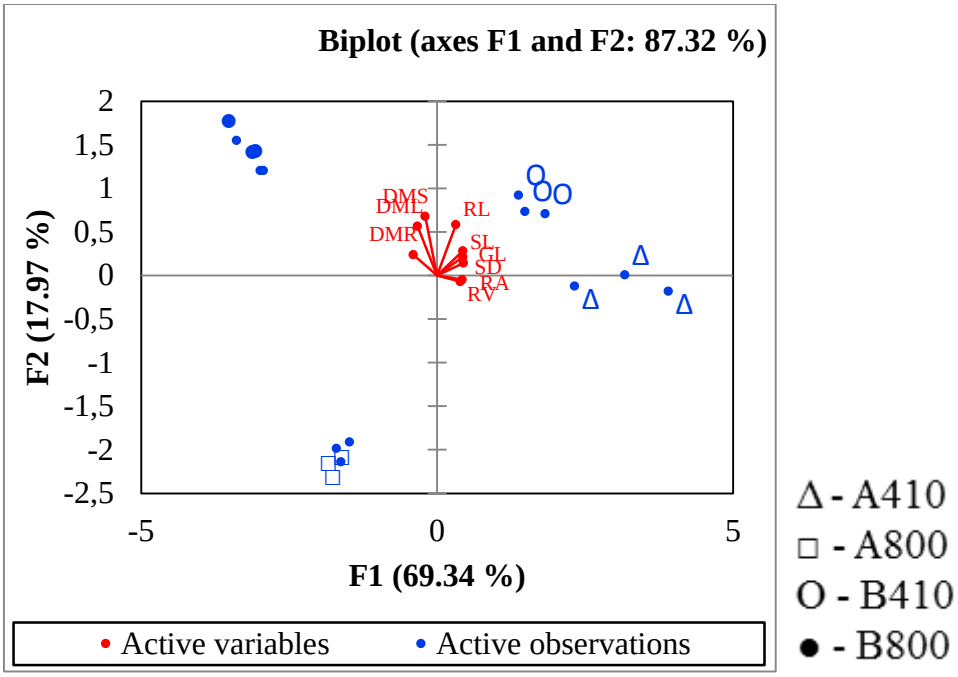
*Δ A410; □ A800; O B410; ● B800. A410 (genotype A control); A800 (genotype A under stress); B410 (genotype B control); and B800 (genotype B under stress). Source: Authors.

Figure 4. Principal component analysis. (A) Scree plot showing eigenvalue and cumulative variability of the nine traits evaluated; (B) Biplot showing the dispersion of the 12 treatments, for the principal components F1 x F2. A410 (genotype A control); A800 (genotype A under stress); B410 (genotype B control); and B800 (genotype B under stress).

(A)



(B)



Source: Authors.

Therefore, the results show that the correlation coefficients, exploratory dissimilarity analyses and principal components are a good strategy to compare and to classify the treatments tested based on the multiple characteristics. This type of analysis is an exploratory method that characterizes the interaction of data in understanding the mutual influence of plants responses to adverse environmental conditions (Queiroz et al., 2011; Silva et al., 2012).

This approach can be useful in the study of the several morphophysiological and molecular characteristics responsive to climate changes expected for the coming years, evaluating, for example, a wider group of sugarcane genotypes and other C_4 crops.

4. Final Considerations

Correlation coefficients between the biometric parameters were positively significant, and these establish a negative correlation with the dry mass measurements. Multivariate analyses successively discriminate the treatments tested. The sugarcane genotypes (IACSP94-2094 and IACSP94-2101) present divergent

performances in the experimental conditions evaluated. As a suggestion for the next researches, we recommend the evaluation of a greater number of sugarcane genotypes. In addition, the determination of physiological and molecular responses may be an important tool for understanding the different biological mechanisms (tolerance) that are regulated in sugarcane under associated abiotic stress.

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CAPÍTULO 4 - MODULATION OF ANTIOXIDANT SYSTEMS AND SECONDARY METABOLISM BY ELEVATED [CO_2] AND DROUGHT IN SUGARCANE

Abstract

Climate change has an important impact on agricultural production. In this context, environmental factors such as drought and an increase in the concentration of atmospheric CO_2 [CO_2], can trigger changes in several agricultural crop performance. The present study investigated morphophysiological, biochemical, and molecular responses of two sugarcane genotypes subjected to elevated [CO_2] and drought. Genotypes IACSP94-2094 and IACSP94-2101 were grown in chambers with 410 ppm [CO_2] (well-watered) and 800 ppm [CO_2] (with drought). After 30 days in these conditions, the plants were evaluated for growth parameters, measurements of total soluble solids, relative water content (RWC), quantum efficiency of photosystem II (Fv/Fm), stomatal density, photosynthetic pigments, H_2O_2 and MDA content, antioxidant enzyme activities, glutathione (GSH), and flavonoids content. Moreover, the transcriptomic analysis was performed. Both sugarcane genotypes showed a considerable reduction in growth when subjected to stress condition. However, IACSP94-2094 genotype showed RWC, Fv/Fm, and stomatal density measurements higher than the values observed for IACSP94-2101 genotype. There was a reduction in the ascorbate peroxidase (APX) activity in both genotypes and contrasting responses in the glutathione cycle. A total of 2,081 and 4,266 genes were differentially expressed (DEGs) in the IACSP94-2094 and IACSP94-2101 genotypes, respectively. The IACSP94-2101 genotype, more responsive to abiotic stress, activates a set of peroxidases and lipoxygenases. On the other hand, in the IACSP94-2094 genotype, we verified a possible transient H_2O_2 and MDA accumulation, signaling to activation of the antioxidant system, genes associated with secondary metabolism, and less severe damage to photosystems. The identified stress-responsive genes can be useful for understanding the adaptive mechanisms of sugarcane against environmental changes.

Key words: Antioxidant enzymes, Climate change, RNA sequencing, *Saccharum* spp., Secondary metabolism.

Introduction

Sugarcane (*Saccharum* spp.), a perennial grass of the family Poaceae (Simpson, 2010) is of considerable importance for Brazilian agribusiness. It is considered a robust and efficient crop in the production of sugar, ethanol and biomass (Silva et al., 2008; Hodgson-Kratky et al., 2019). Most of the world's sugarcane is grown in subtropical and tropical areas, being that adequate available moisture throughout the growing period is important for obtaining maximum yields (FAO, 2021).

The sugarcane (vegetative stage) is significantly dependent on climatic factors (Silva et al., 2010), and its yield is negatively influenced by environmental factors such as drought (Lawlor and Cornic, 2002). Changes in the water regime and global temperature specifically are partly a consequence of climate change, mainly due to the increased emission of gases such as carbon dioxide (CO_2) (Carvalho et al., 2010; NASA, 2020). The current reported level of atmospheric CO_2 concentration ([CO2]) is in the range of 411.2 ppmv (parts per million volume) (NOAA, 2020), with forecasts for levels above 900 ppmv for the next 80 years (Meehl et al., 2007).

In several experiments (Anderson et al., 2001; Souza et al., 2008; Souza et al., 2015; Khan et al., 2020), it was observed that plants with C_4 photosynthetic metabolism have shown different responses to the increase in [CO_2]. Studies carried out with sugarcane (Vu et al., 2006; Souza et al., 2008) and sorghum (*Sorghum bicolor*) (Vara Prasad et al., 2009) indicates the increase in photosynthetic rates. In maize (*Zea mays*), changes in transpiration rate and carbohydrate metabolism were observed (Prins et al., 2010).

Associated with the [CO_2] effects it is known that other environmental conditions cause drastic changes in the morphophysiological and genetic responses of plants. Water deficiency in the soil is one of the main factors limiting the sugarcane growth and production (Silva and Costa, 2004; Flexas et al., 2006; Solari et al., 2006). In sugarcane subjected to water deficiency, more

than 1,600 differentially expressed genes were identified and involved, e.g., in different stress response mechanisms (Rodrigues et al., 2011).

What would be the main effects of combining the different abiotic stresses? In sugarcane and other C_4 plants there are few studies that report these responses (Silva et al., 2020). The lack of information on the effects of cultivars and climate on productivity and quality of sugarcane crop has been the main impact factor in the sugar and alcohol sector in Brazil (Cardozo and Sentelhas, 2013). In durum wheat (*Triticum turgidum*), a C_3 plant, the interactive effects of water deficiency and the increase in [CO_2] were studied by Medina et al. (2016). The authors found that the effects vary according to the plant genotype and severity of water stress, especially when observing the expression of genes responsive to oxidative stress.

Plants have developed several strategies to deal with oxidative stress (Dumont and Rivoal, 2019). Antioxidant mechanisms can minimize the damage resulting from this stress, by eliminating the free reactive oxygen species (ROS) in the cells and maintaining the cellular redox state (Gratão et al., 2015). Oxidative stress is caused by the increase in ROS levels that leads to the generation of superoxide (O2- •), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·) (Barbosa et al., 2014; Sies et al, 2017).

The primary line of defense at the cellular level comprises antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9) (Das and Roychoudhury, 2014; Hippler et al., 2015; Noctor and Foyer, 2016). Other mechanisms, considered non-enzymatic, including compounds such as flavonoids, carotenoids and glutathione (GSH), may be able to neutralize the cytotoxicity of ROS (Agati et al., 2012; Barbosa et al., 2014; Haida and Hakiman, 2019 Nabavi et al., 2020). Processes related to the transport of flavonoids from their biosynthesis site (endoplasmic reticulum) to different cell compartments been demonstrated (Agati et al., 2012), as well as that their biosynthesis has been reported by oxidative stress stimulus, which is triggered by ROS generated by the metabolic functions of plants (Nabavi et al., 2020).

Although plant responses to different abiotic stresses have been the subject of study in recent years, few studies have been carried out combining

the action of different types of stresses on plants. Among the strategies that are used to identify genes that are differentially expressed in the sugarcane genotypes and knowledge about the regulated mechanisms in different stressful conditions, RNA sequencing (RNA-seq), a transcriptomic technology that uses high-throughput sequencing, has been used to analyze tissue or cell cDNA libraries (Rosa-Santos et al., 2020). Studies had been carried out using RNA-seq to identify genes that are differentially expressed in sugarcane under aluminum stress (Rosa-Santos et al., 2020), water stress (Belesini et al., 2017; Nawae et al., 2020), and biotic stress (Malviya et al., 2020).

Thus, in order to understand the major antioxidant mechanisms and stress-responsive genes modulated in sugarcane under water deficiency associated to elevated [CO_2], the study investigate the main morphophysiological, biochemical and gene expression changes in two sugarcane genotypes (IACSP94-2094 and IACSP94-2101). The stress-modulated genes identification expands our knowledge about the adaptive mechanisms and can be useful for genetic improvement of sugarcane.

Materials and methods

Plant material and experimental conditions

Sugarcane seedlings were provided by the Instituto Agronômico de Campinas (IAC), Centro de Cana, Ribeirão Preto - SP, Brazil. Seedlings of contrasting sugarcane genotypes (IACSP94-2094, genotype A; and IACSP94-2101, genotype B) (Supplementary Table S1) were grown for a 50 days period under regular greenhouse conditions. After that plants with similar aerial development characteristics (similar heights) were transferred into 10 L pots (one plant per pot) containing a mixture of sand and soil (1:1, v/v) and kept in growth chambers (Fitotron-Weiss Gallenkamp, UK), under controlled temperature (28 °C), relative humidity (60%), photon flow density (190 $\mu mol.m^2.s^{-1}$) and a 12/12 h dark/light photoperiod for 7 days. After this period, the atmospheric concentrations of CO_2 inside the growth chambers were adjusted to 410 ± 10 ppm (chamber I) and 800 ± 10 ppm (chamber II).

Plants in the chamber I (control treatment) were irrigated over the 30 days of the experiment and the plants located in the chamber II had their irrigation

stopped (stress treatment). The experimental design is shown in Figure 1. After 30 days in the chambers, plants were analyzed for the biometric, physiological, and biochemical parameters described below. Leaves were collected for further analysis by RNA-seq.

Plant growth analysis

After 30 days we evaluated growth characteristics of the aerial part (three plants per treatment), including culm length (cm), culm diameter (mm), and number of leaves. Roots growth was also assessed, using the root length (cm) and total root area (TRA, mm^3). TRA was determined with architectural measurements from images in Safira software (Root and Fibers Analysis System), public-domain software for root image analysis (Jorge and Rodrigues, 2008). The roots sampled (total root of three plants per treatment) were separated from soil by washing with water. For each sample, one image (linearized) was analyzed by Safira.

Chlorophyll a fluorescence and photosynthetic pigment content

Chlorophyll *a* (Chl*a*) fluorescence was measured using a portable fluorometer OS30p (Opti-Sciences, USA) in randomly three points on the leaves (third fully expanded leaf) from five plants per treatment. The photosystem II (PSII) efficiency was measured in dark-adapted leaves (30 min of adaptation). Values of maximum fluorescence yield (F_m) and minimum fluorescence yield (F_0) were used to estimate the maximum quantum yield of PSII [$F_v/F_m = (F_m-F_0)/F_m$] (Maxwell and Johnson, 2000).

Leaves samples (0.5 g), from three plants per treatment, were added into tubes containing 5 ml of acetone 80% (v/v). The tubes were kept in the dark at 8 ºC for 48 h and the absorbance was measured with a spectrophotometer – CCD array (GeneQuant 1300 Spectrophotometer, BioChrom, USA) at 663 nm, 647 nm, and 470 nm. The contents (µg. g^{-1} FW) of Chl*a*, Chl*b*, total chlorophyll (Chl*a* + Chl*b*), and carotenoids were determined through equations established by Lichtenthaler (1987).

Relative water content

Leaf relative water content (RWC, %) was determined for ten leaf discs (Ø = 1 cm) collected for each expanded leaf (from the three plants per treatment). Fresh (FW), dry (DW), and turgid weight (TW) for all samples plants were measured using an precision analytical balance and RWC calculated according to Barrs and Weatherley (1962), using the formula: RWC (%) = [(FW−DW)/ (TW−DW)] × 100.

Total soluble solids

Total soluble solids (Brix, %) was determined with a portable/optical LED-RHB0-90 brix refractometer (YHequipment Co., Shenzhen, China), accuracy ±0.5Brix, equipped with built-in ATC compensation range (automatic temperature compensation), used to measure the refractive index of soluble solids in the juice extracted from sugarcane stalk. The sugar broth was extracted by pressing 5-8 cm of culm, from five plants per treatment. Droplets from the broth were deposited in the refractometer reader.

Stomatal density

To assess stomatal density, stomatal impressions were made in regions of the middle third of adult leaves, four regions on each expanded leaf (adaxial and abaxial surface), with three plants per treatment. For the printing of the leaf, a drop of colorless adhesive was added on a microscopic slide and the leaf surface was pressed for approximately 3-5 sec. The stomata count (density per mm^2) was estimated using an Optical microscope (Eclipse E200, Nikon, Tokyo, Japan), with a 10x magnification objective and determination of the 0.5 x 0.5 mm analysis area. ImageJ software was used to count stomata in each sampled field.

Lipid peroxidation and H_2O_2 content

Lipid peroxidation was estimated by the substances reactive content to thiobarbituric acid (TBARS), as described by Shimizu et al. (2006). Malondialdehyde (MDA) equivalents concentration was determined using an extinction coefficient of 1.55 x 10-5 $mol^{-1}cm^{-1}$, with readings (in triplicate) on the spectrophotometer between 535 and 600 nm and expressed in µmoL mg^{-1} FW.

H_2O_2 content was estimated according to Alexieva et al. (2001) method. After maceration, homogenization, and obtaining the supernatant, 100 mM potassium phosphate buffer (pH 7.5) and 1 M potassium iodide was added to the final solution, followed by incubation in ice for 1 h without light. Absorbance was read at 390 nm on a spectrophotometer (in triplicate). H_2O_2 content was determined using a standard curve of known concentration and result expressed in μmoL mg^{-1} FW.

Enzyme extraction and protein determination

Leaves samples (1 g) of three plants per treatment were macerated in liquid N_2 and homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol (DTT), and 5% (w/v) polyvinylpolypyrrolidone insoluble (PVPP) (Boaretto et al., 2014). After homogenization, the solution was centrifuged at 10,000 x g for 30 min at 4 ºC, and the supernatant (divided into aliquots – leaves extracts) stored at -80 °C for later enzymes activities determination. Protein concentration was determined by the Bradford (1976) method, using bovine serum albumin (BSA) as the standard.

Superoxide dismutase (SOD, EC 1.15.1.1) assay

SOD activity was determined using non-denaturing PAGE as described by Azevedo et al. (1998). Leaves extracts (70 μg) were submitted to gel separation. The gels were rinsed in distilled-deionized water and kept in the dark for 30 min in 50 mM potassium phosphate buffer (pH 7.8) with 0.05 mM riboflavin, 0.1 mM nitro blue tetrazolium, 1 mM EDTA, and 0.3% N,N,N´,N´-tetramethylethyllenediamine. After, the gels were again rinsed in distilled-deionized water and from the lighting it was possible to observe the achromatic bands of SOD isoenzymes. These isoenzymes were distinguished and classified by sensitivity to inhibition by 5 mM hydrogen peroxide (H_2O_2) or 2 mM potassium cyanide. The relative abundance of the bands was calculated using the ImageJ software.

Ascorbate peroxidase (APX, EC 1.11.1.11) assay

APX activity was measured according to (Gratão et al., 2012; Alves et al., 2018). Reaction consisted of leaf extract (50 μL), 50 mM potassium phosphate

buffer (pH 7.0), 0.5 mM ascorbate, and 0.1 mM H_2O_2. APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm, 30 ºC, for 1 min. APX activity was expressed as µmol min^{-1} mg^{-1} protein.

Glutathione peroxidase (GPX, EC 1.11.1.9) assay

GPX activity was determined by estimating the decrease in absorbance at 340 nm during the first minute of reaction. Leaf extract was added to a solution containing 100 mM potassium phosphate buffer (pH 7.0), 3 mM EDTA, 0.24 U GR/mL, 10 mM GSH and 1 mM sodium azide (Nagalakshmi and Prasad, 2001), with adjustments. The reaction was catalyzed by the addition of 1.5 mM NADPH and 1.5 mM H_2O_2, and the oxidation of NADPH. The activity calculation was made using the molar extinction coefficient of 6.2 mM^{-1} cm^{-1} (Anderson and Davis, 2004). GPX activity was expressed as µmol min^{-1} mg^{-1} protein.

Glutathione reductase (GR, EC 1.6.4.2) assay

GR activity was measured spectrophotometrically (412 nm) at 30ºC as described in Carvalho et al. (2013). Leaf extract was added to a solution containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5,5'-dithio-bis (2-nitrobenzoic acid, NBT), 1 mM oxidized glutathione (GSSG), and 0.1 mM NADPH. GR activity was estimated by reducing the GSSG over 1 min and expressed as µmol min^{-1} mg^{-1} protein.

Glutathione-S-transferase (GST, EC 2.5.1.18) assay

GST activity was determined as described by Zablotowicz (1995), with adjustments. The test was conducted by adding 5 mM GSH and 25 µL of leaf extract in a mixture containing 100 mM potassium phosphate buffer (pH 6.5) and 0.04 mM CDNB (1-chloro-2,4-dinitrobenzene). The absorbance reading in a spectrophotometer was done for 5 min at 340 nm. GST activity was expressed as µmol min^{-1} mg^{-1} protein.

Glutathione (GSH) content

GSH content was determined as described by Israr et al. 2006, with adjustments (Alves et al. 2018) using DTNB (5.5 '–dithio-bis (2-nitrobenzoic acid),

NADPH, sodium phosphate buffer, glutathione reductase (GR), EDTA and leaf extract. Two readings were performed on a spectrophotometer (412 nm). In the first reading, the reaction medium contained a mixture of 0.1 M sodium phosphate buffer (pH 7.0), 3 mM DTNB, 0.5 mM EDTA and the extract; in the second reading, made on the same sample, where 0.4 mM NADPH and 2 µl GR were added to the reaction. GSH content was expressed as nmol g^{-1} FW.

Flavonoids analysis

A summary of the flavonoids extraction and analysis from leaves extract (three plants per treatment) evaluated in the present study is found in the Supplementary Fig. S1. Powdered leaf material (300 mg) was mixed with 6 ml of MeOH:H_2O (50%) (methanol: J.T. Baker HPLC grade; Avantor Performance Materials, Center Valley, PA, USA) in a flask and submitted to sonication (3 x 20 sec/25 °C). The mixture was filtered through a nylon filter (Millipore, 0.45 µm) and the filtrate dried in a fume cupboard. Triplicate extractions were performed for each of the treatments. Samples (30 mg) of dried extracts were dissolved in 1 ml of an 80:20 (v/v) mixture of methanol (J.T. Baker HPLC grade) and Milli-Q Ultrapure water (Merck Millipore, Darmstadt, Germany) and applied to Supelco LC-18 solid-phase extraction (SPE) tubes (Sigma, St. Louis, MO, USA) that had been previously eluted with 1 ml of methanol followed by 1 ml of 80:20 (v/v) methanol:water mixture. 20 µl aliquots were analyzed by HPLC on a Shimadzu (Kyoto, Japan) model LC-10ADvp instrument coupled to an SPD-M10Avp diode array detector (DAD).

Separations were carried out at room temperature (20 ± 2°C) on a Luna® C18 column (5 µm, C18 100 Å, LC Column 250 x 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phase comprised methanol/water/acetic acid (80: 20: 0.1, v/v /v) and was supplied at a continuous flow rate of 1 mL/min, 116 Kgf cm^{-2}, with linear gradient: 10% B (min 0.01-32.00), 66% B (min 32.01-35.00), and 10% B (min 35.01-45.00). The detection wavelength was set at 245 nm and the acquired data were processed using Shimadzu LabSolutions Multi LC-PDA software.

Flavonoids content was determined using external standards: luteolin (Sigma-Aldrich, St. Louis, MO, EUA; #BCBN2483V), quercetin (Sigma-Aldrich,

St. Louis, MO, EUA; #SLBD8415V), and rutin (Sigma-Aldrich, St. Louis, MO, EUA; #71H0815) (Supplementary Fig. S2). Standard solutions containing flavonoids at concentrations of 500 to 1 µg/mL were prepared and calibration curves constructed by subjecting each solution to HPLC analysis in triplicate.

RNA extraction

Green and completely open leaves (3 cm length) were excised per plant and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until further use. The leaves samples were collected after 30 days of stress. Total RNA of sugarcane leaves samples was extracted using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. Four samples (treatments), each with three biological replicates were subjected to RNA extraction. After the extraction, samples were treated with DNAse (Turbo DNA-free Kit, Ambion, USA) according to the manufacturer's instructions. RNA was then quantified and assessed for integrity using the NanoDrop Spectrophotometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent, USA), following the manufacturer's protocol. Agilent 2100 Bioanalyzer (Agilent, USA) was used for the 28S/18S test (average 2.3) and RIN test (average 8.0).

cDNA library preparation

Approximately 1 µg of total RNA was initially used for BGISEQ-500 library preparation. The PE100 (paired-end 100 bp reads) strand-specific library preparation involved the steps described by Zhu et al. (2018a). At the end of the transcriptome pipeline, DNBs (DNA nanoball) loaded into the patterned nanoarrays and 2x reads of 100 bp read through on the BGISEQ-500 platform for subsequent data analysis.

RNA-seq data analysis

Raw sequencing reads were filtered to get clean reads by using SOAPnuke (v2.1.0 - https://github.com/BGI-flexlab/SOAPnuke). The clean reads were quality checked using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mapping was

performed with STAR version 2.7.0f_0328 (Dobin et al., 2013). Afterwards mapped reads were assigned to genomic features to obtain read counts for each gene, using FeatureCounts version 1.6.4 (Liao et al., 2014). Sequence annotation was carried out from monoploid reference genome sequence obtained from sugarcane R570 cultivar (https://sugarcane-genome.cirad.fr/) (Gasmeur et al., 2018) and the unmapped reads were further mapped against the *Sorghum_bicolor*_NCBIv3.47 genome (Sbi). In order to obtain the best Blast hits for sugarcane genes in sorghum genome, the transcript sequences of all the sugarcane genes were used as a query for Blastx (Blast version 2.9.0) against the *Sorghum* predicted protein sequences, using an e-value threshold of 1e-3.

The DESeq analysis was used to find differentially expressed genes (Love et al., 2014), as well as the 'apeglm' algorithm for LFC shrinkage (Zhu et al., 2018b). We considered genes with an adjusted p-value <0.01 and a log2FoldChange > |1| as being differentially expressed genes (DEGs) between stressed and control treatments (Genotype A: stress vs control; Genotype B: stress vs control). RNA-seq data have been deposited in the NCBI's (accession number).

Functional annotation and GO enrichment analysis

Functional annotation of DEGs between control and stress treatments were performed based on gene ontology (GO) enrichment analysis, using agriGO tool version 1.2 with default settings (http://bioinfo.cau.edu.cn/agriGO/index.php) (Zhou et al., 2010). GO annotation carried out considering all categories (Biological process, Cellular component, and Molecular function), *Sorghum bicolor* V2.1 as annotated reference and a false discovery rate cut-off of 0.05% probability (FDR ≤0.05).

Validation by RT-qPCR

In order to validate the RNA-seq data, 10 DEGs with a two-fold change (or more) and a baseMean >150 were selected for RT-qPCR analysis. Selected genes are involved in antioxidant metabolism (Peroxidase, Sh01_g036120; Linoleate 9S-lipoxygenase 2, Sh05_g011820; Glutathione-S-transferase, Sh04_g011700; Catalase isozyme 3, Sh04_g000610; and

Monodehydroascorbate reductase, Sh07_g013240); ABA signaling/response to stimulus (Abscisic acid receptor PYL5, Sh01_g032260); photosynthesis and other processes (NADP-dependent malic enzyme, Sh03_g020720); lignin metabolism (Laccase, Sh01_g033820); and flavonoids biosynthesis (Chalcone-flavonone isomerase, Sh08_g002490; and Flavonoid 3'5' hydroxylase, Sh01_g027870).

The cDNA of the first strand was synthesized from total RNA using the RevertAid kit (Thermo Fisher Scientific, USA), following the manufacturer's recommendations. KAPA SYBR Fast qPCR (2X) Universal Master Mix kit (Kapa Biosystems, USA) was used for RT-qPCR reactions using 10 ng cDNA and 200 nM of each specific primer (Supplementary Table S2). Reactions were conducted using a Stratagene MX3005P (California, USA) thermocycler with the following cycle conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. RT-qPCR reactions were conducted in triplicate. After the efficiency of primer amplification being verified, the gene expression results were analyzed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). The *GAPDH* gene (CA254672.1) was selected as internal control.

Statistical analysis

Data obtained in the previous analyses were subjected to variance analysis by the F-test and the significant differences were evaluated to the means comparison test, applying the Tukey's test ($P \leq 0.05$). Statistical analyses were performed using the AgroEstat version 1.1.0 software (Barbosa and Maldonado, 2010).

Results

Increase [CO_2] and drought reduced growth of sugarcane plants

Studying the combined effect of elevated atmospheric concentration of CO_2 ([CO_2]) and water deficiency (drought) in sugarcane genotypes, for 30 d, a drastic reduction in plant growth of both genotypes is observed (IACSP94-2094, genotype A; IACSP94-2101, genotype B) (Fig. 2A). The effects of stress reduced culm diameter by 37% and 42% in genotypes A and B, respectively (Fig. 2B).

Culm height and number of leaves were also affected. The reductions were significant, being 41% (culm height) and 46% (number of leaves) for genotype A, and 37% (culm height) and 51% (number of leaves) for genotype B (Fig. 2C, D).

Other growth parameters related to root growth were also severely affected by stress condition. In 30 d of elevated [CO_2] and drought, it is possible to observe similar root development (with roots shortening). Genotype A shows thicker roots (Fig. 3A), however, the reduction (44%) in root growth was more significant in this genotype (versus control plants), compared to 30% observed in plants of genotype B (Fig. 2B). Moreover, sugarcane genotypes show a significant reduction in the root area, 59% and 49% for genotype A and B, respectively (Fig. 3C).

Contrasting physiological responses differentiate sugarcane genotypes

Despite the similar effects observed in the growth among the sugarcane genotypes, there is a contrast in physiological performance. Genotypes A and B subjected to stress condition showed a considerable increase in the total soluble solids production, with an increase greater than 50% (Fig. 4A). The relative water content (RWC) and photosystem II efficiency (Fv/Fm) on sugarcane genotypes were affected by stress. Reduction was expressive mainly in genotype B. Plants of this genotype under stress showed a reduction of 28% RWC (genotype A with 16%) and 5% Fv/Fm (genotype A with 2%) in relation to plants subjected to control condition (Fig. 4B, C).

Another important difference between the sugarcane genotypes was observed for stomatal density parameter. In plants of genotype A subjected to stress, there was a significant increase in the number of stomata. This increase was 21% for stomatal density surface adaxial (Fig. 4D) and 72% for stomatal density surface abaxial (Fig. 4E).

Plants of genotype A had higher total chlorophyll content (total Chl) than that found in plants of genotype B under stress. However, there was a significant reduction in total Chl in both sugarcane genotypes compared to control condition. In genotype A the reduction was 34%, and in genotype B of 27% (Fig. 4F). Reductions are observed in the values obtained for chlorophyll *a* (Ch*a*) and *b* (Ch*b*), in plants of genotype A (above 30%) and genotype B (above 24%). There

were no significant differences for carotenoids content (Supplementary Table S3).

Sugarcane genotypes displays different antioxidant strategies to alleviate oxidative stress

Parameters related to oxidative stress were measured in leaves of sugarcane plants (genotype A and B) after 30 d under control and stress conditions. H_2O_2 and MDA content accumulated in plants of sugarcane genotype A subjected to stress condition were significantly expressive (versus control). The increase in H_2O_2 and MDA content for this genotype was 315% and 103%, respectively (Fig. 5A, B). An increase of 67% for MDA content was observed in genotype B under stress (Fig. 5B).

The increase in lipid peroxidation (expressed as MDA content) seen in both genotypes under stress condition may had implied an increase in antioxidant enzyme activities. Two superoxide dismutase (SOD) isoenzymes, Mn-SOD (I) and Cu/Zn-SOD (II) were detected in the leaves of sugarcane plants (Fig. 5C). The bands' abundance (Mn-SOD (I) and Cu/Zn-SOD (II) isoenzymes) identified using non-denaturing PAGE was lower in lane 1 (genotype A under control condition) (Fig. 5C), therefore, bands' intensity in this treatment was used as a reference in the comparison with other treatments (relative abundance). An increase in the enzymatic activity of the Mn-SOD and Cu/Zn-SOD isoenzymes is observed in sugarcane genotypes when submitted to elevated [CO_2] and drought (A/800 – lane 2, and B/800 – lane 4; Fig. 5C, D).

Effects of elevated [CO_2] and drought on the antioxidant metabolism exhibited interesting results. The ascorbate peroxidase enzyme activity (APX) was drastically reduced (79%) in plants of sugarcane genotype A and also significantly reduced (51%) in plants of genotype B under stress condition (Fig. 6A).

Glutathione enzyme (glutathione reductase, GR; glutathione peroxidase, GPX; and glutathione-S-transferase, GST) activities are crucial for the redox status control in cells. These enzyme activities in response to oxidative stress were found to be different among sugarcane genotypes. GPX activity was maintained in genotype B and significantly reduced in genotype A (55%) (Fig.

6B). However, the GR activity was maintained in genotype A and significantly reduced in genotype B (29%) (Fig. 6C). In genotype A, there was a 35% increase in GST activity, which was not observed in genotype B (Fig. 6D). This increase in GST activity is considerably associated with the increase in the H_2O_2 content (Pearson's correlation, r = 0.95) and MDA content (r = 0.81) (more information can be found in Supplementary Fig. S3).

Glutathione enzyme activities are directly related to glutathione (GSH) content measured in sugarcane plants. GSH content was altered in both genotypes subjected to stress condition. In plants of genotype A, there is an increase of 49% of this content, while in genotype B, a decrease of 13% (Fig. 8A). The GSH regeneration formed from oxidized glutathione (GSSG) is catalyzed by GR using NADPH as a reducing agent (Alves et al., 2016). Based on the results, there is a greater GSH regeneration in plants of genotype A under stress condition, mediated by GR enzyme activity (maintenance), GPX enzyme activity (reduction), and GSSG supply through the use of H_2O_2 by GST enzyme (Fig. 7B). This response was different in plants of genotype B, mainly because the GR enzyme has reduced activity and consequently the GSH compound production was also reduced (Fig. 7B).

Increase [CO2] and drought alters flavonoids content on sugarcane plants

Flavonoids quantification in the leaf extract of sugarcane plants was performed by HPLC analysis (more information in Supplementary Fig. S1 and S2). The flavonoids quercetin, luteolin, and rutin were identified and quantified in plants of sugarcane genotypes A and B subjected to control and stress conditions. In general, plants have differences in flavonoids content. Despite the significant decrease (52%) in quercetin content in genotype A under stress condition, in genotype B, there was a significant reduction of 77% for the same metabolite (Fig. 8A). Moreover, luteolin and rutin content was maintained in plants of genotype A and significantly reduced (77% and 36%, respectively) in plants of genotype B under stress condition (Fig. 8B, C). Nevertheless, in sugarcane genotype B subjected to stress condition, quercetin and rutin content was higher than that observed for genotype A (Fig. 8A, C).

RNA-seq data analysis

A transcriptome profiling was performed to identify DEGs on sugarcane plants (IACSP94-2094, genotype A; IACSP94-2101, genotype B) subjected to control condition (410 ppm [CO_2]; well-watered), and stress condition (800 ppm [CO_2]; drought), for 30 d. There were about 588 million total reads, an average of 49 million reads per sample generated by BGISEQ-500 platform.

Read mapping efficiency against the sugarcane monoploid reference genome (Gasmeur et al., 2018) ranged between 58.91% and 61.59%, and around 34% unmapped. An average of 73.43% of uniquely mapped reads was assigned to exon features (using FeatureCounts, version 1.6.4). Part of the 34% of the reads that did not find a match in the sugarcane genome could be originated from unresolved regions of this genome. To tackle this hypothesis, these unmapped reads were further mapped against the *Sorghum bicolor*_NCBIv3.47 genome (Sbi).

Mapped reads were then assigned to exon features using the Sbi reference annotation. Around 96% of the reads were successfully assigned to the reference annotation (Sbi). Using this approach, mapping against sugarcane and Sbi genome, we obtained a total of 183 million reads mapped and assigned (average of 62.25% HQ read pairs). More information can be found in Supplementary Table S4.

Unraveling transcriptome: identification of differentially expressed genes (DEGs) on sugarcane genotypes

To prove the consistency of the data across all sequenced samples, a PCA plot (Fig. 9A) and Distance heatmap (Supplementary Fig. S4) of the transcriptional profiles was performed. Both PCA plot and Distance heatmap confirm the samples taken as biological replicates are in fact more similar among themselves than with other treatment/genotype. There is a clear separation of the sample according to experimental condition (control or stress) and sugarcane genotype.

Independent of stress, for adjusted p-value <0.01 and focusing only on DEGs with log2FoldChange >| 1 | (genes with more than 2-fold difference between genotypes) we obtained 2171 more abundant genes in genotype B (up-

regulated, 44%), and 2804 more abundant genes in genotype A (down-regulated, 56%) (Fig. 9B). When analyzing the first 40 DEGs (with lower adjusted p-value), we observed that in addition to the expected difference between sugarcane genotypes A and B, most of the represented genes (i.e.: *Catalase isozyme 3*, Sh04_g000610; *Transcription factor bHLH153*, Sh06_g008410; and *Chitin-inducible gibberellin-responsive protein 2*, Sh02_g026060) respond to elevated [CO_2] and drought (Fig. 9C).

Considering the effect of stress in the DEGs analysis, we identified 1294 genes up-regulated and 787 genes downregulated by stress condition in sugarcane genotype A. Of this total, 557 and 528 DEGs we found up and down-regulated, respectively, are exclusive to genotype A under stress condition (Fig. 10A). In genotype B, 2712 up-regulated genes and 1554 down-regulated genes were identified. A greater amount of DEGs is reported for this genotype, therefore, more responsive to stress condition, with 1941 and 1329 DEGs up and down-regulated, respectively (Fig. 10A). Common DEGs (intersection in Venn diagrams) represent 700 up-regulated, 188 down-regulated and 108 genes with contrasting expression (Fig. 10A). The list of DEGs found in leaves of sugarcane plants (genotype A and B; considering stress versus control) subjected to elevated [CO_2] and drought is available in Supplementary Tables S5 and S6.

Stress-responsive genes annotated for sugarcane genotypes

To identify the stress-responsive genes through the RNA-seq performance on sugarcane genotypes, GO enrichment analysis was performed and the GO grouped up-regulated/down-regulated genes in three main categories: biological process (P), cellular component (C), and molecular function (F). We found that most enriched significant GO terms (32; with 308 annotated genes) from up-regulated genes in sugarcane genotype A under stress condition, especially associated with biological processes like fatty acid biosynthetic process (GO:0006633; FDR = 6e-06), and steroid biosynthetic process (GO:0006694; FDR = 0.0068). Among the down-regulated genes (11 significant GO terms; with 288 annotated genes) in this genotype, the response to oxidative stress (GO:0006979; FDR = 0.0072), and cellular polysaccharide metabolic process

(GO:0044264; FDR = 0.011) were identified (Fig. 10B and more information in Supplementary Table S7).

In sugarcane genotype B under stress condition, we found most enriched significant GO terms (17; with 653 annotated genes) from down-regulated genes, associated with oxidation reduction (GO:0055114; FDR = 0.0069), and protein amino acid phosphorylation (GO:0006468; FDR = 0.036). In the grouped up-regulated genes (12; significant GO terms; with 1053 annotated genes), genotype B plants showed that biological processes as cellular carbohydrate metabolic process (GO:0044262; FDR = 0.016), and polysaccharide metabolic process (GO:0005976; FDR = 0.011), were positively affected by stress (Fig. 10B and more information in Supplementary Table S8).

Regarding the molecular function category, in sugarcane genotype A under stress condition, iron ion binding (GO:0005506; FDR = 0.006), oxidoreductase activity (GO:0016491; FDR = 0.00016), and others subcategories as coenzyme binding and transferase activity were up-regulated (Fig. 10B and Supplementary Table S7). In sugarcane genotype B, molecular function categories that were up-regulated are represented by hydrolase activity (GO: GO:0016787, FDR = 3.6e-07), transferase activity (GO:0016747, FDR = 0.013), and cellulose synthase (UDP-forming) activity (GO:0016760, FDR = 0.044) (Fig. 10B and Supplementary Table S8).

The most important cellular components identified in sugarcane plants subjected to stress condition were membrane (GO: 0016020; FDR = 0.0063 and apoplast (GO: 0048046; FDR = 0.026) in genotype A (Fig. 10B and Supplementary Table S7); and thylakoid (GO: 0044436; FDR = 0.00029) and photosystem I (GO: 0009522; FDR = 1.4e-05) in genotype B (Fig. 10B and Supplementary Table S8). Note that genes belonging to cellular component thylakoid and photosystem I in genotype B were down-regulated.

DEGs stress-modulated in sugarcane

Looking at the set of DEGs in both sugarcane genotypes submitted to stress condition, and based on the main regulated biological processes identification (see Supplementary Tables S7-S9), we found DEGs of interest that are

associated with different pathways such as signaling, response to stimulus, fatty acid, pyruvate and lignin metabolism, and other important processes in sugarcane plants.

Genes associated with signaling (abscisic acid/ABA-mediated) such as protein phosphatase 2C 56 (Sh03_g016710), CBL-interacting protein kinase 4-like (SORBI_3008G158100), CBL-interacting protein kinase 6 (SORBI_3007G140900), receptor-like serine/threonine-protein kinase SD1-8 (SORBI_3006G230100), and aquaporin PIP2-5 (Sh06_g009750) were up-regulated in sugarcane genotype A. However, SORBI_3008G158100, SORBI_3006G230100, and Sh06_g009750, were down-regulated in sugarcane genotype B (information in Supplementary Fig. S5A). Also related to ABA-mediated signaling and response to stimulus, abscisic acid receptor PYL5 (Sh01_g032260) was suppressed in both genotypes (expression confirmed by RT-qPCR, see Fig. 11 and Supplementary Fig. 5D). Moreover, dehydrin DHN1 (SORBI_3009G116700) and dehydrin COR410 (SORBI_3004G286600) genes were induced, mainly in genotype B plants (Supplementary Fig. S5B).

DEGs involved with fatty acid, pyruvate, and lignin metabolism showed up-regulated in genotype A plants. The bisdemethoxycurcumin synthase-like (SORBI_3005G135000) and 3-ketoacyl-CoA synthase 6 (Sh01_g042260) genes induced in genotype A under stress, were not expressed in genotype B. However, laccases were induced (Sh01_g033820 and Sh03_g025910), as well as, NADP-dependent malic enzyme (Sh03_g020720) (see Supplementary Fig. S5C). The laccase and NADP-ME expression were confirmed by RT-qPCR (Supplementary Fig. S5E, F).

Regarding the photosynthesis genes (identified in plants subjected to stress condition), there is a down-regulation of photosystem I and II genes (Sh02_g002310 and Sh03_g031790), especially in sugarcane genotype B; and up-regulation of genes such as phosphoenolpyruvate carboxylase kinase 1 (Sh04_g025950), phosphoenolpyruvate carboxylase (SORBI_3010G160700), and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SORBI_3008G019100) in sugarcane genotype A (Supplementary Fig. S6).

Antioxidant metabolism-related genes with distinct expression in sugarcane genotypes

Comparing a set of the antioxidant metabolism-related genes (Supplementary Fig. S7), we found genes considerably more expressed in sugarcane genotype B under stress condition, including peroxidases (Sh01_g036120 and Sh01_g021030), lipoxygenases (Sh03_g028350 and SORBI_3003G385500), and L-ascorbate (Sh03_g001010). The peroxidases Sh01_g036120, Sh01_g021030 (as well as, Sh09_g011450), and L-ascorbate (Sh03_g001010) were down-regulated in sugarcane genotype A (Supplementary Fig. S7).

Another repressed gene, especially in genotype A plants, was glutathione peroxidase (Sh06_g012020). However, similar to Ferredoxin-like (Sh04_g027890), glutathione-S-transferase U17 (Sh01_g024210), and similar to glutathione-S-transferase GST 38 (Sh01_g024180) were up-regulated in this genotype. The Sh04_g027890 and Sh01_g024210 genes were down-regulated in genotype B plants (Supplementary Fig. S7). These results indicate, therefore, an expression profile of antioxidant enzymes (peroxidases and glutathione's) distinct between the two genotypes subjected to stress condition. The expression of some antioxidant metabolism-related genes has been validated by RT-qPCR and the results can be found in Supplementary Fig. S7.

Moreover, DEGs associated with secondary metabolism and involvements in the antioxidant responses modulation were identified in both sugarcane genotypes. We found cytochromes P450 induced in genotype A and with negative expression in genotype B: geraniol 8-hydroxylase (CYP76B6; SORBI_3002G108500) and 4-hydroxyphenylacetaldehyde oxime monooxygenase (CYP71E1; Sh01_g000880). Related to flavonoids biosynthesis, chalcone--flavonone isomerase (Sh01_g002450) and chalcone-flavonone isomerase family protein (Sh08_g002490) were up-regulated in both genotypes (Supplementary Fig. S7). Also related to flavonoid metabolism, the flavonoid 3'5' hydroxylase gene (CYP75A; Sh01_g027870) was up-regulated in sugarcane genotypes A and B. The positive expression of Sh08_g002490 and Sh01_g027870 was confirmed by RT-qPCR (Fig. 11; Supplementary Fig. S8).

RNA-seq validation by RT-qPCR

To confirm the data obtained by RNA-seq, the expression of selected DEGs involved in antioxidant metabolism, signaling/response to stimulus, photosynthesis, and secondary metabolism was assessed by RT-qPCR (primer list used for RT-qPCR analysis is shown in Supplementary Table S2). To allow comparisons between RNA-seq and RT-qPCR results, the relative expression value is presented as log2FoldChange (FC). We observed a positive correlation between the results of RNA-seq and RT-qPCR, with r = 0.84 and r = 0.70, for genotype A and B, respectively (Fig. 11). The decrease of abscisic acid receptor PYL5 (Sh01_g032260) transcripts in leaves of both sugarcane genotypes subjected to stress condition was validated by RT–qPCR, as well as the contrasting peroxidase (Sh01_g036120) expression in the tested genotypes was also confirmed (Fig. 11).

Discussion

Saccharum spp. (sugarcane) growth and production are influenced by climate change, exhibiting positive responses (e.g., increased leaf area and plant height) under elevated atmospheric CO_2 concentration ([CO_2]) (Vu et al., 2006; Souza et al., 2008; Silva et al., 2020). However, it is known that a set of environmental factors can limit plant growth. The water deficit is an important environmental stress that interferes in physiological performance of sugarcane plants, causing changes in photosynthetic efficiency (Zhang et al., 2020a) and consequently in crop growth/productivity. Combined effects of drought and elevated [CO_2] on physiological and genetic responses of C_4 plants have been little studied (Silva et al., 2020). Thus, the present study evaluated the performance of two sugarcane genotypes (IACSP94-2094, genotype A; IACSP94-2101, genotype B) subjected to elevated [CO_2] and drought, investigating morphophysiological and biochemical aspects integrated to analysis transcriptomics, and seeking a better understanding of the mechanisms underlying climate change.

Adverse effects of drought caused a drastic reduction in the sugarcane growth in full early growth stage. Plant growth such as number of leaves, culm height and root development decreased in both sugarcane genotypes (Fig. 2 and

Fig. 3). The reduction in the number of leaves in genotypes A and B, e.g., may be associated with a decrease in the transpiration and metabolic expenditure for tissue maintenance (Inman-Bamber and Smith, 2005; Inman-Bamber et al., 2008). The genotypes tested in this study are contrasting in relation to some agronomic parameters such as responsiveness to abiotic stress (Supplementary Table S1), even so, despite IACSP94-2094 (genotype A) rusticity, no significant differences in growth were observed between genotypes (A and B) after 30 d of elevated [CO_2] and drought. However, a study observed a significant increase in photosynthesis and biomass production of sugarcane plants under well-watered conditions grown in elevated [CO_2] (~720 ppm) (Souza et al., 2008).

It is known that other C_4 plants such as *Zea mays* (maize) and *Sorghum bicolor* (sorghum) subjected to elevated [CO_2], do not respond with accelerated biomass production in condition of sufficient water supply (Kellner et al., 2019). However, elevated [CO_2] (561 µmol mol^{-1}) increases sorghum biomass under drought conditions (Ottman et al., 2001), and maize hybrids grown under elevated [CO_2] (750 µmol mol^{-1}) with drought had negative effects on stem elongation and leaf area showing reductions in average plant height compared to the control (400 µmol mol^{-1} [CO_2]) (Wijewardana et al., 2016). Plants have different dynamics to respond to changing environmental conditions. Environmental stresses trigger a wide variety of responses, ranging from altered cellular and metabolic responses such as oxidative stress, a reduced net photosynthesis rate, a decrease in stomatal conductance, changes in stress-related gene expression, and consequently variations in growth rates and crop yields (Reddy et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2006; Wijewardana et al., 2016).

Water deficiency in sugarcane reduces culm elongation and leaf development, and more sucrose (sugars produced in the leaves) becomes available for storage in the culm (Robertson et al., 1999; Watt et al., 2014). Plants of both genotypes (A and B) showed a significant increase in the total soluble solids content (Fig. 4A). Sugarcane genotype drought-tolerant exhibited a significant enhancement in soluble sugar contents at moderate drought treatment (Zhang et al., 2020a). This soluble solids accumulation in sugarcane genotypes A and B is instigated by drought to maintain osmotic balance and to improve

plants' defense system against reactive oxygen species (ROS)-induced oxidative damage (Khedr et al., 2003; Ahmed et al., 2013; Zhang et al., 2020a).

Significant reduction in relative water content (RWC), photosynthetic efficiency of photosystem II in dark-adapted state (Fv/Fm), and total chlorophyll content (total Chl) (Fig. 4), in plants subjected to elevated [CO_2] and drought (versus control plants), it indicates the increased effects of oxidative stress in both sugarcane genotypes. The chlorophyll content reduction under drought is considered a characteristic of oxidative stress caused by photo oxidation and pigments degradation (Farooq et al., 2009).

The expressive decrease observed mainly in the average values of RWC and Fv/Fm in sugarcane genotype B (Fig. 4B and 4C) can be an indication that after 30 days under elevated [CO_2] and drought, plants of this genotype are showing more marked changes in water status and on photosynthetic apparatus, since Chl content and Fv/Fm is an indicator for photosynthetic performance (Kitajima and Butler, 1975; Nageswara Rao et al., 2001), and RWC is an important indicator of water status and also measure to evaluate tolerance of plants to drought stress (Cia et al., 2012; Zhang et al., 2020a).

As observed in the present study (Fig. 4C), the decrease in Fv/Fm was also reported in maize genotypes subjected to elevated [CO_2] and drought (Wijewardana et al., 2016). The ability of sugarcane plants to maintain high Fv/Fm under drought indicates an efficiency of radiation use possibly for photochemistry and carbon assimilation (Silva et al., 2007). Also according to Wijewardana et al. (2016), maize plants showed an increase in the total Chl content and a decrease in the carotenoid content, what we didn't found in sugarcane (genotypes A and B) (Fig. 4F and Supplementary Table S3). Moreover, as reported by Cia et al. (2012), it is clear the differences in leaf RWC in sugarcane genotypes under water scarcity. The RWC decreased in sugarcane during the drought (20 days of stress), especially in sugarcane sensitive-drought genotype.

Among the main differences observed between sugarcane genotypes A and B, we found a significant increase in stomatal density (adaxial and abaxial surfaces) in genotype A (Fig. 4D e 4E). According to Pincelli and Silva (2012), in drought-tolerant genotypes, it may be possible to observe an increase in stomatal density. Thus, increases in density stomata leads to an increase in the rate of

photosynthesis, since stomata plays an important role in keep status hydration on the leaves and causes the entry of more carbon dioxide into the plant (Zhang et al., 2014; Avivi et al., 2016). Moreover, according to Xu et al. (2016), elevated [CO_2] may lead to reduction in stomatal density by inhibition of guard cells and promote leaf enlargement. On the other hand, a severe abiotic stress factor such as water deficit may diminish leaf enlargement and increase stomatal density.

In this context, maintaining water status is essential for preservation of plant cellular processes (Silva et al., 2007; Davies et al., 2011). The changes reported in sugarcane genotypes A and B, in RWC and photosynthetic apparatus (Fv/Fm and total Chl), follow the increase measured for lipid peroxidation (MDA content) (Fig. 5B). This association was observed in other sugarcane genotypes submitted to drought, where the genotypes exhibited reduced RWC, increased H_2O_2 content, and lipid peroxidation (MDA content) (Cia et al., 2012).

Based on our results, we found that the increase in H_2O_2 was very significant in sugarcane genotype A (Fig. 5A), and this may be related to a signaling mechanism for oxidative protection (Scandalios, 2005; Slesak et al., 2007). The stress-signaling or cell damage due to excess ROS, as seen in genotype A, depends on a balance between the oxidative components and antioxidant substances production (Gratão et al., 2005). A significant increase in MDA content was also detected with variations in different sugarcane genotypes subjected to multiple environmental stresses (Morais et al., 2018), moreover, drought stress increased ROS level and MDA content in leaves of drought-tolerant and sensitive sugarcane genotypes (Zhang et al., 2020a). Therefore, high H_2O_2 and MDA content levels do not necessarily indicate a lesser capacity for antioxidant defense in plants (Morales and Munné-Bosch, 2019). Our results show that sugarcane genotypes use different defense strategies and thus try to attenuate the effects of oxidative stress.

Despite the similar growth among the sugarcane genotypes we observed contrast on physiological attributes and gene expression between genotypes A and B. In the transcriptomic analysis, we found a broader set of differentially expressed genes (DEGs) in sugarcane genotype B (4266 DEGs; more stress-responsive) subjected to elevated [CO_2] and drought compared to DEGs identified in sugarcane genotype A (2081 DEGs; more robust) (Fig. 10A). Distinct

transcriptomic response was also observed in sugarcane genotypes contrasting for lignin content (Vicentini et al., 2015), under to biotic stress (Wang et al., 2019), aluminum stress (Rosa-Santos et al., 2020), and water deficit (Nawae et al., 2020; Selvi et al., 2020).

Looking at the DEGs identified in both sugarcane genotypes (A and B), we found genes that are related to changes in stomatal closure, stomatal density, and leaf water homeostasis. These processes can be regulated by ROS accumulation and ABA-mediated signaling (see Supplementary Fig. S9). Mittler and Blumwald (2015) proposed the model of basic regulatory events involved in the regulation of stomatal movement by ABA (increase under stress conditions). Thus, we suggest that in sugarcane under elevated [CO_2] and drought, stomatal responses are related to interactions between ROS and ABA. The mechanism involves the differential expression of genes: abscisic acid receptor (PYL); protein phosphatase 2C (PP2C); G-type lectin S-receptor-like serine/threonine-protein kinase (G-lecRKs); respiratory burst oxidase (RBOH); calcineurin B-like protein (CBL); and CBL-interacting protein kinase (CIPK); as well as other important genes related to ion transport/water and intracellular signaling in plants, such as aquaporin's and calcium-dependent protein kinase (CPK) (Maurel et al., 2015; Durian et al., 2020) (More information in Supplementary Fig. S9; Supplementary Tables S5 and S6).

Several previous studies discuss the role of these genes in environmental stresses, e.g., Chater et al. (2015) reports that leaf changes in stomatal apertures and stomatal density require the generation of ROS, ABA receptors (PYL genes) and ABA itself. Other authors reports that CBL-CIPK network is vital regulatory mechanisms which decode calcium signals triggered by stresses (Drerup et al., 2013; Mao et al., 2016); G-lecRKs with play vital roles in sensing outside signals (Sun et al., 2013), and RBOH, a critical components contributing to the generation of ROS (Drerup et al., 2013), acts in the regulation of Na^+/K^+ homeostasis of the plant under abiotic stress and is a major component mediating systemic ROS signaling in plants (Miller et al., 2009; Ma et al., 2012).

Regarding to GO analysis, we observed that biological processes associated with fatty acid and steroid biosynthesis were up-regulated in sugarcane genotype A under to elevated [CO_2] and drought, and

carbohydrate/polysaccharide metabolic processes in genotype B (Fig. 10B; Supplementary Tables S7 and S8). According to Sui et al. (2018), previous studies had shown that environmental stresses, such as drought, cold, and salt, can induce changes in fatty acid composition. In maize under water deficit, most of the detected DEGs, e.g., were annotated to secondary metabolic pathways and fatty acid metabolism, which indicated that these genes may play roles in drought stress-response (Zheng et al., 2020).

DEGs associated with oxidoreductase activity were mainly up-regulated in sugarcane genotype A and down-regulated in genotype B (Fig. 10B). Interestingly, in common reed (*Phragmites australis*, family Poaceae), from the pattern of enrichment of transcripts involved in salinity response, the oxidoreductase activity and glutathione metabolism genes showing significantly higher expression (Zhang et al., 2020b). In sugarcane genotype A, we observed an up-regulation of glutathione metabolism and secondary metabolic processes (Fig. 12 and 13). Similar to our results, Li et al. (2019) report through transcriptome analysis that some stress-related genes, including glutathione's, flavonoid biosynthesis, metabolic processes, and oxidoreductase activity were induced by salt in *Arabidopsis* transgenic plants.

In Fig. 12, it is shown processes related to secondary metabolism that have been up-regulated in sugarcane genotypes subjected to elevated [CO_2] and drought. DEGs associated with changes in very-long-chain fatty acid (VLCFA) levels were identified in both genotypes (A and B). Fatty acid elongase activity results in successive action of 3-ketoacyl-CoA synthase (KCS5), 3-oxoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and enoyl-CoA reductase (ECR). These participate in a cycle that is initiated by malonyl-CoA condensation to long-chain acyl-CoA (Granrut and Cacas, 2016). Some genes related to fatty acid elongation and phenylpropanoids biosynthesis, such as glycerol-3-phosphate 2-O-acyltransferase 6 (GPAT6; SORBI_3001G250200), fatty acyl-CoA reductase 1 (FAR1; Sh08_g005200, Sh07_g013180, and Sh07_g013130), and bisdemethoxycurcumin synthase (PKS; SORBI_3005G135700, SORBI_3005G135300, and SORBI_3005G135000) were exclusively induced in sugarcane genotype A (see Supplementary Tables S5 and S6).

An increase of VLCFA was reported in *Arabidopsis thaliana* leaves under oxidative stress (Nagano et al., 2012), and rice (*Oryza sativa*) leaves under drought (Zhu and Xiong, 2013). The increase reported here in the gene expression associated with VLCFA may indicate a change in sugarcane lipid metabolism and an attempt to adapt to the stressful condition. In addition, the positive expression of GPAT and FAR genes in sugarcane genotype A indicates a possible drought-tolerance strategy. GPAT gene overexpression can increase tolerance to salt-stress in tomato (*Lycopersicon esculentum*) and *Arabidopsis* (Sun et al., 2010; Sui et al., 2017). Still according to Sui et al. (2017), this increased GPAT expression is related to attenuation of the photoinhibition of PSI and PSII. Moreover, in wheat (*Triticum aestivum*), FAR5 transcript levels were regulated in response to drought, cold, and ABA (Wang et al., 2015).

Another gene considerably up-regulated in genotype A (stress versus control), the *PKS* gene (Supplementary Table S5), is involved in the flavonoid biosynthesis pathway (Morita et al., 2010). Knowing that flavonoids content (quercetin, luteolin, and rutin) was differentially altered in sugarcane genotypes A and B (Fig. 8), and that these may be associated with ROS elimination in cells (Das and Roychoudhury, 2014), these compounds can have an important role in modulating antioxidant defense under elevated [CO_2] and drought. Flavonoids have been considered fundamental (secondary system) in the plant's ROS elimination and protection of damage to photosynthetic apparatus (Fini et al., 2011). Stable luteolin and rutin levels, and even least significant reduction of quercetin content in sugarcane genotype A (stress versus control) (Fig. 8), suggest that in this genotype, flavonoids (or secondary metabolism) possibly are an important response in mitigating oxidative stress.

DEGs associated with lignin metabolism and flavonoids biosynthesis were up-regulated in both sugarcane genotypes (A and B) and indicate that plants activate secondary mechanisms to defend against oxidative stress. The lacasses Sh03_g025840, Sh03_g025910, and Sh01_g033820, among others, were highly induced in the sugarcane genotypes. Laccases participate in flavonoid metabolism in *Arabidopsis* (Pourcel et al. 2005), and lignin biosynthesis (Cesarino et al. 2013). Results obtained by Li et al., (2020), indicated that overexpression of laccase increased the content of lignin in transgenic

Arabidopsis and improved the adaptability to drought. Additionally, we found key genes involved with flavonoids biosynthesis up-regulated in both genotypes (e.g., PAL, Sh06_g009640; and *CHI*, Sh08_g002490) (Fig. 12; Supplementary Tables S5 and S6). Increased levels of individual flavonoid metabolites were found under elevated [CO_2] and/or drought in ragweed (*Ambrosia artemisiifolia*) (El Kelish et al., 2014).

It is emphasized that genes participate in integrated pathways and, therefore, not necessarily their increased expression results in an increase in all flavonoids content. There was a decrease in quercetin, luteolin, and rutin content in sugarcane genotype B (Fig. 8), but it should be considered that other flavonoids have already been identified in sugarcane, such as schaftoside and vitexin (Colombo et al., 2006), and can be useful against abiotic stress (Agati et al., 2012). According to França et al. (2001), from a prediction study, the profile of isoprenoid and phenylpropanoid metabolism in sugarcane is regulated during the adaptive response to abiotic stress. Moreover, results by Ma et al. (2014), suggest that flavonoids pathway gene expression may be closely associated with drought tolerance in wheat.

As summarized in Fig. 12, we observed that the dhurrin biosynthesis pathway is activated, mainly in sugarcane genotype A subjected to elevated [CO_2] and drought. Cytochromes CYP79A1 (Sh01_g015030 and SORBI_3005G157600) and CYP71E1 (Sh01_g000880, Sh05_g008020, Sh05_g008030, and Sh02_g007020) were up-regulated in genotype A. On the other hand, CYP71E1 was differentially expressed in genotype B (Sh08_g004400 up-regulated; Sh01_g000880 and Sh05_g008030 down-regulated) (Supplementary Tables S5 and S6). The expression of dhurrin biosynthesis genes indicates a possible metabolic adjustment in sugarcane plants, including sugars accumulation, depletion of water, and conditions suitable for safe-storage of both enzymes and natural products such as dhurrin (Knudsen et al., 2020). Dhurrin, cyanogenic glucosides, have important roles in plant resistance against abiotic and biotic stresses (Emendack et al., 2018). Sorghum plants accumulate large amounts of dhurrin after exposure to water stress and can use these secondary metabolites as a mechanism to mitigate oxidative stress (O'Donnell et al., 2013).

We observed DEGs of thylakoid part and photosystem I (GO analysis: cellular components) were down-regulated in sugarcane genotype B subjected to elevated [CO_2] and drought (Fig. 10B; Supplementary Table S8). These changes correlate with the major physiological changes at 30 days observed in sugarcane genotype B (RWC, Fv/Fm, and stomatal density; Fig. 4). Oxidative stress is known to modulate plant physiology in several aspects. In this context, severe water stress can affect the photosynthetic apparatus, which may indicate that water use efficiency does not increase with elevated [CO_2], as well as cause stomatal closure, and significantly decreased net photosynthesis (Zhao et al., 2004; Warren et al., 2011; Bauweraerts et al., 2013).

Plants have different systems for dealing with oxidative stress effects. In the first-line defense antioxidants act in preventing ROS formation (Netto, 2001; Gratão et al., 2005). However, once they are formed, antioxidant enzymes will act by disrupting uncontrolled oxidation (Medici et al., 2004). In our study, isoenzyme superoxide dismutase (SOD) activity, Mn-SOD (I) and Cu/Zn-SOD (II) (Fig. 5C), was increased in both sugarcane genotypes submitted to elevated [CO_2] and drought, especially in genotype B (Fig. 5C e 5D). These isoenzymes were also characterized in sugarcane genotypes subjected to drought (Cia et al., 2012).

The product resulting from dismutation by SOD is H_2O_2, which is eliminated by enzymes ascorbate peroxidase (APX) and catalase (CAT) activity (Morais et al., 2018). APX activity on genotype B (Fig. 6A) may be resulting in less H_2O_2 production (compared to genotype A). The obtained values for SOD and APX enzymes activities in genotype A (Fig. 5C and Fig. 6A) do not explain a primary and effective action of this antioxidant system on oxidative stress. However, oxidative protection in sugarcane genotype A may be more associated with the other defense mechanisms activation. An increase in SOD and APX activity was reported in IACSP94-2094 (genotype A) subjected to water deficit, but by stress cycles that ranged from 5-10 days (Marcos et al., 2018).

Important differences between genotypes A and B related to the enzymes glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) activities were detected (Fig. 6). This difference was also observed in the compound glutathione production (GSH) (Fig. 7A). Among the

non-enzymatic molecules that play antioxidant roles in the cell, we know the GSH, flavonoids, phenolic compounds, among others (Gratão et al., 2005; Das and Roychoudhury, 2014). In response to plant stress, it may be possible to identify an increase in enzymes activities that participate in GSH biosynthesis (Noctor et al., 2002).

GPX activity was reduced, GR was maintained and GST increased, in the sugarcane genotype A subjected to elevated [CO_2] and drought, that can be correlated with an increase in H_2O_2 and MDA content (Fig. 6; Supplementary Fig. S3). GR is the enzyme responsible for GSH regeneration by new redox cycles (Netto, 2001). GSH content was significantly increased in genotype A (Fig. 7). In genotype B, H_2O_2 is eliminated by GPX and GST action (Fig. 7B). It is known that GSTs help oxidative stress regulation (Kumar and Trivedi, 2018), modulating different aspects of plant development (Chen et al., 2012). Thus, our results may indicate a signaling mediated by increase of H_2O_2 in sugarcane genotype A plants, and consequent induction of glutathione's activities.

Based on DEGs identified and associated with antioxidant metabolism, we proposed a signaling model mediated by ROS (H_2O_2) and MDA accumulation in sugarcane genotypes (Fig. 13). Intra and intercellular H_2O_2 levels increase during environmental stresses and activate different signaling pathways, triggering changes in gene expression and cell-cycle processes (Ślesak et al., 2007). Formation of MDA can be induced by ROS or enzymatically by lipoxygenase activity (Farmer and Mueller, 2013). MDA content was increasing in sugarcane genotypes A and B (Fig. 5B), in addition, we found a positive expression of lipoxygenases in genotype B (e.g., Sh05_g011820, Sh03_g028360, SORBI_3003G385500, and SORBI_3003G385900), and a significant increase in H_2O_2 content in genotype A (Fig. 5A).

The increase in H_2O_2-MDA content in sugarcane genotype A possibly indicate a transient accumulation and that despite the damage in plant growth (in 30 days), activation of antioxidant system occurs (e.g., glutathione cycle activate: GST, Sh01_g024180 and Sh04_g011700 up-regulated; GPX, SORBI_3006G173900 down-regulated), and less severe damage to PSI / PSII (Fig. 13). According to Morales and Munné-Bosch (2019), increases in MDA content may represent acclimatization processes, since it can play a positive role

in activating regulatory genes involved in granting cell protection under oxidative stress. In a previous study, the improved performance of sugarcane IACSP94-2094 (genotype A) under drought (mild stress) was associated with a more efficient antioxidant system response (Boaretto et al., 2014).

In sugarcane genotype B, we verified up-regulation of lipoxygenases, increased MDA content, and down-regulation of PSI / PSII genes by stress. Plants of this genotype activate mainly a set of peroxidases (Sh01_g025130, Sh03_g010780, Sh10_g016150, and Sh02_g000190, among others) to mitigate oxidative stress. However, the inactivation of photosystems in this genotype, suggests that MDA levels are maintained, leading to more expressive long-term cell damage, since the balance between MDA production, elimination, and signaling is an important factor that can determine plant survival under stress (Morales and Munné-Bosch, 2019). Therefore, as presented in Fig. 13, we summarize the major changes observed in antioxidant metabolism-related genes expression in sugarcane genotypes IACSP94-2094 (genotype A) and IACSP94-2101 (genotype B) subject to elevated [CO_2] and drought. Sugarcane genotypes activate similar and contrasting primary/secondary antioxidant mechanisms to adapt to environmental conditions. Signaling mediated by ROS-MDA content in sugarcane plants in this condition can be an indicator of redox biology and adaptability to abiotic stress.

Supplementary data

Table S1. Information on the contrasting sugarcane genotypes used in the study.

Table S2. Primer list used for RT-qPCR analysis.

Table S3. Effect of elevated [CO_2] and drought on photosynthetic pigment content.

Table S4. Summary of RNA sequencing by BGISEQ-500 platform.

Table S5. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (genotype A) subjected to elevated [CO_2] and drought.

Table S6. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (genotype B) subjected to elevated [CO_2] and drought.

Table S7. Gene ontology (GO) enrichment analysis of specific DEGs found in sugarcane genotype A.

Table S8. Gene ontology (GO) enrichment analysis of specific DEGs found in sugarcane genotype B.

Table S9. Gene ontology (GO) enrichment analysis of intersection (common) DEGs found in sugarcane genotypes A and B.

Table S10. References used in the construction of schematic overview of pyruvate and fatty acid metabolism, lignin metabolism, flavonoids, and dhurrin biosynthesis.

Figure S1. Methodology for extracting flavonoids from sugarcane leaves.

Figure S2. Flavonoids (external standards) used in HPLC analysis.

Figure S3. Correlation between oxidative stress indicators (MDA and H_2O_2 content) and antioxidant enzyme activities.

Figure S4. Distance heatmap to test the consistency of the data across all sequenced samples.

Figure S5. DEGs associated to different biological processes regulated in leaves of sugarcane plants.

Figure S6. DEGs associated to photosynthesis.

Figure S7. DEGs associated to antioxidant metabolism.

Figure S8. RT-qPCR expression analysis of genes involved in flavonoids biosynthesis.

Fig. S9. A proposed model for stomatal responses on sugarcane by ROS accumulation and ABA-mediated signaling.

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Figure Legends

Fig 1. Experimental design. (A) Plants of both sugarcane genotypes (IACSP94-2094, genotype A; and IACSP94-2101, genotype B) were grown in Fitotron chambers under two conditions. In the control condition, plants were subjected to 410 ppm [CO_2] and well-watered (I), for 30 d. Plants subjected to stress condition were kept at 800 ppm [CO_2] with watering suspension (II), for 30 d. (B) Soil moisture indicating percentage of field capacity was measured (five points).

Fig. 2. Effect of elevated [CO_2] and drought on sugarcane growth. (A) Morphological aspect of genotype A (IACSP94-2094) plants, and genotype B (IACSP94-2101) plants subjected to 410 ppm [CO_2] + well-watered (control: I, III), and 800 ppm [CO_2] + drought (treatment: II, IV), for 30 d. (B) Culm diameter, (C) culm height, and (D) number of leaves measured for sugarcane plants. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for $p \leq 0.05$ (*symbol).

Fig. 3. Effect of elevated [CO_2] and drought on root growth of sugarcane. (A) Root growth of genotype A (IACSP94-2094) plants, and genotype B (IACSP94-2101) plants subjected to 410 ppm [CO_2] + well-watered condition (control: I, III), and 800 ppm [CO_2] + drought (treatment: II, IV), for 30 d. (B) Root length, and (C) root area measured for sugarcane plants. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for $p \leq 0.05$ (*symbol). Different letters indicate a significant difference between sugarcane genotype A and B plants under stress condition by Tukey's test ($p \leq 0.05$).

Fig. 4. Effect of elevated [CO_2] and drought on physiological parameters of sugarcane. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) Total soluble solids, (B) relative water content

(RWC), (C) Fv/Fm, (D) stomatal density adaxial surface, (E) stomatal density abaxial surface, and (F) chlorophyll content (total Chl) measured for sugarcane plants. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for p≤0.05 (*symbol). Different letters indicate a significant differences between sugarcane genotype A and B plants under stress condition by Tukey's test (p≤0.05).

Fig. 5. Measurement of oxidative stress parameters and SOD activity on sugarcane. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) H_2O_2 content, (B) MDA content, (C) superoxide dismutase (SOD) activity, and (D) relative abundance of Cu/Zn-SOD and Mn-SOD, measured for sugarcane plants. In (C), arrow I indicates the Mn-SOD isoform and II the Cu/Zn-SOD isoform. The lanes listed are: (P) SOD standard, (1) A410, (2) A800, (3) B410, and (4) B800. The relative abundance of isoforms was calculated in relation to the A410 bands (#). Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for p≤0.05 (*symbol). Different letters indicate a significant differences between sugarcane genotype A and B plants under stress condition by Tukey's test (p≤0.05).

Fig. 6. Antioxidant enzyme activities on sugarcane. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) Ascorbate peroxidase (APX) activity, (B) glutathione peroxidase (GPX) activity, (C) glutathione reductase (GR) activity, and (D) glutathione-S-transferase (GST) activity. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for p≤0.05 (*symbol). Different letters indicate a significant differences between sugarcane genotype A and B plants under stress condition by Tukey's test (p≤0.05).

Fig. 7. GSH content on sugarcane. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) GSH content, and (B) schematic representation of the effect of stress condition on GSH content and specific glutathione activities. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for p≤0.05 (*symbol).

Fig. 8. Flavonoids content on sugarcane. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) Quercetin content, (B) luteolin content, and (C) rutin content. Data represents means ± SE of three independent biological replicates. Differences between control (A410, B410), and respective stress condition (A800, B800) were considered significant according to Tukey's test for p≤0.05 (*symbol). Different letters indicate a significant differences between sugarcane genotype A and B plants under stress condition by Tukey's test (p≤0.05).

Fig. 9. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (IACSP94-2094, genotype A; IACSP94-2101, genotype B) independent of stress. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) PCA plot for biological replicates (genotype/treatment), (B) DEGs of genotype BvsA comparison, with log2FoldChange >|1|, and (C) hierarchical clustering of the top 40 DEGs (adjusted p-value <0.01) considering genotype BvsA comparison. A1-A3: genotype A under control condition; A7-A9: genotype A under stress condition; B4-B6: genotype B under control condition; and B10-B12: genotype B under stress condition. Colours represent normalized expression.

Fig. 10. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (IACSP94-2094, genotype A; IACSP94-2101, genotype B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. (A) DEGs in genotype A and B regulated by stress condition (p-value < 0.01 and log2FoldChange > |1|), with Venn diagrams showing number of specific and common DEGs, and (B) Functional annotation and gene ontology (GO) enrichment analysis of DEGs (specific and common), with FDR ≤0.05. Red: up-regulated, blue: down-regulated, and yellow: contrasting expression. More information in Supplementary Tables S5 – S9.

Fig. 11. RNA-seq validation by RT-qPCR. Expression analysis of 10 DEGs associated with different biological processes, on sugarcane leaves of (A) genotype A (IACSP94-2094), and (B) genotype B (IACSP94-2101). Treatments consisted of plants subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. The analysis was performed considering the expression of the plants under stress condition in relation to control condition. The GAPDH gene (CA254672.1) was selected as internal control. Pearson's correlation (r) between log2FoldChange (FC) from RNA-seq and RT-qPCR was carried out by the statistical analysis software Analyse-it®.

Fig. 12. Schematic overview of pyruvate and fatty acid metabolism, lignin metabolism, flavonoids, and dhurrin biosynthesis up-regulated in sugarcane genotype A (IACSP94-2094) subjected to elevated [CO_2] and drought. The scheme organizes the results based on several studies, available in Supplementary Table S10. Represented genes, in (I) pyruvate and fatty acid metabolism, are: NADP-dependent malic enzyme (NADP-ME), 3-ketoacyl-CoA synthase (KCS5), 3-oxoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD/PAS 2), glycerol-3-phosphate 2-O-acyltransferase 6 (GPAT6), fatty acyl-CoA reductase 1 (FAR1), and bisdemethoxycurcumin synthase (PKS); in (II) lignin metabolism, laccase; in (III) flavonoids biosynthesis, are: phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), flavonol synthase (FLS1), flavonoid 3',5'-hydroxylase (CYP75A), anthocyanidin

reductase (ANR), and anthocyanidin 5,3-O-glucosyltransferase (GT1); in (IV) dhurrin biosynthesis, tyrosine N-monooxygenase (CYP79A1) and 4-hydroxyphenylacetaldehyde oxime monooxygenase (CYP71E1). Genes: NADP-ME, KCS5, KCR, GT1, laccase, PAL, CHI, FLS1, CYP75A, ANR, GT1, and CYP71E1 were also up-regulated in sugarcane genotype B (stress versus control). The arrows indicate selected steps for each process. Up-regulated genes are marked in red. *symbol in red indicates that genes associated with carbohydrates metabolism were differentially expressed. See Supplementary Tables S5 and S6.

Fig. 13. Summary of stress-responsive genes expressed on sugarcane genotypes. This scheme represents the effects of elevated [CO_2] and drought (e[CO_2] + drought) on the modulation of antioxidant responses by ROS-MDA content. The possible ROS signaling and MDA accumulates in sugarcane plants differentiate genotypes A and B in terms of antioxidant gene expression and photosynthetic efficiency. Red arrows indicate up-regulated genes and blue arrows down-regulated genes. Abbreviations: ROS, reactive oxygen species; MDA, malondialdehyde; GST, glutathione-S-transferase; CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase; MDAR, monodehydroascorbate reductase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxylase kinase; PSI, photosystem I; PSII, photosystem II.

Figures:

Figure 1

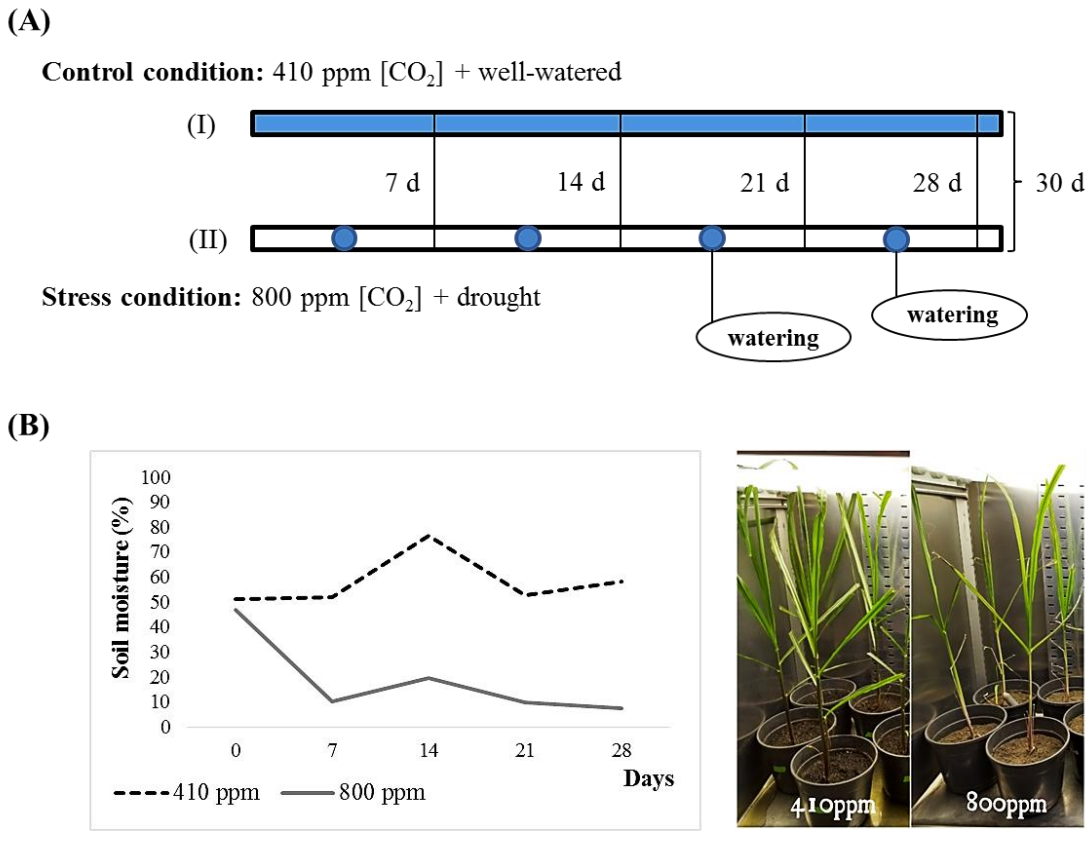


Figure 2

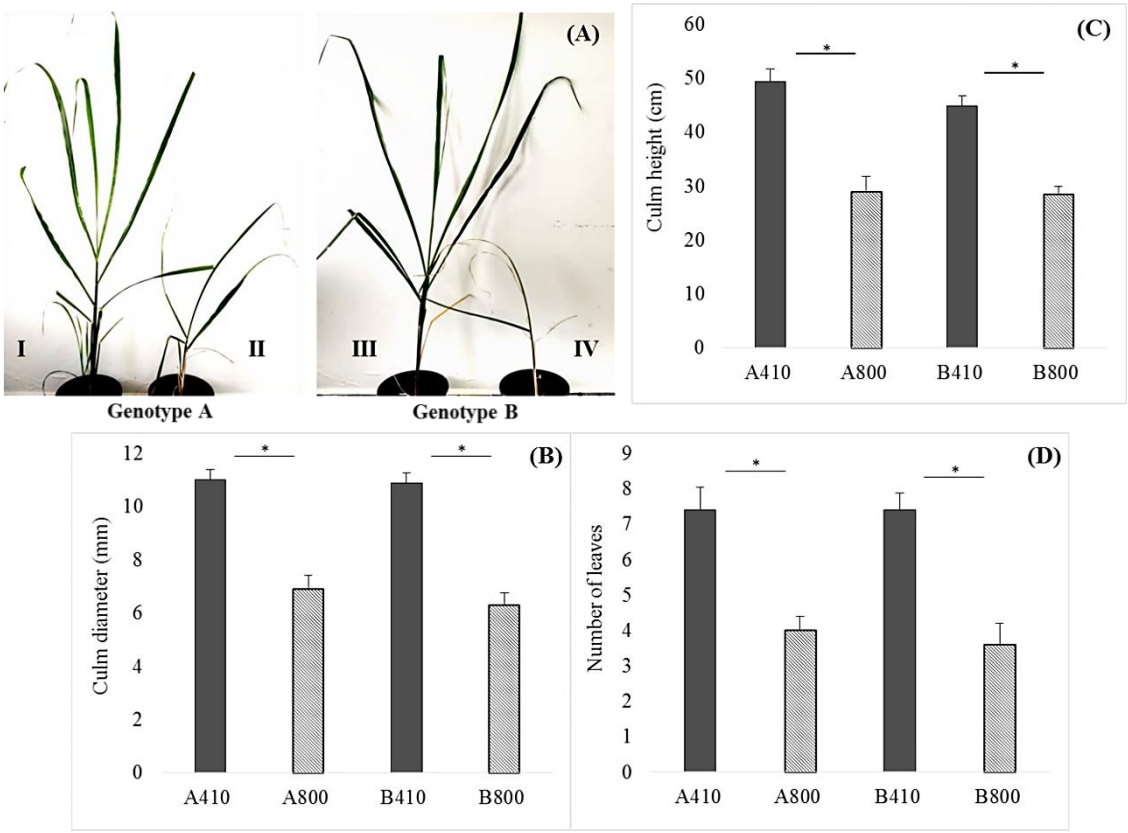

(A)
I
II
III
IV
Genotype A
Genotype B
(C)
Culm height (cm)
60
50
40
30
20
10
0
*
*
A410
A800
B410
B800
(B)
Culm diameter (mm)
12
10
8
6
4
2
0
*
*
A410
A800
B410
B800
(D)
Number of leaves
9
8
7
6
5
4
3
2
1
0
*
*
A410
A800
B410
B800

Figure 3

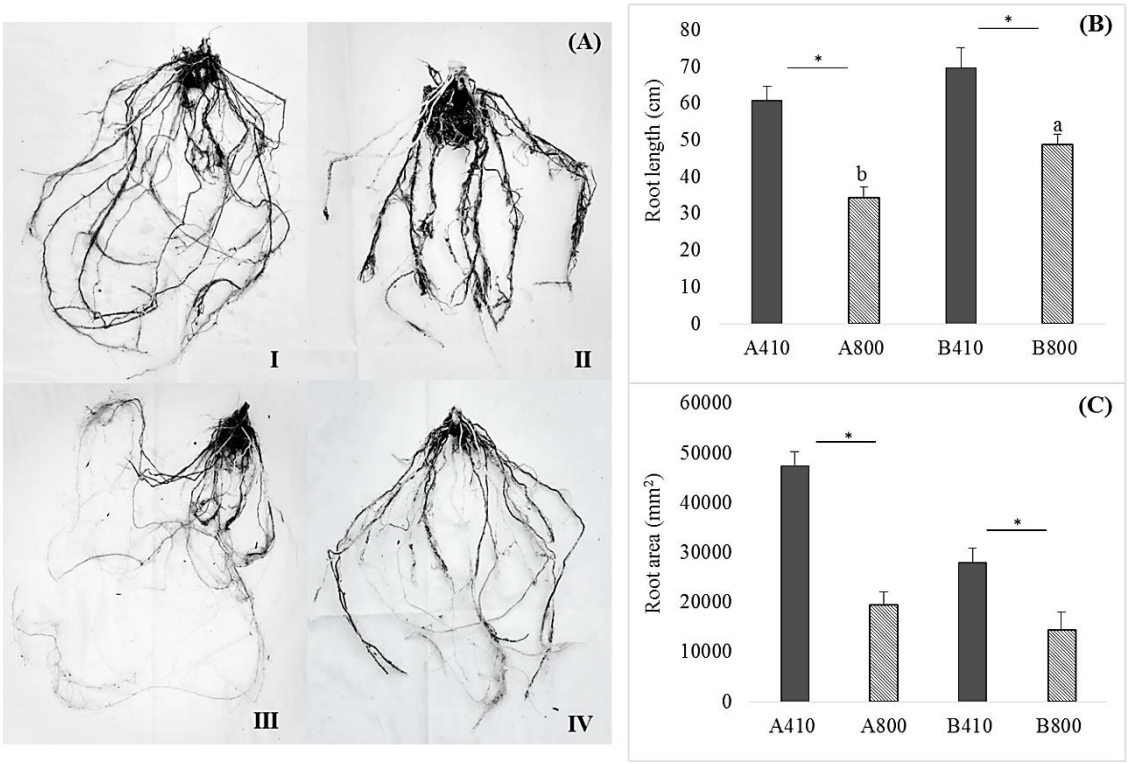

(A)
I
II
III
IV
(B)
Root length (cm)
80
70
60
50
40
30
20
10
0
*
*
b
a
A410
A800
B410
B800
(C)
Root area (mm²)
60000
50000
40000
30000
20000
10000
0
*
*
A410
A800
B410
B800

Figure 4

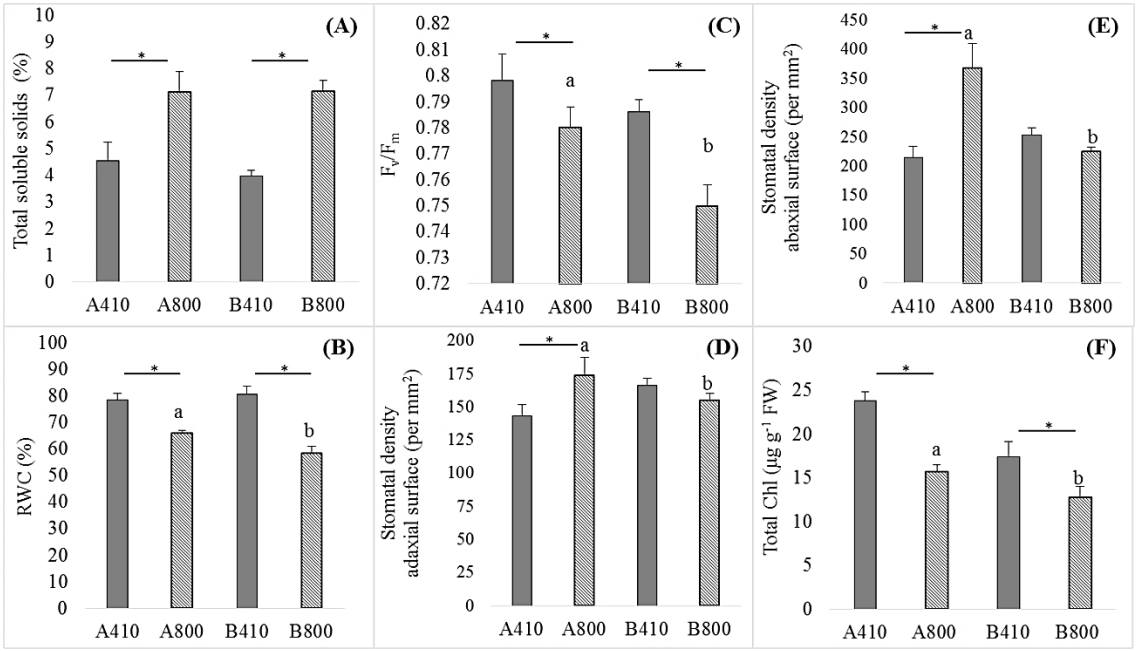

(A)
Total soluble solids (%)
(B)
RWC (%)
(C)
F_v/F_m
(D)
Stomatal density adaxial surface (per mm^2)
(E)
Stomatal density abaxial surface (per mm^2)
(F)
Total Chl (µg g^{-1} FW)
A410
A800
B410
B800
*
a
b

Figure 5

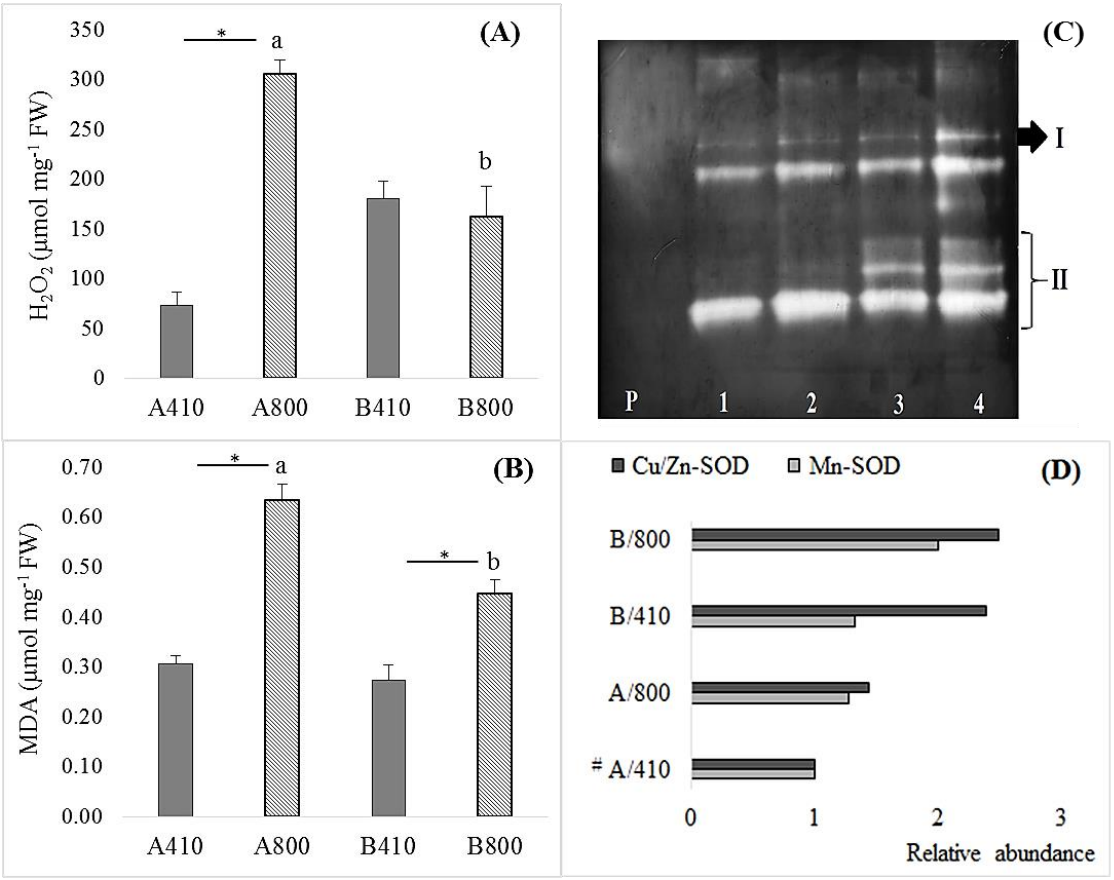

(A)
H_2O_2 (μmol mg^{-1} FW)
350
300
250
200
150
100
50
0
*
a
b
A410
A800
B410
B800
(C)
I
II
P
1
2
3
4
(B)
MDA (μmol mg^{-1} FW)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
a
*
b
A410
A800
B410
B800
(D)
Cu/Zn-SOD
Mn-SOD
B/800
B/410
A/800
A/410
0
1
2
3
Relative abundance

Figure 6

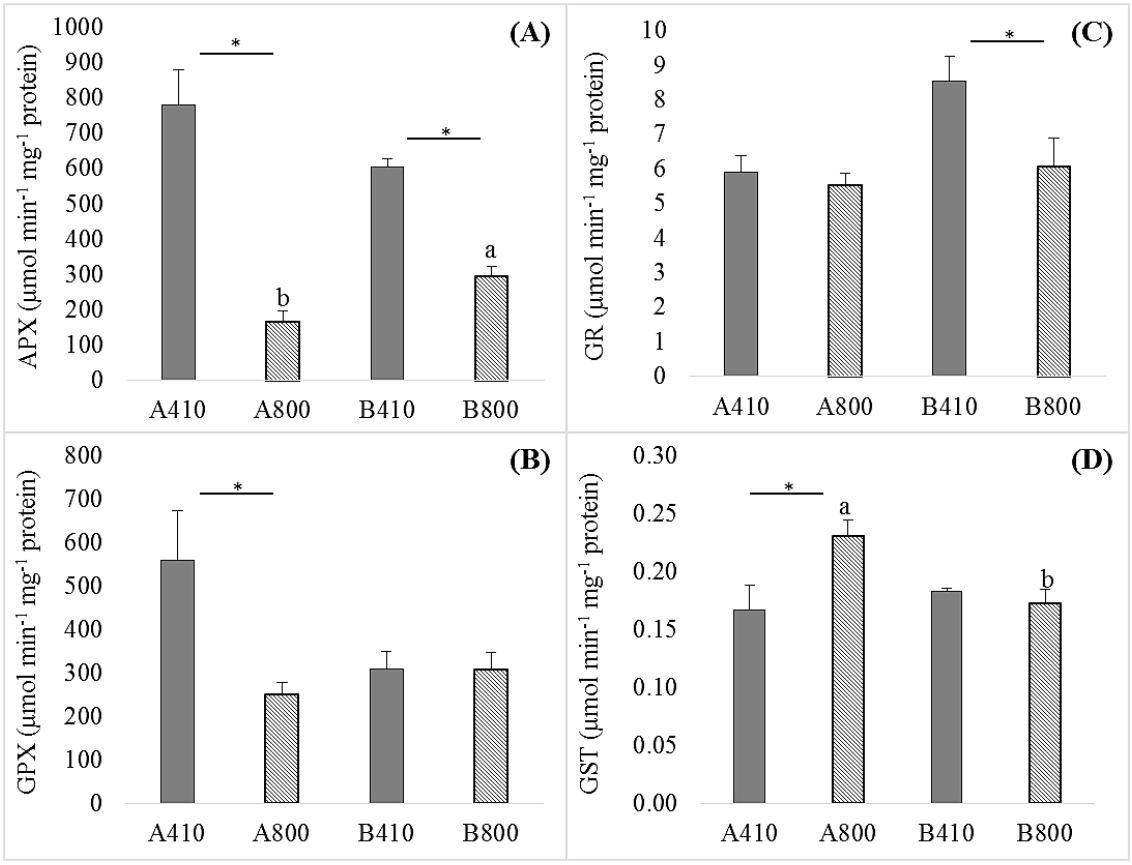

(A)
APX (µmol min-1 mg-1 protein)
1000
900
800
700
600
500
400
300
200
100
0
*
*
b
a
A410
A800
B410
B800
(C)
GR (µmol min-1 mg-1 protein)
10
9
8
7
6
5
4
3
2
1
0
*
A410
A800
B410
B800
(B)
GPX (µmol min-1 mg-1 protein)
800
700
600
500
400
300
200
100
0
*
A410
A800
B410
B800
(D)
GST (µmol min-1 mg-1 protein)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
a
b
A410
A800
B410
B800

Figure 7

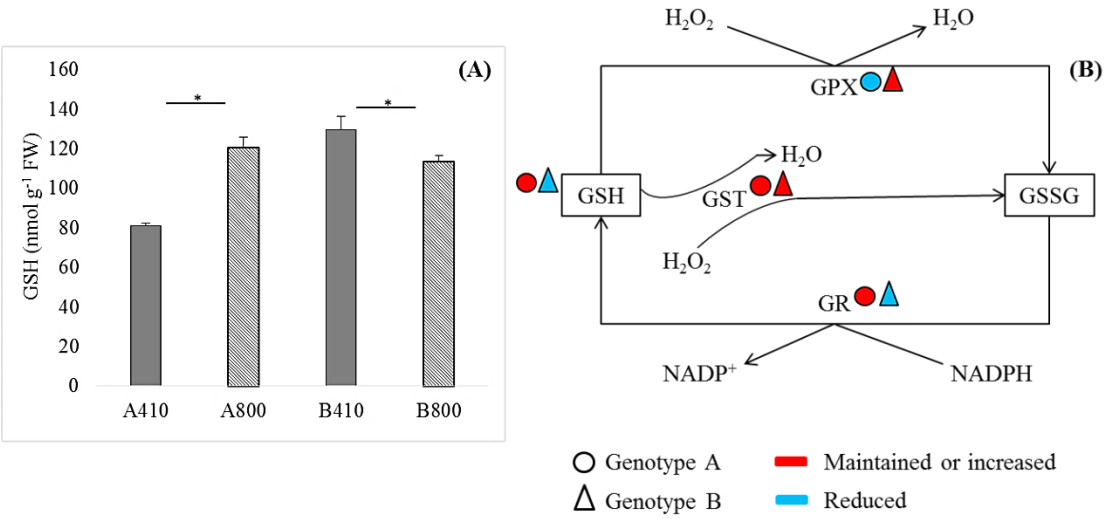

(A)
GSH (nmol g-1 FW)
160
140
120
100
80
60
40
20
0
*
*
A410
A800
B410
B800
(B)
H2O2
H2O
GPX
GSH
H2O
GST
GSSG
H2O2
GR
NADP+
NADPH
Genotype A
Genotype B
Maintained or increased
Reduced

Figure 8

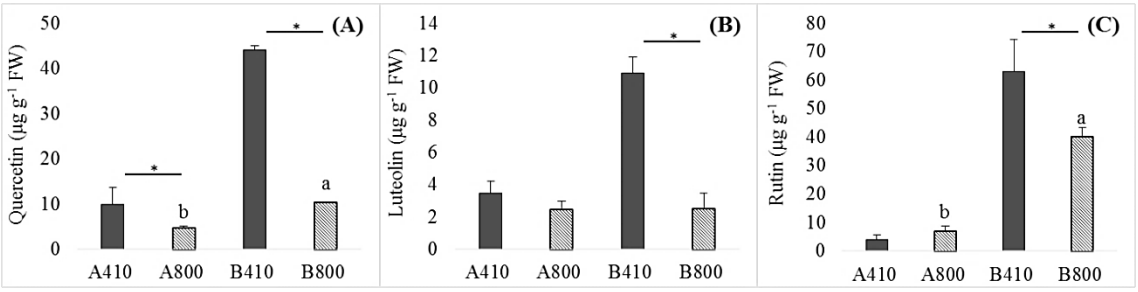

(A)
Quercetin (µg g-1 FW)
50
40
30
20
10
0
*
*
b
a
A410
A800
B410
B800
(B)
Luteolin (µg g-1 FW)
14
12
10
8
6
4
2
0
*
A410
A800
B410
B800
(C)
Rutin (µg g-1 FW)
80
70
60
50
40
30
20
10
0
*
b
a
A410
A800
B410
B800

Figure 9

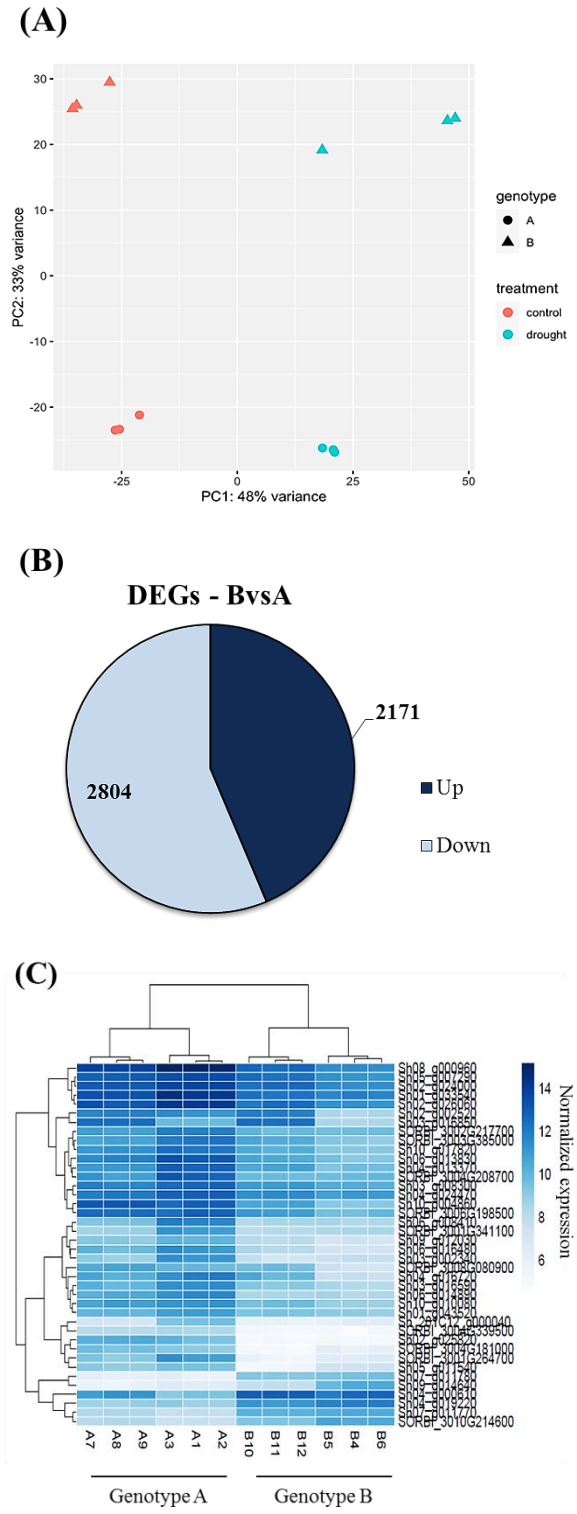

(A)
PC2: 33% variance
PC1: 48% variance
genotype
A
B
treatment
control
drought
(B)
DEGs - BvsA
2171
2804
Up
Down
(C)
Normalized expression
Genotype A
Genotype B

Figure 10

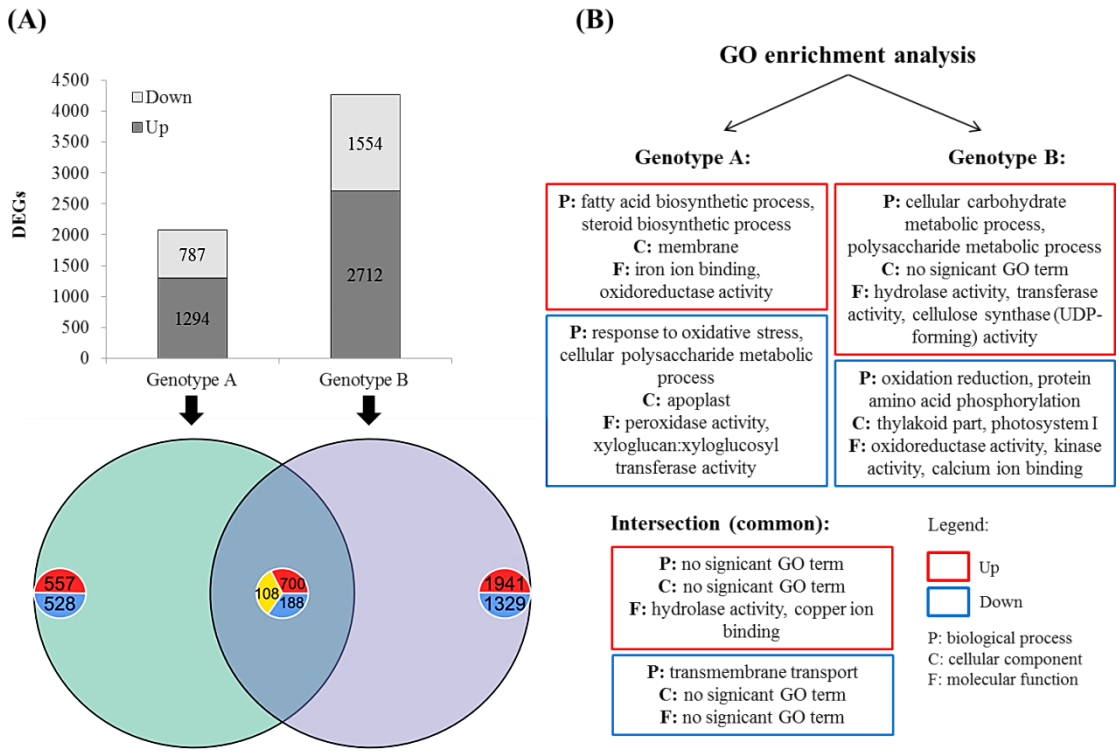

(A)
DEGs
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Down
Up
787
1294
1554
2712
Genotype A
Genotype B
557
528
108
700
188
1941
1329
(B)
GO enrichment analysis
Genotype A:
Genotype B:
P: fatty acid biosynthetic process, steroid biosynthetic process
C: membrane
F: iron ion binding, oxidoreductase activity
P: cellular carbohydrate metabolic process, polysaccharide metabolic process
C: no signicant GO term
F: hydrolase activity, transferase activity, cellulose synthase (UDP-forming) activity
P: response to oxidative stress, cellular polysaccharide metabolic process
C: apoplast
F: peroxidase activity, xyloglucan:xyloglucosyl transferase activity
P: oxidation reduction, protein amino acid phosphorylation
C: thylakoid part, photosystem I
F: oxidoreductase activity, kinase activity, calcium ion binding
Intersection (common):
P: no signicant GO term
C: no signicant GO term
F: hydrolase activity, copper ion binding
P: transmembrane transport
C: no signicant GO term
F: no signicant GO term
Legend:
Up
Down
P: biological process
C: cellular component
F: molecular function

Figure 11

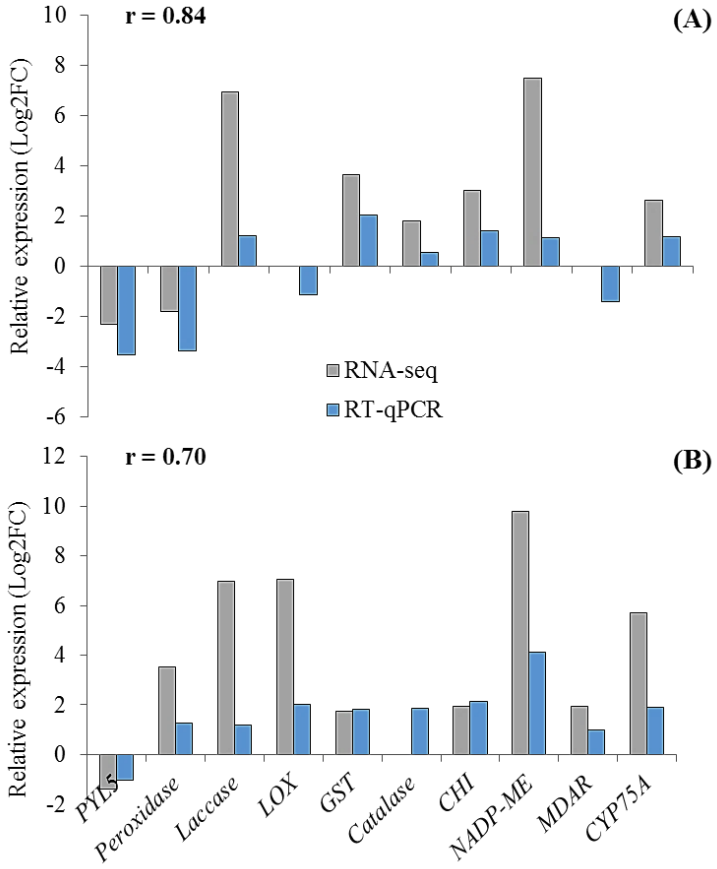

r = 0.84
(A)
Relative expression (Log2FC)
10
8
6
4
2
0
-2
-4
-6
RNA-seq
RT-qPCR
r = 0.70
(B)
Relative expression (Log2FC)
12
10
8
6
4
2
0
-2
PYL5
Peroxidase
Laccase
LOX
GST
Catalase
CHI
NADP-ME
MDAR
CYP75A

Figure 12

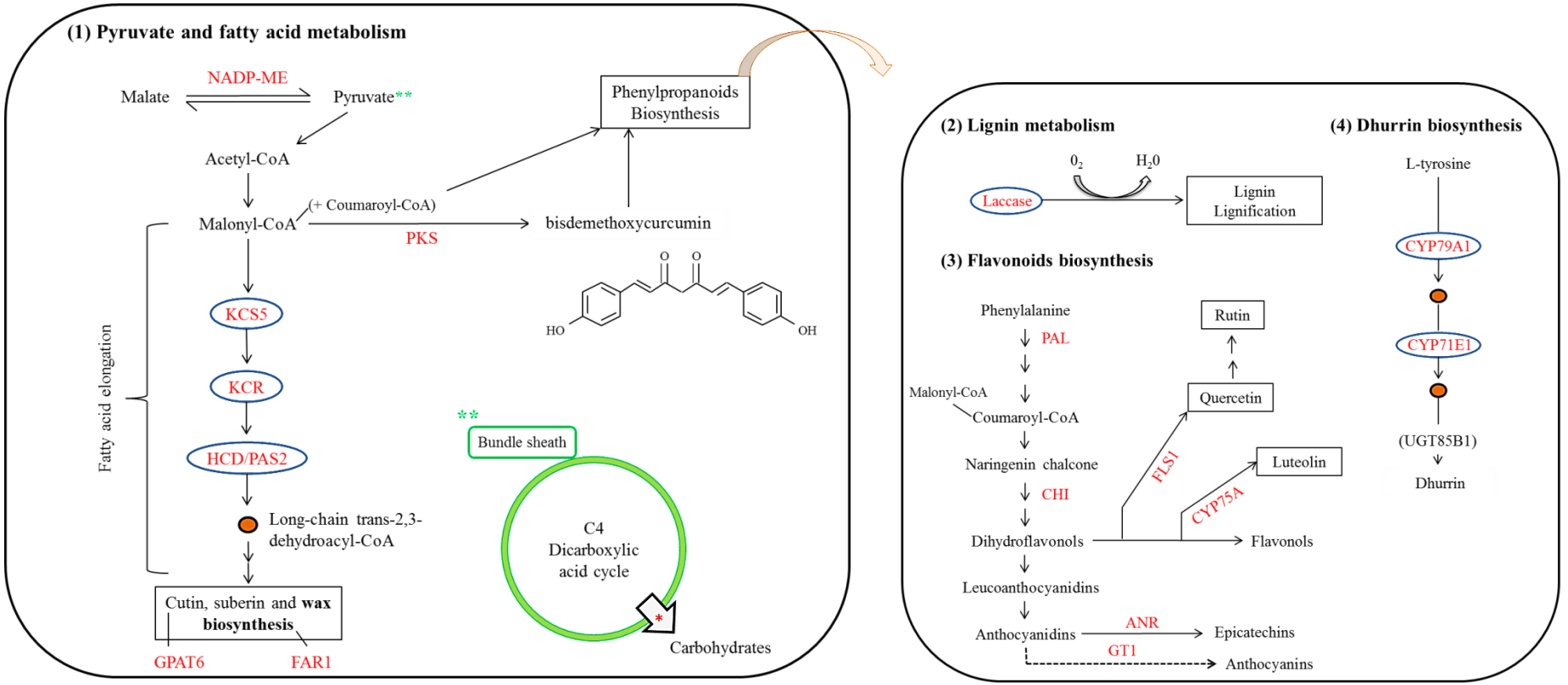
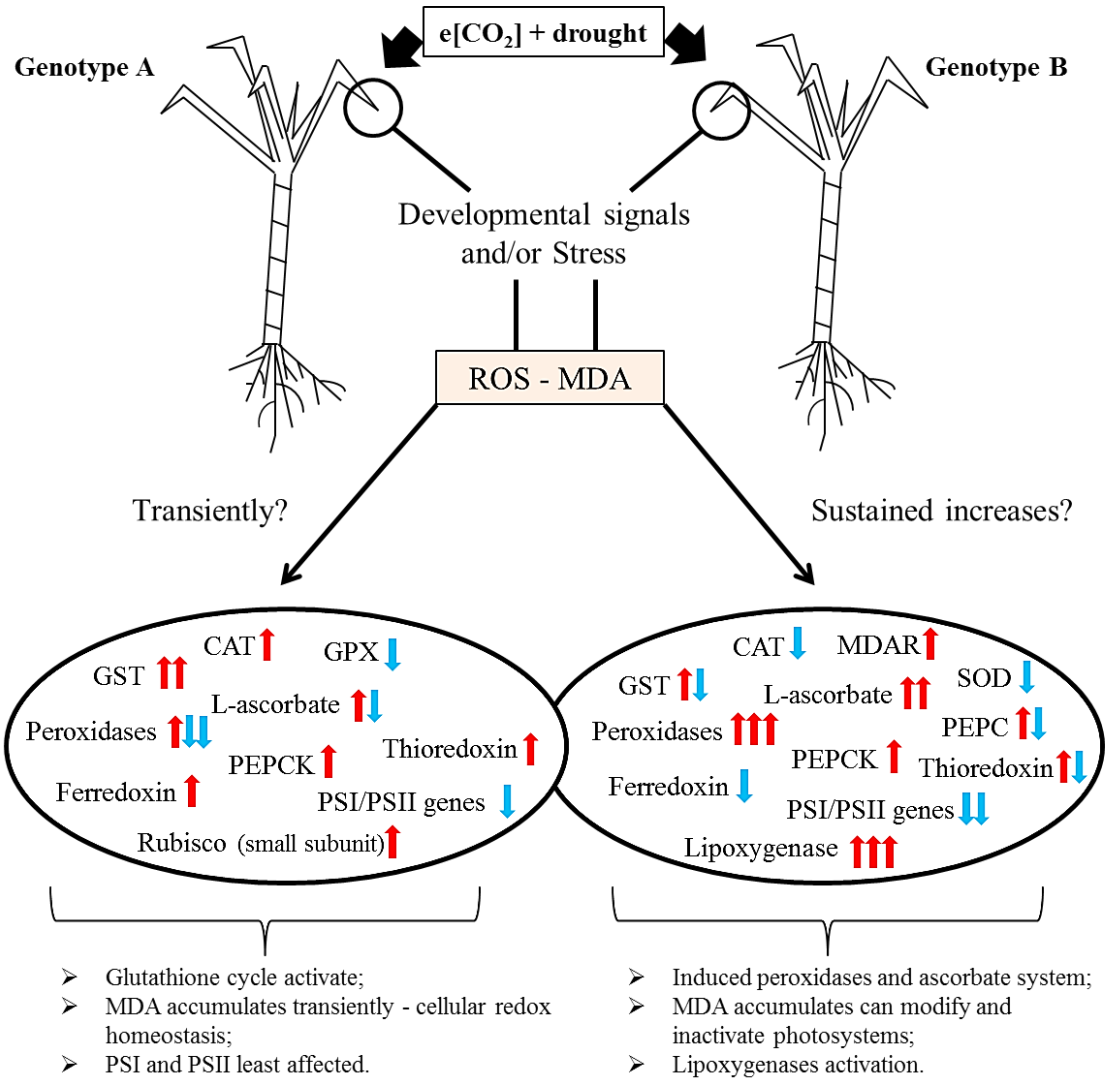

(1) Pyruvate and fatty acid metabolism
Malate
NADP-ME
Pyruvate**
Acetyl-CoA
Malonyl-CoA
(+ Coumaroyl-CoA)
PKS
bisdemethoxycurcumin
Phenylpropanoids Biosynthesis
HO
OH
KCS5
KCR
HCD/PAS2
Fatty acid elongation
Long-chain trans-2,3-dehydroacyl-CoA
Cutin, suberin and wax biosynthesis
GPAT6
FAR1
**
Bundle sheath
C4 Dicarboxylic acid cycle
*
Carbohydrates
(2) Lignin metabolism
0_2
H_20
Laccase
Lignin Lignification
(3) Flavonoids biosynthesis
Phenylalanine
PAL
Malonyl-CoA
Coumaroyl-CoA
Naringenin chalcone
CHI
Dihydroflavonols
Leucoanthocyanidins
Anthocyanidins
ANR
Epicatechins
GT1
Anthocyanins
Rutin
Quercetin
Luteolin
FLS1
CYP75A
Flavonols
(4) Dhurrin biosynthesis
L-tyrosine
CYP79A1
CYP71E1
(UGT85B1)
Dhurrin

Figure 13


e[CO2] + drought
Genotype A
Genotype B
Developmental signals and/or Stress
ROS - MDA
Transiently?
Sustained increases?
CAT
GPX
GST
L-ascorbate
Peroxidases
PEPCK
Thioredoxin
Ferredoxin
PSI/PSII genes
Rubisco (small subunit)
CAT
MDAR
GST
SOD
L-ascorbate
Peroxidases
PEPC
PEPCK
Thioredoxin
Ferredoxin
PSI/PSII genes
Lipoxygenase
Glutathione cycle activate;
MDA accumulates transiently - cellular redox homeostasis;
PSI and PSII least affected.
Induced peroxidases and ascorbate system;
MDA accumulates can modify and inactivate photosystems;
Lipoxygenases activation.

Supplementary Tables and Figures

Table S1. Information on the contrasting sugarcane genotypes used in the study.

Sources: Márcio A. P. Bidoia (technical director), IAC Sugarcane Center (Ribeirão Preto, São Paulo, Brazil); National Bioenergy Union - UDOP (https://www.udop.com.br/index.php?item=variedades_iac); and Feltre Group (http://www.grupofeltre.com.br/arquivos/variedades.pdf).

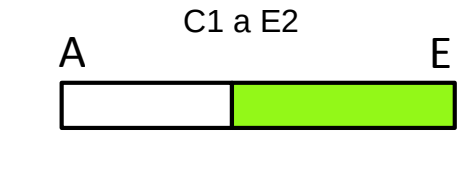
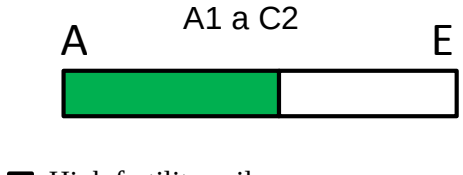
Genotype	Crossing	Characteristics	Production Environment	Indications
IACSP 94-2094 (A)	SP84-7017 x ?	Rustic genotype; Optimum bud sprouting and adapted to restrictive environments (drought tolerant), rare flowering in the conditions of the Center-South of Brazil; Erect size, high number of tillers (13-15/meter), with medium/thin stem diameter.		Suitable for harvesting from April to August, therefore, early-medium cycle.
IACSP 94-2101 (B)	SP77-5181 x RB785148	Responsive genotype; It stands out in favorable environments; It does not flourish in the conditions of the Center-South of Brazil. It presents fast initial vegetative development and excellent budding capacity under straw; Erect size, high number of tillers (12-14/meter), with medium diameter.		Suitable for harvesting from July to October, therefore, medium-late cycle.

Table S2. Primer list used for RT-qPCR analysis.

Gene ID[1]	Description	Primer sequence[2]
CA254672.1	*GAPDH*	Fw: GCTGACAAGGCTGATGGTAA Rv: CGATTCTGACAGTGAGGTCATC
Sh01_g032260	*Abscisic acid receptor PYL5* (*PYL5*)	Fw: AGATCCTGGACGACGAGAG Rv: TGGTGACGGAGAGGTAGTT
Sh01_g036120	*Peroxidase*	Fw: CTGGTGCGTCCCGTCAGT Rv: CTGGCACCGGACTGATTTGC
Sh01_g033820	*Laccase*	Fw: TCCAGCAAAGACACGTTCAG Rv: GTTGGCGATGGAGAAGAAGAG
Sh05_g011820	*Linoleate 9S-lipoxygenase 2* (*LOX*)	Fw: CTCCAGCTCGTAAAGAGTGTC Rv: CGTCATCCAACCTGTCTTGT
Sh04_g011700	*Glutathione-S-transferase* (*GST*)	Fw: CCGTCGTGTACGAGTGCCT Rv: TCGAGCACCTTGCTGAGCTT
Sh04_g000610	*Catalase isozyme 3* (*Catalase*)	Fw: GGCAGGAGAGGTTCGTCAGAA Rv: TGGAGAGGAGGTCGATCCAGA
Sh08_g002490	*Chalcone-flavonone isomerase* (*CHI*)	Fw: GAAGGGCAAGACGGCGGA Rv: TCTCCTTGATGACCACCACGC
Sh03_g020720	*NADP-dependent malic enzyme* (*NADP-ME*)	Fw: AGACACTCCAGCCGTTTAAG Rv: TTAGTGCAGTCGGTCTGATAAC
Sh07_g013240	*Monodehydroascorbate reductase* (*MDAR*)	Fw: AAGTGTTCTCGACGCTGATATT Rv: GGTCTTGAGTCCACCTTTCTC
Sh01_g027870	*Flavonoid 3'5' hydroxylase* (*CYP75A*)	Fw: CATCACCGAGGTCAACATCAA Rv: ATTTCAGACATCGCCCACTC

[1]ID number (Sh_) according to https://sugarcane-genome.cirad.fr/ (Gasmeur et al., 2018). Reference: Garsmeur O, Droc G, Antonise R, et al. 2018. A mosaic monoploid reference sequence for the highly complex genome of sugarcane. Nature Communications 9, 2638.

[2]Primers were designed with the software Primer 3 (Untergrasser et al., 2012). Fw: forward primer; Rv: reverse primer. Reference: Untergrasser A, Cutcutache I, Koressaar T, et al. 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Research 40(15), e115.

Table S3. Effect of elevated [CO_2] and drought on photosynthetic pigment content. Treatments consisted of sugarcane plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. The contents (µg. g^{-1} FW) of Chl*a*, Chl*b*, and carotenoids were determined through equations established by Lichtenthaler (1987). Data represents means ± SE of three independent biological replicates. Differences between control condition and respective stress condition were considered significant according to Tukey's test for p≤0.05 (*symbol). Different letters indicate a significant difference between sugarcane genotype A and B plants under stress condition by Tukey's test (p≤0.05).

Genotype	Condition	Chl*a*[1]	Chl*b*[1]	Carotenoids[1]
A	410 ppm [CO_2] + well-watered	16,30 ± 0,57*	7,53 ± 0,43*	3,14 ± 0,12
	800 ppm [CO_2] + drought	11,21 ± 0,55[a]	4,53 ± 0,18[a]	2,79 ± 0,19
B	410 ppm [CO_2] + well-watered	11,98 ± 1,10*	5,40 ± 0,64*	2,69 ± 0,35
	800 ppm [CO_2] + drought	9,08 ± 0,89[b]	3,68 ± 0,34[b]	2,16 ± 0,30

[1]Values in µg g^{-1} fresh weight.

Table S4. Summary of RNA sequencing by BGISEQ-500 platform. HQ raw reads were mapped against sugarcane reference genome (https://sugarcane-genome.cirad.fr/, Gasmeur et al., 2018) and assigned to exon features using the sugarcane_stp.gff3 annotation. HQ raw reads, that were unmapped in the sugarcane genome were mapped against *Sorghum bicolor* (Sbi) reference genome and assigned to exon features using the corresponding annotation.

Genotype / Treatment[1]	Sample ID[2]	Total HQ reads	Mapping against sugarcane and Sbi	
			Total number of reads mapped and assigned	% HQ read pairs
A / control	A1	49091860	15442604	62.91
	A2	48812306	15251962	62.49
	A3	49194280	15118897	61.47
A / stress	A7	49458780	15017727	60.73
	A8	49105582	15460186	62.97
	A9	49316112	15402366	62.46
B / control	B4	48822912	15016128	61.51
	B5	49065444	15517799	63.25
	B6	48803162	15170722	62.17
B / stress	B10	49016808	15268393	62.30
	B11	49167974	15347746	62.43
	B12	48902782	15221018	62.25
				Average: 62.25

[1]Three biological replicates. Treatments consisted of sugarcane plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. [2]Number identified for BGI sequencing.

Table S5. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (genotype A) subjected to elevated [CO_2] and drought. DEGs were considered if the adjusted p-value < 0.01 and log2 FoldChange > |1| (stress versus control). Blue: down-regulation; Red: up-regulation.

(Archive: Table S5 and S6_DEGs genotype A and B_DvsC)

Table S6. Differentially expressed genes (DEGs) found in leaves of sugarcane plants (genotype B) subjected to elevated [CO_2] and drought. DEGs were considered if the adjusted p-value < 0.01 and log2 FoldChange > |1| (stress versus control). Blue: down-regulation; Red: up-regulation.

(Archive: Table S5 and S6_DEGs genotype A and B_DvsC)

Table S7. Gene ontology (GO) enrichment analysis of specific DEGs found in sugarcane genotype A. Functional annotation of genotype A DEGs (stress versus control; genotype A_DvsC) were performed using agriGO tool version 1.2. GO annotation carried out considering all categories (Biological process, P; Cellular component, C; and Molecular function, F), *Sorghum bicolor* V2.1 as annotated reference and FDR ≤0.05.

Genotype A_DvsC			
Down-regulation			
GO term	**Ontology**	**Description**	**FDR**
GO:0006979	P	Response to oxidative stress	0.0072
GO:0044264	P	Cellular polysaccharide metabolic process	0.011
GO:0042221	P	Response to chemical stimulus	0.011
GO:0005975	P	Carbohydrate metabolic process	0.011
GO:0005976	P	Polysaccharide metabolic process	0.011
GO:0016684	F	Oxidoreductase activity, acting on peroxide as acceptor	0.007
GO:0004601	F	Peroxidase activity	0.007
GO:0016209	F	Antioxidant activity	0.016
GO:0016762	F	Xyloglucan:xyloglucosyl transferase activity	0.04
GO:0048046	C	Apoplast	0.026
GO:0005576	C	Extracellular region	0.026
Up-regulation			
GO:0006631	P	Fatty acid metabolic process	7.2e-07
GO:0006629	P	Lipid metabolic process	2.3e-06
GO:0032787	P	Monocarboxylic acid metabolic process	2.3e-06
GO:0008610	P	Lipid biosynthesis process	4.4e-06
GO:0006633	P	Fatty acid biosynthetic process	6e-06
GO:0044255	P	Cellular lipid metabolic process	0.00023
GO:0019752	P	Carboxylic acid metabolic process	0.00024
GO:0043436	P	Oxoacid metabolic process	0.00024
GO:0042180	P	Cellular ketone metabolic process	0.00024

GO:0006082	P	Organic acid metabolic process	0.00024
GO:0055114	P	Oxidation reduction	0.00033
GO:0016053	P	Organic acid biosynthetic process	0.00033
GO:0046394	P	Carboxylic acid biosynthetic process	0.00033
GO:0044283	P	Small molecule biosynthetic process	0.0062
GO:0006694	P	Steroid biosynthetic process	0.0068
GO:0008202	P	Steroid metabolic process	0.0091
GO:0003824	F	Catalytic activity	0.00016
GO:0016491	F	Oxidoreductase activity	0.00016
GO:0016614	F	Oxidoreductase activity, acting on CH-OH group of donors	0.0043
GO:0005506	F	Iron ion binding	0.006
GO:0003854	F	3-beta-hydroxy-delta5-steroid dehydrogenase activity	0.0074
GO:0016747	F	Transferase activity, transferring acyl groups other than amino-acyl groups	0.0074
GO:0016616	F	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.0074
GO:0016229	F	Steroid dehydrogenase activity	0.0074
GO:0033764	F	Steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.0074
GO:0048037	F	Cofactor binding	0.0074
GO:0050662	F	Coenzyme binding	0.0096
GO:0016746	F	Transferase activity, transferring acyl groups	0.013
GO:0016705	F	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.014
GO:0005215	F	Transporter activity	0.033
GO:0009055	F	Electron carrier activity	0.035
GO:0016020	C	Membrane	0.0063

Table S8. Gene ontology (GO) enrichment analysis of specific DEGs found in sugarcane genotype B. Functional annotation of genotype A DEGs (stress versus control; genotype A_DvsC) were performed using agriGO tool version 1.2. GO annotation carried out considering all categories (Biological process, P; Cellular component, C; and Molecular function, F), *Sorghum bicolor* V2.1 as annotated reference and FDR ≤0.05.

Genotype B_DvsC			
Down-regulation			
GO term	**Ontology**	**Description**	**FDR**
GO:0015979	P	Photosynthesis	0.00099
GO:0008152	P	Metabolic process	0.0024
GO:0006796	P	Phosphate metabolic process	0.0024
GO:0006793	P	Phosphorus metabolic process	0.0024
GO:0016310	P	Phosphorylation	0.0046
GO:0055114	P	Oxidation reduction	0.0069
GO:0006468	P	Protein amino acid phosphorylation	0.036
GO:0043687	P	Post-translational protein modification	0.045
GO:0005509	F	Calcium ion binding	4.9e-06
GO:0016491	F	Oxidoreductase activity	0.00039
GO:0016301	F	Kinase activity	0.041
GO:0009579	C	Thylakoid	6.8e-06
GO:0034357	C	Photosynthetic membrane	1.e-05
GO:0009522	C	Photosystem I	1.4e-05
GO:0009521	C	Photosystem	2.1e-05
GO:0044436	C	Thylakoid part	0.00029
GO:0044425	C	Membrane part	0.012
Up-regulation			
GO:0005975	P	Carbohydrate metabolic process	1.e-11
GO:0005976	P	Polysaccharide metabolic process	0.011
GO:0044262	P	Cellular carbohydrate metabolic process	0.016
GO:0008152	P	Metabolic process	0.043

GO:0016798	F	Hydrolase activity, acting on glycosyl bonds	2.1e-11
GO:0004553	F	Hydrolase activity, hydrolyzing O-glycosyl compounds	1.6e-10
GO:0003824	F	Catalytic activity	3.3e-09
GO:0016787	F	Hydrolase activity	3.6e-07
GO:0016746	F	Transferase activity, transferring acyl groups	0.0092
GO:0016747	F	Transferase activity, transferring acyl groups other than amino-acyl groups	0.013
GO:0016760	F	Cellulose synthase (UDP-forming) activity	0.044
GO:0016759	F	Cellulose synthase activity	0.044

Table S9. Gene ontology (GO) enrichment analysis of intersection (common) DEGs found in sugarcane genotypes A and B. Functional annotation of common DEGs (stress versus control; intersection between genotype A and B - DvsC) were performed using agriGO tool version 1.2 (Zhou et al., 2010). GO annotation carried out considering all categories (Biological process, P; Cellular component, C; and Molecular function, F), *Sorghum bicolor* V2.1 as annotated reference and FDR ≤0.05.

Intersection between Genotype A and B - DvsC			
Down-regulation			
GO:0055085	P	Transmembrane transport	0.038
Up-regulation			
GO:0016798	F	Hydrolase activity, acting on glycosyl bonds	9.4e-05
GO:0004553	F	Hydrolase activity, hydrolyzing O-glycosyl compounds	0.0016
GO:0005507	F	Copper ion binding	0.0016
GO:0048037	F	Cofactor binding	0.0016
GO:0003824	F	Catalytic activity	0.003
GO:0016491	F	Oxidoreductase activity	0.0096
GO:0019842	F	Vitamin binding	0.023
GO:0050662	F	Coenzyme binding	0.049

Table S10. References used in the construction of the schematic overview of pyruvate and fatty acid metabolism, lignin metabolism, flavonoids, and dhurrin biosynthesis. These processes were up-regulated in sugarcane genotype A (IACSP94-2094) subjected to elevated [CO_2] and drought.

Pyruvate, Fatty acid metabolism, and Phenylpropanoid biosynthesis
Thomas. 2010. Phenylpropanoid biosynthesis. Molecular Plant 3, 2-20. DOI: 10.1093/mp/ssp106
Petit et al. 2016. The glycerol-3-phosphate acyltransferase GPAT6 from tomato plays a central role in fruit cutin biosynthesis. Plant Physiology 171, 894-913. DOI: 10.1104/pp.16.00409
Kassab et al. 2019. GFP scaffold-based engineering for the production of unbranched very long chain fatty acids in *Escherichia coli* with oleic acid and cerulenin supplementation. Frontiers in Bioengineering and Biotechnology 7, 408. DOI: 10.3389/fbioe.2019.00408
Lignin metabolism
O'Malley et al. 1993. The role of of laccase in lignification. The Plant Journal 4, 751-757. DOI: 10.1046/j.1365-313X.1993.04050751.x.
Liu et al. 2018. Lignins: biosynthesis and biological functions in plants. International Journal of Molecular Sciences 19, 335. DOI: 10.3390/ijms19020335
Flavonoids biosynthesis
Tian et al. 2017. McMYB12 transcription factors co-regulate proanthocyanidin and anthocyanin biosynthesis in *Malus* Crabapple. Scientific reports 7, 43715. DOI: 10.1038/srep43715
Ahmad et al. 2017. Isoflavone malonyltransferases GmIMaT1 and GmIMaT3 differently modify isoflavone glucosides in soybean (*Glycine max*) under various stresses. Frontiers in Plant Science 8:735. DOI: 10.3389/fpls.2017.00735
Dhurrin biosynthesis
Blomstedt et al. 2016. Metabolic consequences of knocking out UGT85B1, the gene encoding the glucosyltransferase required for synthesis of dhurrin in *Sorghum bicolor* (L. Moench). Plant & Cell Physiology 57, 373-786. DOI: 10.1093/pcp/pcv153
Knudsen et al. 2020. Stabilization of dhurrin biosynthetic enzymes from *Sorghum bicolor* using a natural deep eutectic solvent. Phytochemistry 170, 112214. DOI: 10.1016/j.phytochem.2019.112214

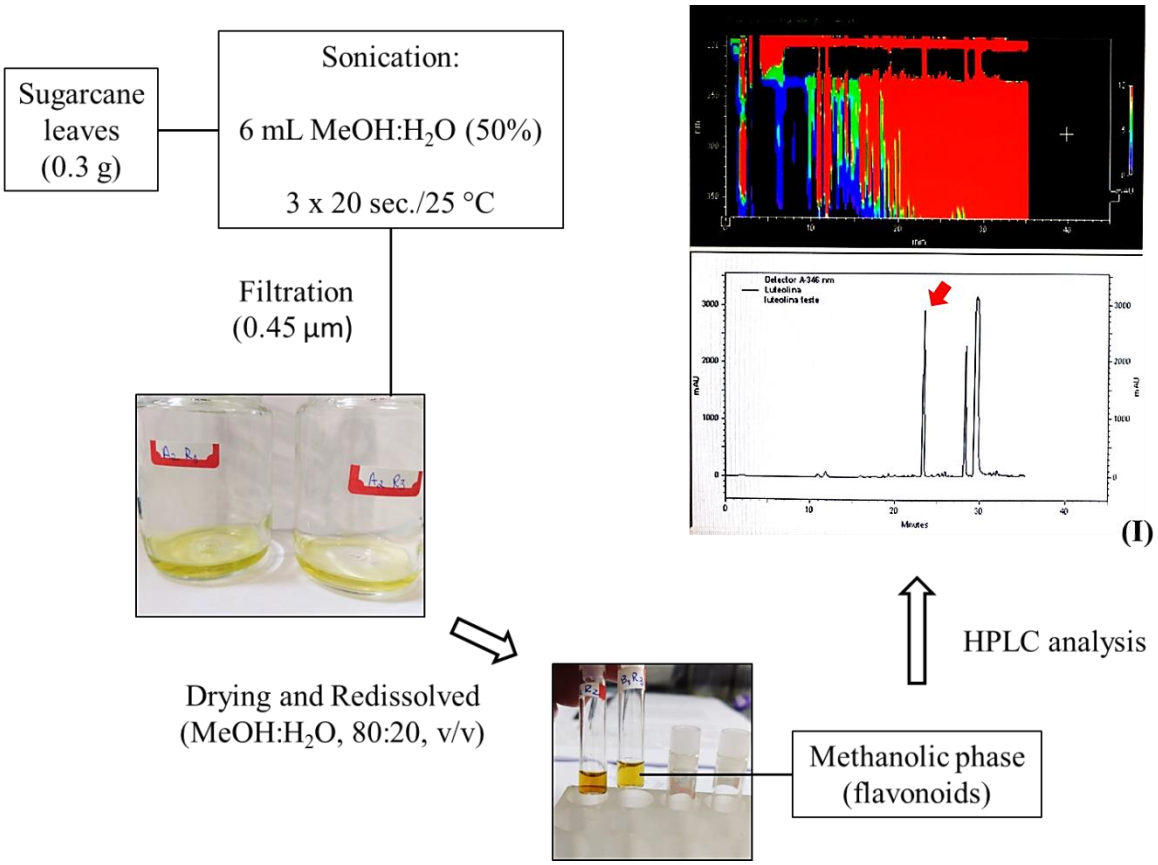
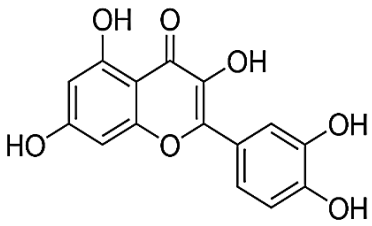
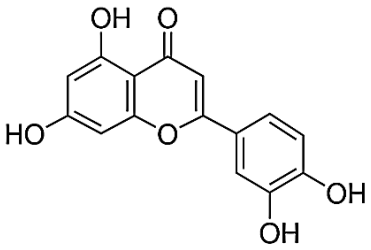


Figure S1. Methodology for extracting flavonoids from sugarcane leaves. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. Three independent biological replicates. After obtaining the methanolic phase, the luteolin (# BCBN2483V), quercetin (# SLBD8415V), and rutin (# 71H0815) flavonoids were quantified based on standard curves (I). Red arrow: indicates the peak retention of the luteolin flavonoid.

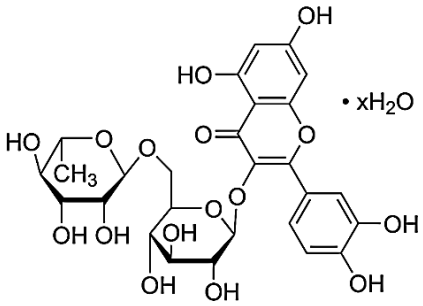
Flavonoids (external standards)



Quercetin (MW*: 302.24)



Luteolin (MW*: 286.24)



Rutin (MW*: 610.52)

*MW: Molecular Weight

Figure S2. Flavonoids (external standards) used in HPLC analysis. The chemical structure and molecular weight of the flavonoids are indicated. Standards: luteolin (# BCBN2483V), quercetin (# SLBD8415V), and rutin (# 71H0815). Source: https://www.sigmaaldrich.com/.

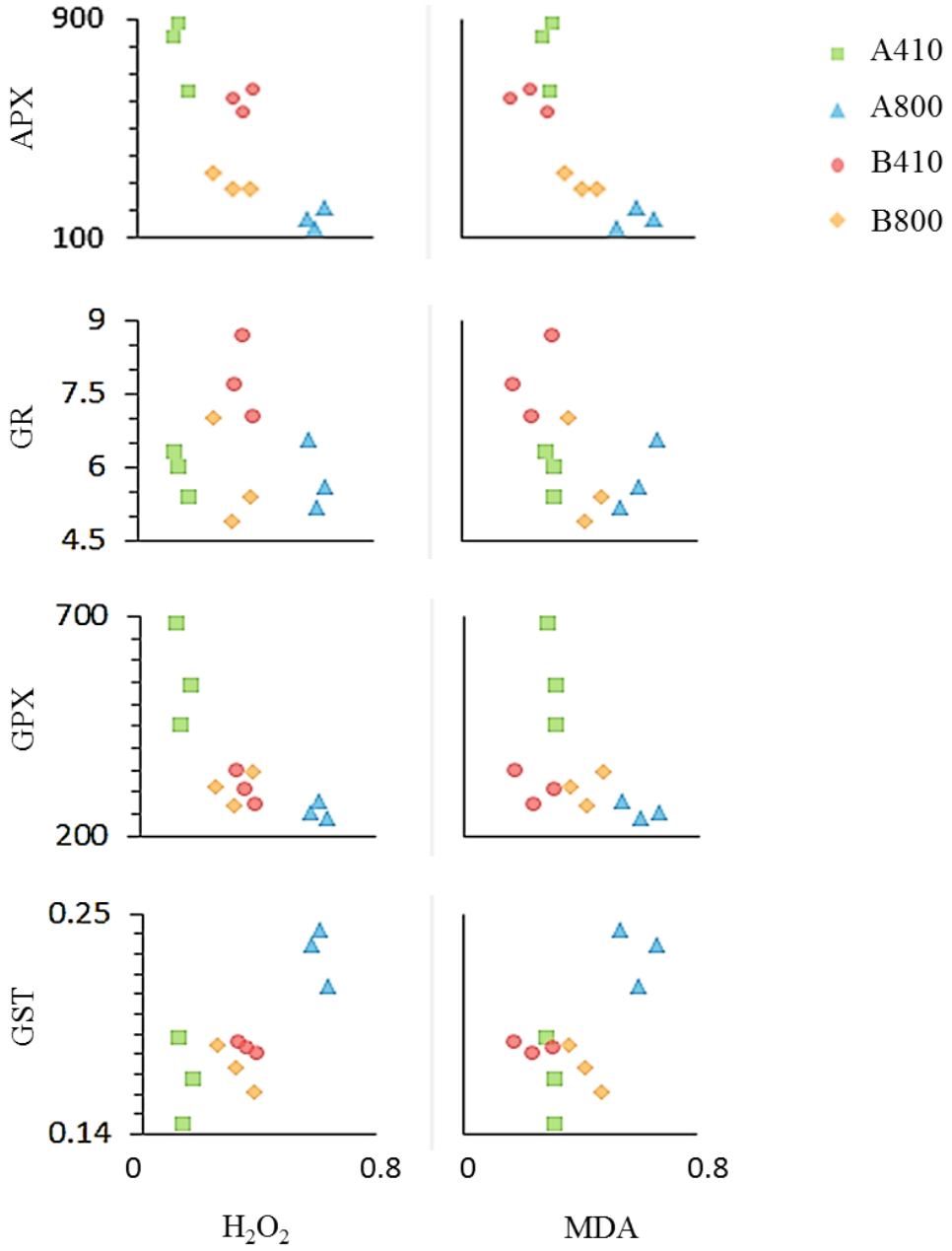


Figure S3. Correlation between oxidative stress indicators (MDA and H_2O_2 content) and antioxidant enzyme activities. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. Three independent biological replicates. Enzymes: ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), glutathione peroxidase (GPX, EC 1.11.1.9), and glutathione-S-transferase (GST, EC 2.5.1.18). Pearson's correlation (data analyzed by statistical analysis software Analyse-it®) resulted in r = 0.95 (GST activity x H_2O_2 content), and r = 0.81 (GST activity x MDA content).

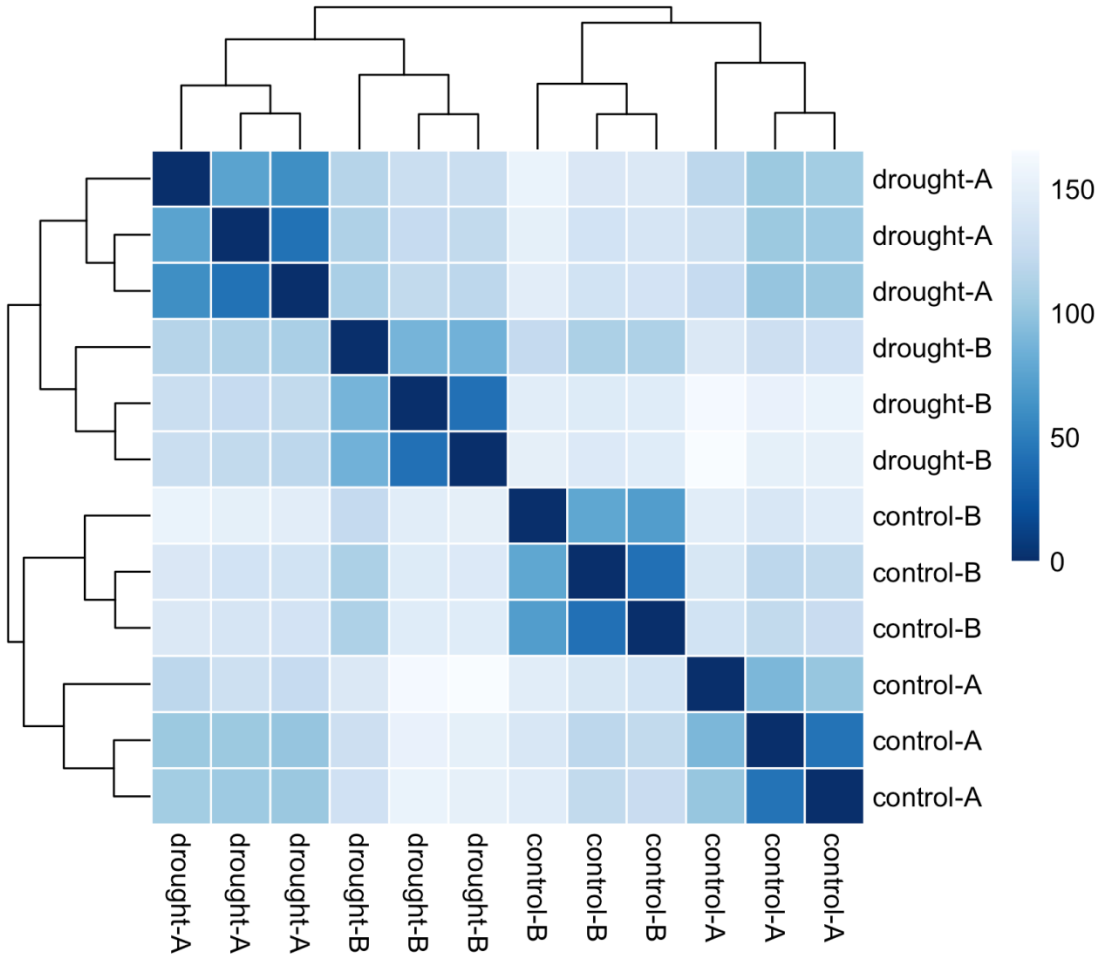


Figure S4. Distance heatmap to test the consistency of the data across all sequenced samples. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. Twelve biological samples (replicates) were used in the sequencing. There is a clear separation of the sample according to experimental condition and genotype.

Figure S5

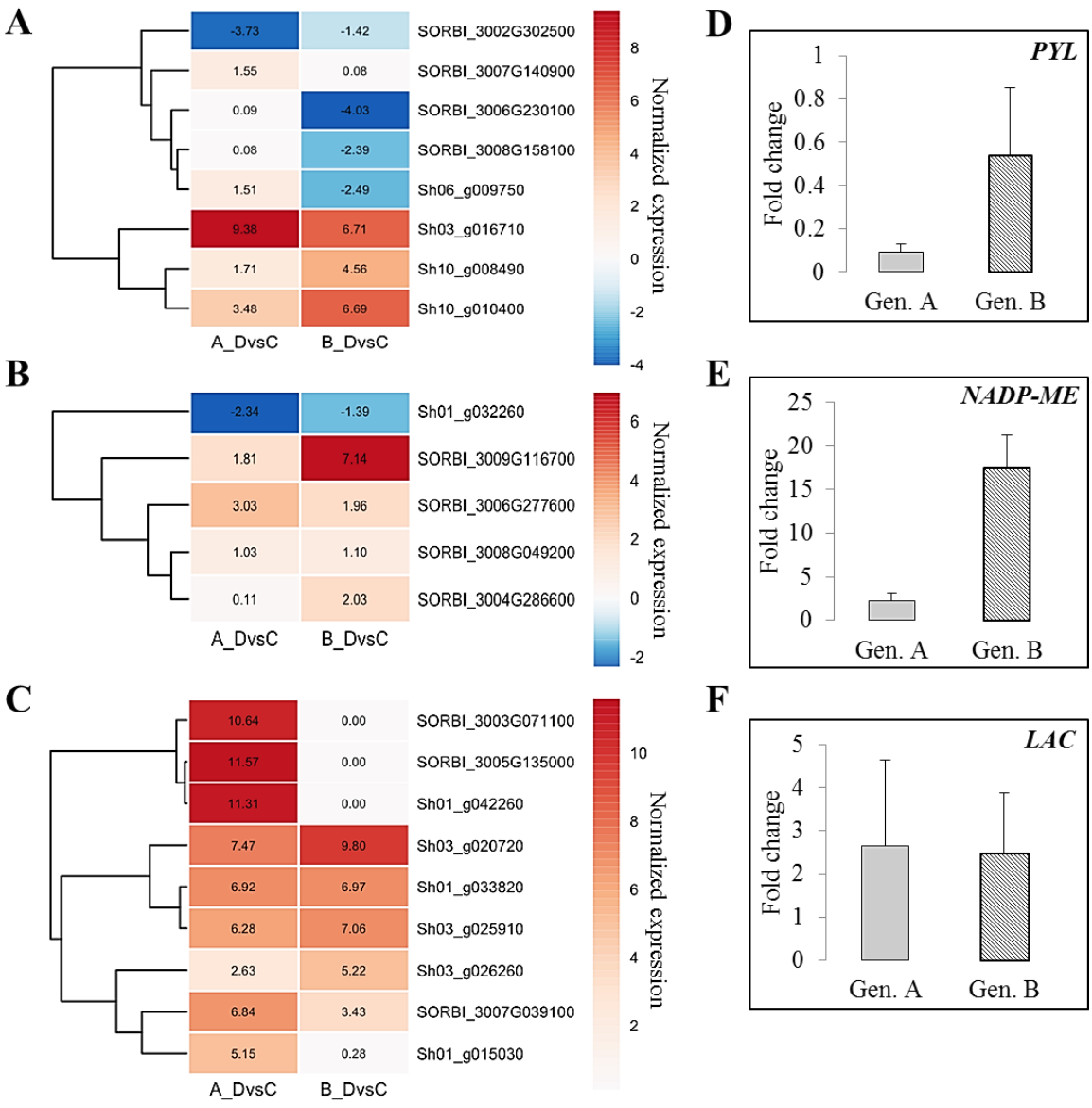

A
-3.73
-1.42
SORBI_3002G302500
1.55
0.08
SORBI_3007G140900
0.09
-4.03
SORBI_3006G230100
0.08
-2.39
SORBI_3008G158100
1.51
-2.49
Sh06_g009750
9.38
6.71
Sh03_g016710
1.71
4.56
Sh10_g008490
3.48
6.69
Sh10_g010400
A_DvsC
B_DvsC
Normalized expression
8
6
4
2
0
-2
-4
B
-2.34
-1.39
Sh01_g032260
1.81
7.14
SORBI_3009G116700
3.03
1.96
SORBI_3006G277600
1.03
1.10
SORBI_3008G049200
0.11
2.03
SORBI_3004G286600
A_DvsC
B_DvsC
Normalized expression
6
4
2
0
-2
C
10.64
0.00
SORBI_3003G071100
11.57
0.00
SORBI_3005G135000
11.31
0.00
Sh01_g042260
7.47
9.80
Sh03_g020720
6.92
6.97
Sh01_g033820
6.28
7.06
Sh03_g025910
2.63
5.22
Sh03_g026260
6.84
3.43
SORBI_3007G039100
5.15
0.28
Sh01_g015030
A_DvsC
B_DvsC
Normalized expression
10
8
6
4
2
D
PYL
Fold change
1
0.8
0.6
0.4
0.2
0
Gen. A
Gen. B
E
NADP-ME
Fold change
25
20
15
10
5
0
Gen. A
Gen. B
F
LAC
Fold change
5
4
3
2
1
0
Gen. A
Gen. B

Figure S5. DEGs associated to different biological processes regulated in leaves of sugarcane plants. A) RNA-seq normalized expression profiles of DEGs associated to signaling. B) RNA-seq normalized expression profiles of DEGs associated to response to stimulus. C) RNA-seq normalized expression profiles of DEGs associated to fatty acid metabolism, pyruvate metabolism, lignin metabolism and others biological processes in sugarcane plants. The profile was obtained considering the expression in plants under stress versus control (A_DvsC; and B_DvsC). Colours represent normalized expression. D) RT-qPCR expression (fold change) analysis of *abscisic acid receptor* (*PYL5*, Sh01_g032260). E) RT-qPCR expression (fold change) analysis of *NADP-dependent malic enzyme* (*NADP-ME*, Sh03_g020720). F) RT-qPCR expression (fold change) analysis of *laccase* (*LAC*, Sh01_g033820). The *GAPDH* gene (CA254672.1) was selected as internal control. RT-qPCR analysis was performed comparing plants under stress versus control (A_DvsC; and B_DvsC). Gen.A = genotype A; Gen.B = genotype B.

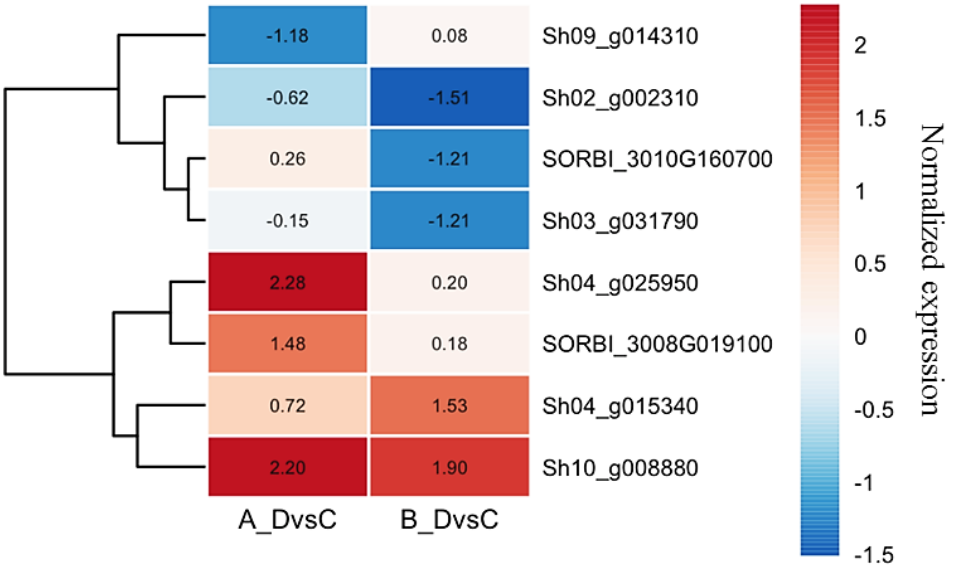


Figure S6. DEGs associated to photosynthesis. RNA-seq normalized expression profiles of DEGs in leaves of sugarcane plants (IACSP94-2094, genotype A; IACSP94-2101, genotype B) subjected to elevated [CO_2] and drought. The profile was obtained considering the expression in plants under stress versus control (A_DvsC; and B_DvsC). Colours represent normalized expression.

Figure S7

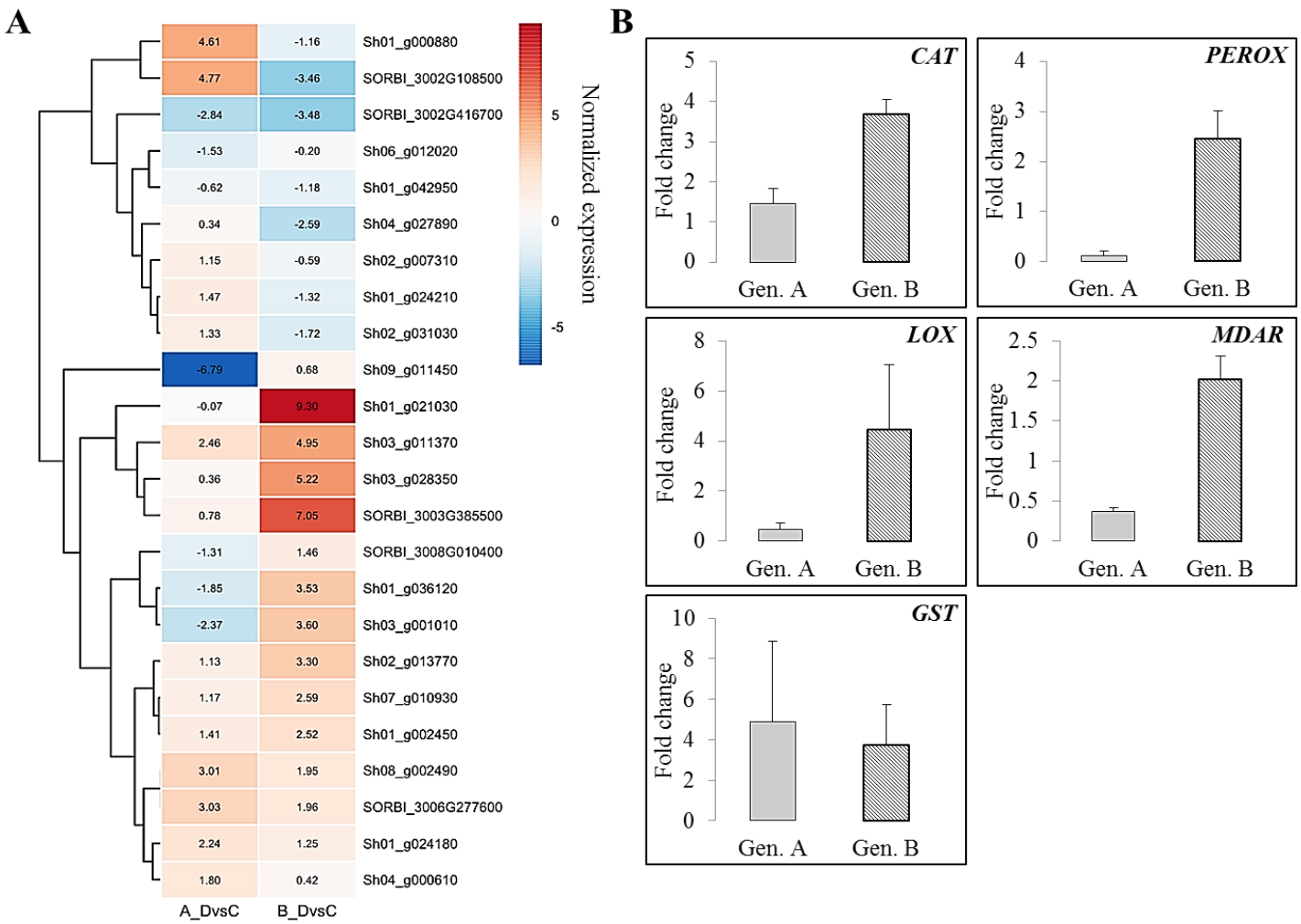

A
4.61 -1.16 Sh01_g000880
4.77 -3.46 SORBI_3002G108500
-2.84 -3.48 SORBI_3002G416700
-1.53 -0.20 Sh06_g012020
-0.62 -1.18 Sh01_g042950
0.34 -2.59 Sh04_g027890
1.15 -0.59 Sh02_g007310
1.47 -1.32 Sh01_g024210
1.33 -1.72 Sh02_g031030
-6.79 0.68 Sh09_g011450
-0.07 9.30 Sh01_g021030
2.46 4.95 Sh03_g011370
0.36 5.22 Sh03_g028350
0.78 7.05 SORBI_3003G385500
-1.31 1.46 SORBI_3008G010400
-1.85 3.53 Sh01_g036120
-2.37 3.60 Sh03_g001010
1.13 3.30 Sh02_g013770
1.17 2.59 Sh07_g010930
1.41 2.52 Sh01_g002450
3.01 1.95 Sh08_g002490
3.03 1.96 SORBI_3006G277600
2.24 1.25 Sh01_g024180
1.80 0.42 Sh04_g000610
A_DvsC
B_DvsC
Normalized expression
5
0
-5
B
CAT
PEROX
LOX
MDAR
GST
Fold change
Gen. A
Gen. B

Figure S7. DEGs associated to antioxidant metabolism. A) RNA-seq normalized expression profiles of DEGs in leaves of sugarcane plants (IACSP94-2094, genotype A; IACSP94-2101, genotype B) subjected to elevated [CO_2] and drought. The profile was obtained considering the expression in plants under stress versus control (A_DvsC; and B_DvsC). Colours represent normalized expression. B) RT-qPCR expression (fold change) analysis of *catalase isozyme 3* (*CAT*, Sh04_g000610), *peroxidase* (*PEROX*, Sh01_g036120), *linoleate 9S-lipoxygenase 2* (*LOX*, Sh05_g011820), *monodehydroascorbate reductase* (*MDAR*, Sh07_g013240), and *glutathione-S-transferase* (*GST*, Sh04_g011700). The *GAPDH* gene (CA254672.1) was selected as internal control. RT-qPCR analysis was performed comparing plants under stress versus control (A_DvsC; and B_DvsC). Gen.A = genotype A; Gen.B = genotype B.

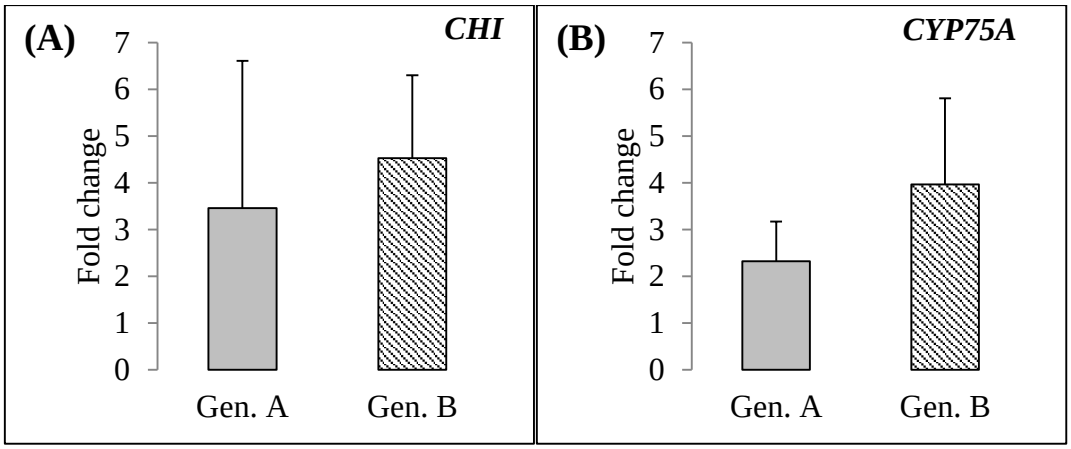


Figure S8. RT-qPCR expression analysis of genes involved in flavonoids biosynthesis. Treatments consisted of plants (genotype A and B) subjected to control condition (410 ppm [CO_2] + well-watered), and stress condition (800 ppm [CO_2] + drought), for 30 d. A) RT-qPCR expression (fold change) analysis of *chalcone-flavonone isomerase* (*CHI*, Sh08_g002490), and B) *flavonoid 3'5' hydroxylase* (*CYP75A*, Sh01_g027870). The *GAPDH* gene (CA254672.1) was selected as internal control. RT-qPCR analysis was performed comparing plants under stress versus control (A_DvsC; and B_DvsC). Gen.A = genotype A; Gen.B = genotype B.

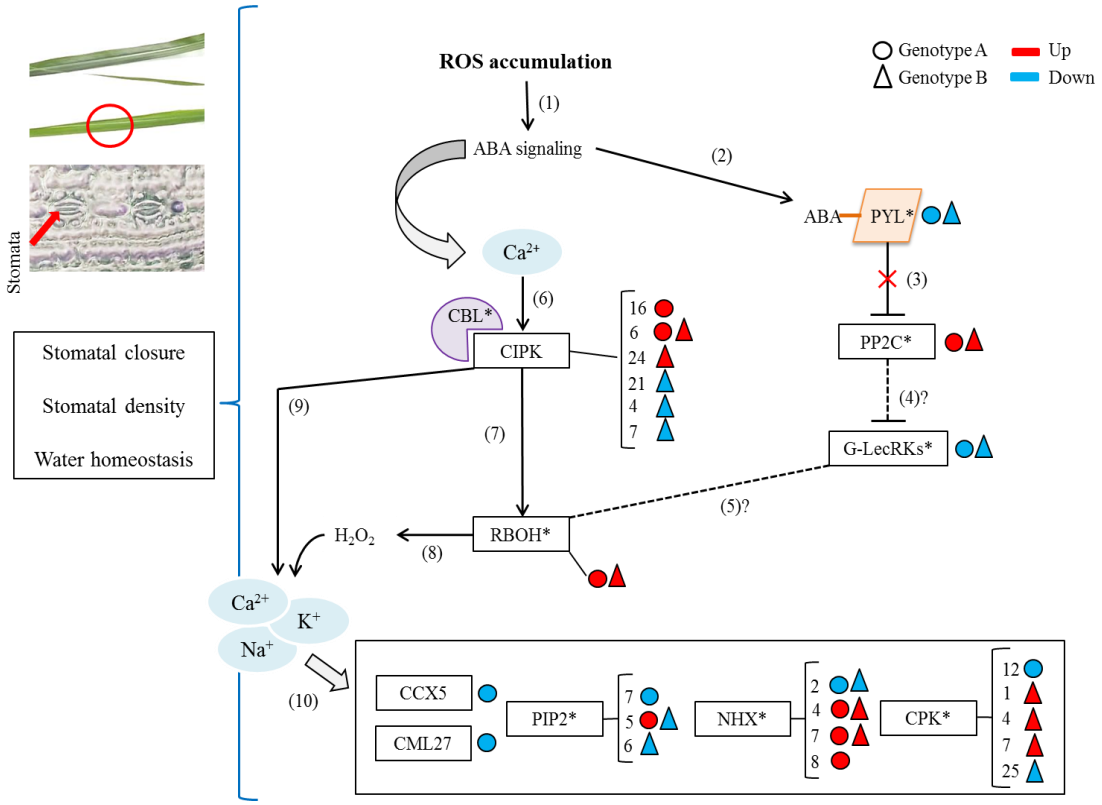


Fig. S9. A proposed model for stomatal responses on sugarcane by ROS accumulation and ABA-mediated signaling. This model can to explain the main changes in stomatal closure and density, and leaf water homeostasis in sugarcane genotypes A and B subjected to elevated [CO_2] and drought. (1) ABA can be synthesized in response to ROS accumulation (H_2O_2); (2) ABA interacts with *abscisic acid receptor* (*PYL*, down-regulated); thus, (3) the inhibition of *protein phosphatase 2C* (*PP2C*) does not happen (up-regulated). However some *PP2C* isoforms have been down-regulated in both sugarcane genotypes, possibly in response to H_2O_2 accumulated; (4) *PP2C* possibly (?) leads to inactivation of *G-LecRKs* (down-regulated); Activated *G-LecRKs* would result in (5) activation of *respiratory burst oxidase* (*RBOH*) (up-regulated), however, *RBOH* may be being activated by other protein kinases (?); (6) ABA activates Ca^{2+} channels, promoting the accumulation of Ca^{2+} in the cytosol of guard cells, which in turn activate (7) *RBOH* mediated by the *CBL-CIPK* complex (up and down-regulated); (8) H_2O_2 generated from *RBOH*, or (9) the direct action of Ca^{2+} sensors proteins (*CBL-CIPK*) regulate channels opening, leading to (10) changes in transporters expression (e.g., *cation/calcium exchanger 5* - *CCX5*, and *sodium/hydrogen exchanger – NHX*), and aquaporin's (e.g., *PIP2-5* was up-regulated in genotype A and down-regulated in genotype B).*symbol indicates more than one identified isoform.