UNIVERSIDADE ESTADUAL DE CAMPINAS
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MEMÓRIA DE PLANTAS DE CANA-DE-AÇÚCAR À SECA

MEMORY OF SUGARCANE PLANTS TO DROUGHT

Campinas
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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em BIOLOGIA VEGETAL.

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RESUMO

A maior parte do cultivo da cana-de-açúcar é feita sem irrigação e desta forma as plantas estão constantemente expostas a ciclos de seca e reidratação. Os efeitos da seca desencadeiam uma série de eventos de sinalizações nas plantas que induzem modificações que podem ser armazenadas e recuperadas como memórias por alterações nas concentrações de alguns metabólitos ou epigenéticas. Como os eventos de seca podem induzir a memória de estresse, nossa hipótese é que plantas previamente expostas a ciclos de déficit hídrico terão melhor desempenho do que plantas que nunca enfrentaram déficit hídrico quando ambas estão sujeitas à baixa disponibilidade hídrica. Para isso, utilizamos o genótipo de cana-de-açúcar IACSP94-2094 (*Saccharum* spp.) em dois experimentos diferentes. No primeiro as plantas de cana-de-açúcar foram cultivadas em solução nutritiva e expostas a um (1WD), dois (2WD) ou três (3WD) ciclos de déficit hídrico com adição de polietilenoglicol (PEG8000). Como referência (Ref.), as plantas foram cultivadas em solução nutritiva sem adição de PEG8000. Sob déficit hídrico, as trocas gasosas foliares foram significativamente reduzidas em plantas de 1WD e 2WD. No entanto, as plantas 3WD mostraram similar assimilação de CO_2 e menor condutância estomática em comparação com as plantas de Referência, com aumento na eficiência de uso da água. A concentração de ácido abscísico, um sinal de seca que poderia levar à memória de estresse, foi maior em 1WD do que em plantas 3WD. Essas plantas 3WD apresentaram maior proporção de massa seca da raiz e maior relação raiz: parte aérea em comparação com a Ref., bem como maior produção de biomassa em condições bem irrigadas. Nossos dados sugerem que as plantas de cana-de-açúcar armazenaram e recuperaram informações de eventos estressantes anteriores, com a melhoria do desempenho das plantas sob déficit hídrico. Os resultados do primeiro experimento indicaram uma nova perspectiva prática para o uso da memória vegetal para melhorar o crescimento de plantas cultivadas. No segundo experimento, testou-se a hipótese de que plantas obtidas de plantas-mãe previamente expostas ao déficit hídrico teriam melhor desempenho sob déficit hídrico quando comparadas com plantas obtidas de plantas-mãe que não enfrentaram condições estressantes. As plantas-mãe da cana-de-açúcar foram cultivadas bem-hidratadas e sob condições de casa de vegetação até que um grupo de plantas continuou sob irrigação diária (W) e outro grupo foi submetido a três ciclos de déficit hídrico (D) por restrição hídrica. Em seguida, novas plantas foram produzidas por meio de propagação vegetativa das plantas-mãe que experimentaram ou não ciclos de déficit hídrico. Após a brotação, plantas de 1 mês de idade foram colocadas em solução nutritiva e transferidas para uma câmara de crescimento. O déficit hídrico foi imposto

pela adição de PEG8000 em solução nutritiva em um grupo de plantas, então tivemos plantas D submetidas a um novo déficit hídrico (D/D), ou mantidas bem irrigadas (D/W); plantas bem irrigadas (W) sujeitas a um déficit hídrico (W/D), ou mantidas bem irrigadas (W/W). Quando estas plantas foram expostas à restrição hídrica, houve uma redução nas trocas gasosas independentemente da origem da planta. As plantas originárias de plantas-mãe submetidas a déficit hídrico (D/D) apresentaram uma recuperação mais rápida da assimilação de CO_2 e da eficiência de carboxilação em comparação com plantas W/D. Alguns metabólitos das plantas tiveram uma concentração diferente relacionada com o tratamento das plantas-mãe. O teor de prolina foliar aumentou sob deficiência hídrica, as plantas D/W tiveram maior teor de sacarose foliar do que as W/W. As plantas D/W apresentaram maior concentração de H_2O_2 na raiz e maior atividade na raiz de CAT do que plantas W/W. A sacarose nas folhas e o H_2O_2 nas raízes foram os sinais químicos da memória de estresse transgeracional em cana-de-açúcar sob condições bem irrigadas. Nossos resultados mostram que o crescimento da cana-de-açúcar é melhorado em plantas obtidas de plantas-mãe que tinham enfrentado déficit hídrico. Isso traz uma nova perspectiva para a produção de cana-de-açúcar, favorecendo a expansão do plantio para áreas desfavoráveis, pois a memória transgeneracional de estresse pode melhorar o desempenho da planta em condições de campo devido a um maior sistema radicular e recuperação mais rápida da fotossíntese após déficit hídrico.

Palavras-chave: ciclos de seca e reidratação, EROS, fotossíntese, *Saccharum* spp, clones.

ABSTRACT

Most of the sugarcane cultivation is done without irrigation and in this way the plants are constantly exposed to cycles of drought and rehydration. The effects of drought trigger a series of signaling in plants that induce modifications that can be stored and retrieved as memories by changes in the concentrations of some metabolites or epigenetics. As drought events can induce stress memory, we hypothesized that sugarcane plants previously exposed to cycles of water deficit will perform better than plants that never faced water deficit when both are subjected low water availability. For this, we used the sugarcane (*Saccharum* spp.) *cv*. IACSP94-2094 in two different experiments. In the first one, sugarcane plants were grown in nutrient solution and exposed to one (1WD), two (2WD) or three (3WD) water deficit cycles. As reference (Ref.), plants were grown in nutrient solution without adding polyethyleneglycol (PEG8000). Under water deficit, leaf gas exchange was significantly reduced in 1WD and 2WD plants. However, 3WD plants showed similar CO_2 assimilation and lower stomatal conductance as compared to reference ones, with increases in water use efficiency. Abscisic acid concentration, a drought signal that could lead to stress memory, was higher in 1WD than in 3WD plants. These 3WD plants presented higher root dry matter and root:shoot ratio as compared to reference ones, as well as higher biomass production under well-watered conditions. Our data suggest that sugarcane plants stored and recovered information from previous stressful events, with plant performance being improved under water deficit. The results of the first experiment indicated a new practical perspective for using plant memory to improve the growth of cultivated plants. In the second experiment, we tested the hypothesis that plants obtained from others previously exposed to water deficit will perform better under water deficit as compared to plants obtained from material that did not face stressful conditions. Mother-plants of sugarcane were grown well-hydrated and under greenhouse conditions until one group of plants continued under daily irrigation (W) and another group was subjected to three cycles of water deficit (D) by water withholding. Then, plants were produced through vegetative propagation from those plants that experienced or not cycles of water deficit. After sprouting, 1-month old plants were placed in nutrient solution and transferred to a growth chamber. Water deficit was imposed by adding PEG8000 in nutrient solution in one group of plants, then we had D plants subjected to a new water deficit (D/D), or kept well watered (D/W); well watered plants (W) subjected to a water deficit (W/D), or kept well watered (W/W). When these plants were exposed to water withholding, there was a reduction in gas exchange regardless of the plant origin. Plants

originated from mother-plants that experienced water deficit (D/D) presented a faster recovery of CO_2 assimilation and carboxylation efficiency as compared to W/D plants. Some plant metabolites had a different concentration related to mother-plants treatment, leaf proline content was increased under water deficit, D/W plants had higher leaf sucrose content than W/W ones. As well as D/W plants had higher root H_2O_2 concentration and higher root CAT activity than W/W plants. The sucrose in leaves and H_2O_2 in roots were the chemical signals of these transgenerational stress memory in sugarcane under well-watered conditions. Our findings show that sugarcane growth is improved in plants obtained from mother-plants who had faced water deficit. These results bring a new perspective to sugarcane production by favoring the expansion to unfavorable areas, since transgenerational stress memory can improve plant performance under field conditions due to a large root system and faster recovery of photosynthesis after water deficit.

Key words: drought and recovery cycles, ROS, photosynthesis, *Saccharum* spp, clonal plants.

Sumário

Introdução Geral

Estresses abióticos são as principais causas de perdas agrícolas. Dentre eles, a falta de água impõe grande redução na produtividade e limita a expansão agrícola para áreas menos favoráveis. Nesses ambientes, as plantas estão expostas a ciclos naturais de seca e reidratação ao longo do desenvolvimento. Embora os estudos que abordam os efeitos de eventos únicos de seca sejam bastante comuns, os efeitos de eventos recorrentes de seca são menos abordados e, portanto, ainda de difícil compreensão (Walter et al., 2013).

Em resposta a menor disponibilidade de água, as plantas apresentam estratégias para superar o período estressante. Essas estratégias envolvem alterações morfológicas, fisiológicas, metabólicas e genéticas, permitindo a aclimatação das plantas e as tornando capazes de manter o desenvolvimento durante a situação de estresse (Chaves et al., 2002). As modificações decorrentes da aclimatação podem levar à memória do estresse, caso as alterações persistam mesmo após a ausência do agente estressor (Gagliano et al. 2014). Como consequência, as plantas podem responder de modo mais rápido, aumentando a tolerância em um evento estressante subsequente (Walter et al., 2013). Apesar das vantagens que o armazenamento da informação pode trazer para as plantas, isso tem um custo que poderia não ser tão vantajoso. O aumento da sensibilidade e os efeitos danosos com a diminuição da fotossíntese e do crescimento (Skirycz and Inzé, 2010) seriam desvantagens. As plantas ainda teriam um mecanismo para apagar possíveis modificações e assim evitar os danos citados anteriormente (Crisp et al. 2016). Portanto, entender como as plantas respondem ao estresse e quais mudanças estão associadas com o ganho de performance numa nova situação estressante são questões importantes na compreensão da memória vegetal.

Deficiência hídrica e a fisiologia das plantas

Aos primeiros sinais de mudança na disponibilidade hídrica, ocorre o fechamento estomático para evitar a perda de água pela transpiração foliar (Chaves, 1991). Esse fenômeno está associado à sinalização entre raiz e parte aérea de plantas sob restrição hídrica (Davies & Zhang, 1991), mas também pode ocorrer em função da desidratação foliar ocasionada por baixa umidade na atmosfera. Sinais químicos transportados pelo xilema, como a diminuição da concentração de cátions e ânions, variação do pH e dos teores de aminoácidos e de ácido abscísico (ABA) estabelecem a comunicação entre raízes e folhas em condições de déficit

hídrico. A concentração de ABA no xilema se eleva quando a planta é exposta a estresse hídrico (Wilkinson & Davies, 2002). Uma vez ligado ao seu receptor, o ABA induz uma cascata de transdução de sinal envolvendo o aumento da concentração de cálcio (Ca^{2+}) citoplasmático, o qual pode promover a saída de K^+ e Cl^- das células guardas e assim causar fechamento estomático (Wilkinson & Davies, 2002). Além dos sinais químicos, existe a sinalização hidráulica capaz de induzir o fechamento estomático (Christmann et al., 2007), com a diminuição do potencial da água e do turgor nos tecidos foliares levando à menor abertura estomática. Independente de qual é o primeiro sinal a induzir o fechamento estomático (hidráulico ou químico), esse fenômeno tem grandes consequências para a fisiologia das plantas sob déficit hídrico.

Em relação à fotossíntese, limitações de origem difusiva, bioquímica e fotoquímica são ocasionadas pelo déficit hídrico. Como uma das primeiras respostas a falta de água é o fechamento estomático, há limitação na difusão do CO_2 até os sítios de carboxilação da ribulose-1,5-bisfosfato carboxilase/oxigenase (Rubisco) e consequentemente redução da assimilação de carbono em plantas C3 (Chaves & Oliveira, 2004). Apesar do fechamento estomático apresentar importante contribuição para a diminuição da fotossíntese em plantas C4, o mecanismo de concentração de CO_2 típico desse metabolismo auxilia no suprimento de CO_2 e assim as limitações difusivas são minimizadas. Associada as limitações difusivas no mesofilo, pode ocorrer redução da atividade de enzimas envolvidas com a fixação de CO_2 e nas reações da cadeia transportadora de elétrons e mudanças na anatomia e ultraestrutura das folhas (Ghannoum, 2009). Em cana de açúcar foi observado que as limitações bioquímicas sob déficit hídrico ocorrem principalmente pela redução na velocidade de carboxilação da fosfoenolpiruvato carboxilase (PEPCase) e da Rubisco (Carmo-Silva, et al. 2008; Machado et al., 2013). Limitações fotoquímicas também ocorrem, com diminuição da eficiência operacional do fotossistema II e no transporte de elétrons em cana-de-açúcar sob déficit hídrico (Sales et al., 2013; Sales et al., 2015).

Plantas submetidas à seca apresentam alterações morfológicas para regular o balanço hídrico, tais como diminuição da área foliar e da razão parte aérea/raiz levando à diminuição da transpiração e aumentando a absorção de água pelas raízes (Pimentel, 2004). Em condição de baixa disponibilidade hídrica, a redução de crescimento da parte aérea está muitas vezes associada ao aumento do crescimento radicular como estratégia para melhorar a absorção de água. As plantas investem nesse processo alterando o padrão de alocação de carbono para permitir a formação de um sistema radicular mais profundo antes que a escassez de água

limite o crescimento (Lopes et al., 2011). A diminuição da área foliar pode ser causada pelo enrolamento das folhas e pela diminuição da expansão foliar afetada pelo status hídrico do tecido. Por exemplo, plantas de cana-de-açúcar tem extensão foliar afetada a partir de um potencial da água na folha de −0,4 MPa e reduzida a praticamente zero em −1,3 MPa (Lopes et al., 2011). No entanto, a taxa de expansão foliar é rapidamente recuperada com a reidratação, podendo inclusive superar em crescimento as plantas que não passaram por estresse (Inman-Bamber & Smith, 2005*).*

Solutos como açúcares, glicina-betaina, prolina e compostos fenólicos podem ser acumulados nas células em resposta ao déficit hídrico no solo, funcionando como osmorreguladores e permitindo a manutenção do teor foliar de água e também protegendo as estruturas e reações celulares de danos induzidos pela deficiência hídrica (Verslues et al., 2006). O aumento da produção e da concentração desses solutos está ligado ao aumento da tolerância a estresses abióticos, visto que além do ajuste osmótico os solutos podem atuar na desintoxicação de espécies reativas de oxigênio, estabilização de membranas e das estruturas de enzimas e de proteínas (Chaves & Oliveira, 2004).

A formação de espécies reativas de oxigênio (ERO_S) é aumentada quando a assimilação de CO_2 diminui e a energia que deveria ser utilizada nas reações bioquímicas de fixação de carbono é direcionada para o oxigênio (Ratnayaka et al., 2003). A planta precisa controlar o balanço energético na folha e EROS e o estado de redox dos componentes fotossintéticos regulam a expressão de vários genes ligados à fotossíntese, causando respostas às alterações ambientais a fim de ajustar o suprimento à demanda de energia (Chaves & Oliveira, 2004). Além do sistema antioxidante enzimático composto pelas enzimas catalase, glutationa redutase, glutationa peroxidase, ascorbato peroxidase e superóxido dismutase, as concentrações intracelulares de EROS são controladas pelos ciclos do ascorbato e da glutationa (Mittler, 2002). Essas EROS podem estar associadas à transdução de sinal, atuando como mensageiros secundários em eventos mediados por hormônios, incluindo o fechamento estomático (Foyer & Noctor, 2003). No entanto, além do aumento das concentrações de compostos antioxidantes durante a fase de estresse, estudos mostram que alguns antioxidantes ou seus transcritos (glutationa redutase ou ascorbato peroxidase) podem ter maiores concentrações durante o período de recuperação, isto é, na ausência do agente estressor, do que no período de estresse (Ratnayaka et al., 2003). De fato, em cana de açúcar observou-se que um genótipo com maior tolerância à seca apresenta maior atividade de enzimas

antioxidantes na reidratação, quando comparado à um genótipo sensível à seca (Sales et al., 2013; 2015).

Os teores de açúcares nas folhas também sofrem alterações em condições de seca, ocorrendo diminuição da concentração de amido em estresse moderado e aumento do teor de açúcares solúveis pela hidrólise de amido (Pimentel, 2004). Essas alterações, em quantidade e qualidade, dos carboidratos podem ser um sinal metabólico ligado à resposta ao estresse e algumas delas estimuladas por ABA (Chaves & Oliveira, 2004). Açúcares ainda estão ligados ao controle da expressão de alguns genes relacionados ao estresse, além de protegerem membranas e macromoléculas contra o estresse oxidativo, apresentando grande contribuição no ajuste osmótico.

Dentre as respostas fisiológicas que acontecem durante o período em que as plantas estão sob estresse, algumas, se não a maioria delas, podem ser vantajosas não só durante o evento estressante, mas também em uma futura exposição ao déficit hídrico (Walter et al., 2011). Entre essas respostas, podemos citar mudanças anatômicas nas folhas que causem menor perda de água sem comprometer a interceptação de energia luminosa, fechamento estomático mais rápido ou mesmo controle estomático mais eficiente da perda de água e melhoria na eficiência do uso da água (EUA), maior desenvolvimento radicular, proteção contra estresse oxidativo pela maior atividade dos sistemas antioxidantes enzimáticos e não-enzimáticos e aumento no teor de moléculas osmoprotetoras. Essas alterações devem ser coordenadas e têm um importante papel na memória ao estresse.

Memória ao estresse hídrico

O conceito de memória implica em reações à sinalização gerada por estímulos ambientais, muitas das vezes estresses ambientais, que provocam um armazenamento da informação, que pode ser recuperada em um novo evento estressante (Trewavas, 2003; Thellier & Lüttge, 2012). O déficit hídrico gera várias respostas fisiológicas e estímulos bioquímicos que são sinalizadores vegetais extremamente importantes para que a planta enfrente a baixa disponibilidade hídrica e possa se recuperar. Essa sinalização induz a produção de fatores de transcrição que podem explicar alterações metabólicas e de expressão gênica, enquanto as mudanças epigenéticas são a mais provável forma de armazenamento da informação, deixando as plantas em estado permissivo e facilitando respostas mais rápidas e potentes (Bruce et al., 2007).

Estudos nos quais as plantas são submetidas a estresses recorrentes fornecem evidências interessantes para a abordagem fisiológica da memória vegetal. Plantas de *Cistus albidus* apresentaram conteúdo relativo de água (CRA) nas folhas superior no segundo ciclo de seca, em função de um possível ajuste osmótico após serem expostas a um ciclo de estresse hídrico e recuperação. Além disso, a recuperação da fotossíntese e a eficiência do uso da água (EUA) foram melhoradas pela exposição prévia à seca. Essas respostas foram atribuídas à menor condutância estomática e manutenção da condutância do mesofilo no segundo ciclo de déficit hídrico (Galle et al., 2011), sugerindo que plantas submetidas à seca teriam uma marca do estresse que garantiria melhor performance em eventos recorrentes. Numa condição de déficit hídrico, plantas de trevo que foram previamente submetidas a dois ciclos de estresse/recuperação apresentaram manutenção do status hídrico, evidenciado por maior potencial da água no xilema e maior CRA do que as plantas que foram submetidas à apenas um ciclo de estresse, sugerindo a ocorrência de memória (Iannucci et al., 2000). Já Villar-Salvador et al. (2004) concluíram que mudas de *Quercus ilex* depois de passar por rustificação à seca, aumentam a tolerância por redução no potencial da água na folha e na transpiração, apresentando também ajuste osmótico.

Muitos genes estão ligados à resposta a estresses abióticos, alguns deles dependentes da ação do ABA, ou de outros hormônios, ou ainda de outras moléculas como cálcio, ácido jasmônico e ácido salicílico (Conrath et al., 2009). O acúmulo de fatores de transcrição em plantas pode ser responsável pelo aumento da transcrição gênica levando à memória ao estresse (Bruce et al., 2007). EROS, além do seu papel sinalizador (Foyer & Noctor, 2005), podem estar ligadas a modificações no padrão de metilação do DNA (Peng & Zang, 2009), sendo essa uma forma de armazenar a informação de um evento estressante. O controle epigenético envolve mudanças na ativação de genes por metilação e acetilação do DNA, modificação das histonas e remodulação da cromatina, que permanecem mesmo após o fim do período de estresse (Allis et al., 2007; Bruce et al., 2007; Hauser et al., 2011). Modificações epigenéticas são induzidas por sinalização hormonal e bioquímica em resposta ao estresse e tais alterações na cromatina auxiliariam na aclimatação das plantas à um novo evento estressante. As mudanças epigenéticas podem levar à memória na geração exposta ao estresse, assim como na geração futura, sendo uma memória transgeracional nesse último caso (Chinnusamy & Zhu, 2009), um aspecto pouco estudado até o momento.

No presente trabalho os experimentos propostos visam avaliar a memória de plantas de cana-de-açúcar a ciclos recorrentes de déficit hídrico/recuperação, utilizando um desenho

experimental que possibilitasse comparar respostas fisiológicas de plantas com a mesma idade, mas com um histórico de vida distinto. No segundo experimento, foi testada a hipótese de memória transgeracional à seca em cana-de-açúcar, com a imposição de ciclos de déficit hídrico/recuperação em plantas-filha advindas de plantas-mãe que passaram ou não por ciclos de déficit hídrico. Como modelo experimental, utilizou-se a cana-de-açúcar, uma planta cultivada, de grande interesse econômico e estratégico para o país.

Hipóteses

Este estudo possui duas hipóteses sobre a memória em plantas. Nesta tese, chamamos de memória à seca quando as plantas têm melhor desempenho quando expostas repetidamente a ciclos de desidratação e reidratação. As hipóteses são: (i) em condição de déficit hídrico, plantas de cana-de-açúcar submetidas previamente a ciclos de déficit hídrico/recuperação terão melhor desempenho do que plantas mantidas sempre bem hidratadas; e (ii) mudas provenientes de plantas-mãe submetidas a ciclos de déficit hídrico/recuperação terão melhor desempenho sob déficit hídrico do que mudas provenientes de plantas-mãe sempre irrigadas. Portanto, o objetivo foi testar as hipóteses citadas usando a variedade de cana-de-açúcar IACSP94-2094 e avaliando alterações morfológicas, fisiológicas e bioquímicas induzidas pelo déficit hídrico.

Chapter I - Stress memory in sugarcane: drought tolerance is improved by previous exposure to water deficit

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Abstract

Under field conditions, plants are exposed to cycles of dehydration and rehydration during their life spam. As drought events can induce stress memory, we hypothesized that sugarcane plants previously exposed to cycles of water deficit will perform better than plants that never faced water deficit when both are subjected low water availability. Sugarcane plants were grown in nutrient solution and exposed to one (1WD), two (2WD) or three (3WD) cycles of water deficit. As reference (REF), plants were grown in nutrient solution without adding polyethyleneglycol. Under water deficit, leaf gas exchange was significantly reduced in 1WD and 2WD plants. However, 3WD plants showed similar CO_2 assimilation and lower stomatal conductance as compared to reference ones, with increases in intrinsic water use efficiency. Abscisic acid concentration, a drought signal that could lead to stress memory, was higher in 1WD than in 3WD plants. Alternatively, we propose root H_2O_2 concentration as an important chemical signal, with the highest values being found in 3WD plants. These plants presented higher root dry matter and root:shoot ratio as compared to reference ones, as well as higher biomass production under well-watered conditions. Our data suggest that sugarcane plants stored and recovered information from previous stressful events, with plant performance being improved under water deficit. In addition, our findings open a new perspective for using stress memory to improve drought tolerance.

Keywords: drought, recovery, ROS, photosynthesis, *Saccharum*

Introduction

In general, plants close stomata to avoid water lost by transpiration under water limiting conditions (Chaves, 1991), being such physiological response related to either hydraulic or chemical signals (Davies & Zhang, 1991; Christmann et al., 2007). Rapid stomatal response to changes in water availability is an important feature in sugarcane (*Saccharum* spp.), preventing excessive loss of leaf turgor and further decreases in leaf water content (Ribeiro et al., 2013). Plant acclimation to water deficit also involves morphological changes as a way to regulate water balance, with plants showing decreases in leaf area and shoot/root ratio (Pimentel, 2004). Cell osmoregulation by solutes such as sugars, glycine-betaine, and proline is another response to water deficit, allowing the maintenance of water content and protecting cellular structures (Verslues et al., 2006). In addition, it is well known that stomatal closure causes low CO_2 availability for photosynthetic enzymes (Du et al., 1996; Chaves et al., 2009; Machado et al., 2013) and then an imbalance between photochemical and biochemical reactions takes place in leaves. As consequence, production of reactive oxygen species (ROS) is enhanced under drought and plants should be able to control such deleterious molecules through the antioxidant system. Such protective system consists of several enzymatic and non-enzymatic compounds, which prevent oxidative damage by scavenging ROS inside cells (Mittler, 2002). For instance, increases in superoxide dismutase and ascorbate peroxidase activities were associated with rapid recovery of leaf gas exchange in sugarcane plants after rehydration (Sales et al., 2013).

All those reported plant responses to a single drought event are quite common; however, plants are exposed to recurrent cycles of drought and rehydration in nature and consequences of such repetitive drought events are less understood (Walter et al., 2013). Plants are able to acclimate to varying water conditions through morphological and physiological changes, which would favor the maintenance of plant growth or survival under stressful conditions (Chaves et al., 2002). Some changes during acclimation period can lead to stress memory, allowing a faster response and increasing plant performance within the next stress event. Ecological stress memory requires not only improvement in plant response to stress but also persistence of acclimation mechanisms after a recovery period, when plants would repair stress-induced damage, recover photoassimilate production and resume assimilatory pathways (Walter et al., 2013). Trewavas (2003) defines plant memory as an ability to access past experience so that new responses incorporate relevant information from the past and plants could retrieve such information at a much later time. Herein, we used this

concept of plant memory to understand how plant performance under water deficit can be improved by previous exposure to drought events.

In fact, an experimental design with repeated cycles of drought is a more realistic approach when considering plants in their natural environment, with improved plant performance under limiting conditions being found in several species by previous exposure to stressful conditions. While *Trifolium alexandrinum* was able to maintain high leaf water potential and relative water content after a second drought event (Iannucci et al., 2000), *Quercus ilex* exhibited reductions in leaf water potential and transpiration accompanied by osmotic adjustment after hardening (Villar-Salvador et al., 2004). Seedlings of *Moringa oleifera* previously subjected to osmotic stress had increases in drought tolerance, with plants showing higher water use efficiency, higher photosynthesis and increases in activity of antioxidant enzymes under water deficit conditions (Rivas et al., 2013). However, most of those studies compared plants differing in age under varying stress intensity and environmental conditions, which turns difficult the study of stress memory.

As a semi-perennial crop grown in rainfed areas, sugarcane may experience seasonal variation of water availability and also unexpected dry periods. In addition, new areas cultivated with sugarcane are located in marginal regions, where water availability is an important issue (MAPA, 2009; Smith et al., 2009). In this study, we used a fine experimental design to understand how sugarcane performance under water limiting conditions is affected by previous exposure to water deficit. We hypothesized that sugarcane plants subjected to previous drought will exhibit improved performance under water deficit, which would be achieved by an imprinted memory of drought stress on sugarcane physiology, biochemistry and morphology.

Material and Methods

Plant material and growth conditions

Drought-tolerant sugarcane (*Saccharum* spp.) variety IACSP94-2094 (Machado et al., 2009; Ribeiro et al., 2013) was used in this study. Plants were propagated using mini-stalks (with one bud) obtained from adult plants, which were planted in trays containing commercial substrate composed by composed of sphagnum peat, expanded vermiculite, limestone dolomite, agricultural gypsum and NPK fertilizer - traces (Carolina Soil of Brazil, Vera Cruz

RS, Brazil). Thirty-five days after planting (DAP), plants were moved to plastic boxes (12 L) containing modified Sarruge (1975) nutrient solution (15 mmol L^{-1} N (7% as NH_4^+); 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 µmol L^{-1} B; 54.0 µmol L^{-1} Fe; 5.5 µmol L^{-1} Mn; 2.1 µmol L^{-1} Zn; 1.1 µmol L^{-1} Cu and 0.01 µmol L^{-1} Mo). To avoid osmotic shock, we diluted the nutrient solution and the initial ionic force was 25%. Then, ionic force was increased to 50% in the second week and to 100% in the following week. The electrical conductivity of nutrient solution was monitored with a conductivimeter (Tec-4MPp, Tecnopon, Piracicaba SP, Brazil) and maintained around 1.5 mS cm^{-1} by replacing the solution once a week. The pH of nutrient solution was 5.4±0.6 and it was monitored with a pHmeter (Tec-3MPp, Tecnopon, Piracicaba SP, Brazil). The osmotic potential of nutrient solution was measured with a C-52 chamber (Wescor Inc, Logan UT, USA) attached to a microvoltmeter HR-33T (Wescor Inc, Logan UT, USA). Nutrient solution with 100% ionic force presented an osmotic potential of −0.12 MPa. After moving to nutrient solution, plants were placed in a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) under 30/20°C (day/night), 80% air relative humidity, 12 h photoperiod (7:00 to 19:00 h) and photosynthetic photon flux density (PPFD) of 800 µmol m^{-2} s^{-1}.

Water deficit treatments

Fifty-five days-old plants were subjected to water deficit cycles by adding polyethylene glycol (Carbowax™ PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential, as follows: -0.27 MPa in the first day and -0.56 MPa in the second day. These values were based in previously experiments with sugarcane (Silveira et al., 2016; 2017). Afterwards, the osmotic potential of -0.56 MPa was maintained by replacing the solution by a new one with the same amount of PEG-8000.

Four groups of plants were formed according to the exposure to water deficit: plants grown under well-watered conditions, i.e. non-exposed to water deficit (Reference); plants that faced water deficit once (1WD); plants that faced water deficit twice (2WD); and plants that faced water deficit thrice (3WD). All the three water deficit cycles were similar in intensity and duration and plants had the same age at the end of the experiment, as shown in Fig. 1. Each water deficit cycle was composed by five days in nutrient solution with -0.56 MPa and other three days of recovery in nutrient solution with -0.12 MPa. During the

experimental phase (Fig. 1), five plants of each treatment were collected at midday, leaves and roots were immediately frozen in liquid nitrogen, and then this material was stored at -80°C for further analyses. Such procedure was done in the fifth day of water deficit, i.e., the maximum water deficit.

Leaf gas exchange and photochemistry

Leaf gas exchange and photochemistry were measured daily with an infrared gas analyzer (LI-6400, LICOR, Lincoln NE, USA) coupled to a modulated fluorometer (6400-40 LCF, LICOR, Lincoln NE, USA) along all the experimental period. The measurements were performed between 10:00 and 13:00 h under PPFD of 2,000 μmol m^{-2} s^{-1} and air CO_2 concentration of 380 μmol mol^{-1}. We measured leaf CO_2 assimilation (A), stomatal conductance (g_S), intercellular CO_2 concentration (C_i) and transpiration (E), with the intrinsic water use efficiency (A/g_S) and the instantaneous carboxylation efficiency ($k = A/C_i$) being calculated according to Machado et al. (2009). The chlorophyll fluorescence was measured simultaneously to leaf gas exchange and the apparent electron transport rate estimated as $ETR = \phi_{PSII} \times PPFD \times 0.85 \times 0.4$, in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light absorption and 0.4 is the fraction of light energy partitioned to PSII in C4 plants (Edwards and Baker, 1993; Baker, 2008). The A and E values were integrated during the experimental period to estimate the total CO_2 gain (A_i), the total water vapor loss (E_i), and the water use efficiency (A_i/Ei) in each treatment. The integrated values were estimated assuming that the values measured between 10:00 and 13:00 h were constant during the 12 hours of photoperiod. In the experimental phase (Fig. 1), the relative recovery of A and g_S after rehydration was evaluated daily, considering values reference plants as 100% (Fig. 3).

Leaf water potential and relative water content

In the experimental phase, pre-dawn leaf water potential (ψ) was evaluated with a pressure chamber model 3005 (Soilmoisture Equipment Corp., Santa Barbara CA, USA). The leaf relative water content (RWC) was calculated using the fresh (FW), turgid (TW) and dry (DW) weight of leaf discs according to Weatherley (1950): $RWC=100\times[(FW-DW)/(TW-DW)]$. Both variables were measured at the fifth day of water deficit, and at the third day of recovery.

Carbohydrates and proline

In leaf and root samples, the extraction of total soluble carbohydrates (SS) was done with a methanol:chloroform:water solution (Bieleski and Turner, 1966) and quantified by the phenol–sulfuric acid method (Dubois et al., 1956). Sucrose (Suc) content was quantified according to van Handel (1968), whereas starch (Sta) content was evaluated by the enzymatic method proposed by Amaral et al. (2007). The concentration of nonstructural carbohydrates (NSC) was calculated as NSC=SS+Sta, as done by Ribeiro et al. (2012). Leaf proline content was determined in test tubes by the reaction with the sample, ninhydrin reagent (ninhydrin, acetic acid and orthophosphoric acid) glycine and acetic acid for 35 minutes at 100°C, and the reaction terminates in an ice bath. The reaction mixture was extracted with toluene and the proline concentration was determined from a standard curve. (Rena and Masciotti, 1976).

Hydrogen peroxide and lipid peroxidation

The hydrogen peroxide (H_2O_2) content in leaves and roots was quantified in 0.16 g fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v) (Alexieva et al., 2001). The extract was centrifuged at 12,000 *g*, 4°C for 15 min. The crude extract was added in the reaction medium (1.2 mL of KI 1 mol L^{-1}, potassium phosphate buffer pH 7.5 at 0.1 mol L^{-1}) and microtubes were incubated on ice under dark for 1 h. After this period, the absorbance was read at 390 nm. The calibration curve was done with H_2O_2 and the results expressed as µmol H_2O_2 g^{-1} FW. The malondialdehyde (MDA) concentration in leaf and root samples was measured and used as a parameter to evaluate lipid peroxidation. Plant tissue (0.16 g) was macerated in 1.5 mL of 0.1% trichloroacetic acid (TCA) (w/v) and centrifuged at 10,000 *g* for 15 min. One aliquot of 0.5 mL of the supernatant was incubated with 0.5% thiobarbituric acid solution in water bath at 90°C for 20 min (Cakmak and Horst, 1991). After 30 min at room temperature, the sample absorbance was read at 532 and 600 nm and the non-specific absorbance at 600 nm discounted. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968) and results were expressed as nmol MDA g^{-1} FW.

Antioxidant enzymes: extraction and activity assays

The enzymatic extract was prepared with 0.2 g of fresh tissue (leaf or root) grounded in liquid nitrogen, with 1% of PVPP and 2 mL of extraction medium composed by 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 0.1 mmol L^{-1} ethylenediaminetetraacetic (EDTA) and 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF). This homogenate was centrifuged at 15.000 *g* for 15 min and 4°C, and the supernatant was collected and preserved on ice.

The analysis of superoxide dismutase (SOD, EC 1.15.1.1) activity was done in a reaction medium with 3 mL of 100 mmol L^{-1} sodium phosphate buffer (pH 7.8), 50 mmol L^{-1} methionine, 5 mmol L^{-1} EDTA, deionized water, crude extract, 100 µmol L^{-1} riboflavin and 1 mmol L^{-1} nitro-blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp of 30 W) for 15 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the amount of enzyme required to inhibit the NBT photoreduction in 50%, being expressed as U min^{-1} mg^{-1} of protein (Giannopolitis and Ries, 1977). Catalase (CAT, EC 1.11.1.6) activity was quantified with a reaction medium of 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.8), deionized water, 125 mmol L^{-1} H_2O_2 and crude extract. The decrease in absorbance at 240 nm was measure to determinate the enzyme activity. We used a molar extinction coefficient of 36 M^{-1} cm^{-1} and CAT activity was expressed as nmol g^{-1}FW min^{-1} (Havir and McHale, 1987). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated within 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.0), deionized water, 10 mmol L^{-1} ascorbic acid, 10 mmol L^{-1} H_2O_2 and crude extract. The decrease in absorbance at 290 nm was measured and a molar extinction coefficient of 2.8 M^{-1} cm^{-1} was used. APX activity was expressed as µmol g^{-1} FW min^{-1} (Nakano and Asada, 1981).

Abscisic acid (ABA) and its metabolites

Fresh leaf tissue samples were grounded using liquid nitrogen, weighted (200 mg) and placed in capped plastic tube. They were extracted with 1.0 mL of methanol:water:acetic acid (10:89:1 v/v) overnight on a shaker at 4ºC under darkness (Silva et al., 2012). After that, samples were centrifuged for 10 min at 12,000 *g* and the supernatant was dried in N_2 stream. The assay was ressuspended in 200 µL of methanol prior analysis. Chromatographic analysis was carried out using a chromatographer UPLC Acquity (Waters, Milford CT, USA) coupled with a TQD mass spectrometer (Micromass-Waters, Manchester, UK) and ESI source. We

used a Waters Acquity column BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm) equipped with a VanGuard pre-column BEH C18 (5 mm × 2.1 mm i.d., 1.7 µm) both kept at 30ºC and full loop precision (10.0 µL) of injection volume. Milli-Q purified water with 0.1% (v/v) of formic acid (A) and acetonitrile (B) were used as solvents. The gradient started with 75% A changing to 65% in 6 min, then ramping to 0% A in 8 min and returning to the initial conditions for re-equilibration until 10 min, at constant flow rate of 0.2 mL min^{-1}. Source and desolvation temperature were set to 150°C and 350°C, respectively. Mass spectra of ABA and its derivatives were acquired by ESI ionization in the negative ion mode using Selected Reaction Monitoring (SRM) and individually optimized using Intellistart Waters software. In total, four compounds were evaluated: phaseic acid (PA), dihydrophaseic acid (DPA), abscisic acid (ABA) and its glucose conjugated form as ABA-β-D-glucosyl ester (ABA-GE).

Biometry

Shoot and root dry matter were evaluated after drying samples in a forced air oven at 65°C. The root/shoot ratio was evaluated. We also calculated the growth in each treatment, using the total dry matter divided by the number of days in which plants remained under well-watered conditions (i.e., nutrient solution with osmotic potential of –0.12 MPa). Biometric evaluations were done at the end of the experimental period.

Statistical analysis

The experimental design was in randomized blocks and the cause of variation was the previous exposure to water deficit, with four levels (Ref, 1WD, 2WD, 3WD). Data were subjected to ANOVA procedure and the mean values (n=4-5) were compared by the Tukey test ($P<0.05$) when significance was detected.

Results

Leaf gas exchange and photochemistry

Leaf gas exchange was evaluated every day along 28 days, including both preparatory and experimental phases. Water deficit caused reductions in leaf CO_2 assimilation, however

plants subjected to the third cycle (3WD) of water deficit had photosynthetic rates similar to those ones of reference plants, which did not experience any drought event (Supplementary Material Fig. S1). When considering the experimental phase, plants subjected to three cycles of water deficit showed photosynthesis similar to one of reference plants after five days of water deficit (Fig. 2A). However, stomatal conductance in plants that experienced three cycles of water deficit was lower than in reference plants and higher than in plants exposed to one or two cycles of water deficit (Fig. 2B). Plants exposed to three cycles of water deficit also showed carboxylation efficiency similar to one of reference plants and higher than ones in 1WD and 2WD treatments (Fig. 2C). Regarding the photochemistry, the apparent electron transport rate was similar between reference plants and those ones subjected to three cycles of water deficit (Fig. 2D). The instrinsic water use efficiency increased as the number of drought events increased, with plants exposed to three cycles of water deficit showing higher A/g_S than reference ones (Fig. 2E). The ratio between the apparent electron transport rate and CO_2 assimilation revealed no differences among treatments, varying between 5.3 (1WD plants) and 4.0 (3WD plants) μmol μmol^{-1}.

Recovery of photosynthesis was also improved by previous exposure to water deficit, with photosynthesis of plants exposed to three cycles of water deficit exceeding the values found in reference plants by 35% at the first day of recovery (Fig. 3A). While plants subjected to two cycles of water deficit also reached full recovery of photosynthesis at the first day of rehydration, plants facing water deficit for the first time showed complete recovery of photosynthesis only after the third day of rehydration (Fig. 3A). Only plants subjected to three cycles of water deficit presented stomatal conductance similar to one found in reference plants during the two first days of rehydration (Fig. 3B).

By integrating *A* and *E* during the experimental phase, we verified that plants exposed to three cycles of water deficit had A_i higher than reference plants and also higher than in plants exposed to one or two cycles of water deficit (Supplementary Material Fig. S2A). E_i was also affected by water deficit, increasing with the occurrence of drought events (Supplementary Material Fig. S2B). As a result of changes in A_i and E_i, plants exposed to three cycles of water deficit had higher integrated water use efficiency than reference plants (Supplementary Material Fig. S2C).

Leaf water status

While the leaf relative water content was not affected by water regimes (Fig. 4A), the leaf water potential was reduced by water deficit (Fig. 4B). During the recovery period, there were no differences among treatments for both variables (Fig. 4).

ABA and its derivatives

Reference plants presented very low concentrations of ABA-GE and DPA and we were not able to detect ABA and PA under well-watered conditions (Fig. 5). In general, plants exposed to water deficit had higher levels of ABA, ABA-GE, PA, and DPA than in references ones (Fig. 5). However, plants exposed to two or three cycles of water deficit exhibited lower concentration of ABA and DPA as compared to plants facing water deficit for the first time (Fig. 5A, D).

Leaf and root carbohydrates and proline

Leaf sucrose concentration was increased by drought, with plants exposed to one, two or three cycles of water deficit showing higher values than reference plants (Fig. 6A). On the other hand, root sucrose concentration was decreased by water deficit and the lowest concentrations were found in plants exposed to two cycles of water deficit (Fig. 6A). Plants exposed to one cycle of water deficit also presented higher leaf content of soluble sugars than reference plants (Fig. 6B). Only plants exposed to two cycles of water deficit presented reduction in root soluble sugars concentration (Fig. 6B). Leaf starch concentration was not changed by treatments; however, plants exposed to one cycle of water deficit showed higher root starch concentration than plants exposed to two or three cycles of water deficit (Fig. 6C). Leaf concentration of non-structural carbohydrates was increased by drought only in plants exposed to one or two cycles of water deficit, whereas only plants exposed to two or three cycles of water deficit presented reduction in root concentration of non-structural carbohydrates (Fig. 6D). Leaf proline concentration was increased by water deficit and the highest values were found in plants exposed to one cycle of water deficit (Fig. 6E). In roots, proline concentration was reduced only in plants exposed to two cycles of water deficit (Fig. 6E).

Antioxidant activity

Leaf H_2O_2 concentration was not affected by treatments, while plants exposed to three cycles of water deficit presented higher root H_2O_2 concentration than reference plants (Fig. 7A). Leaf MDA concentration was reduced by drought only in plants exposed to two cycles of water deficit, whereas root MDA concentration followed the same pattern of root H_2O_2 concentration, i.e., highest values were found in plants exposed to three cycles of water deficit (Fig. 7B). Leaf SOD activity was increased in plants exposed to two cycles of water deficit, whereas leaf APX activity was highest in plants facing water deficit for the first time (Fig. 7C,E). The identification of leaf SOD isoforms was performed and Mn-SOD, Fe-SOD, Cu/Zn-SOD were found in all treatments (data not shown) Root SOD activity was not affected by treatments and root APX activity was increased in plants exposed to one or two cycles of water deficit (Fig. 7C,E). Leaf CAT activity was not changed by treatments (Fig. 7D). Regarding roots, there was a significant increase in CAT activity and the highest values were found in plants exposed to three cycles of water deficit, being 9 times higher than in reference plants (Fig. 7D).

Plant biomass and growth

At the end of the experimental phase, shoot dry matter was similar in Ref, 2WD, and 3WD, with 1WD plants showing higher shoot dry matter (Fig. 8A). Root dry matter and root:shoot ratio were increased in plants exposed to three cycles of water deficit as compared to reference ones (Fig. 8B,C). By considering the time under well-watered conditions (i.e., 28, 22, 16 and 10 days for reference, 1WD, 2WD and 3WD plants respectively), we were able to estimate the growth given by the total biomass production per day (g/day with water). Our data revealed that plants exposed to three cycles of water deficit presented the highest growth efficiency (Fig. 9), i.e., they were more efficient in using water resources.

Discussion

Sugarcane photosynthesis is benefited by repetitive cycles of drought/rehydration

Photosynthetic rates of plants exposed to three cycles of water deficit were similar to ones found in reference plants (Fig. 2A) and even higher when considering integrated CO_2 gain during the experimental period (Supplemental Material Fig. S2A). In fact, such better performance was also caused by higher photosynthetic rates during the recovery period (Fig. 3A). Under water deficit, both photosynthesis and stomatal conductance of plants exposed to three cycles of water deficit were higher than ones found in plants subjected to one or two cycles (Fig. 2A, B), suggesting that stomatal aperture was one factor leading to the better photosynthetic performance of 3WD plants under low water availability.

Higher stomatal conductance of 3WD plants was associated with increases in root biomass (Fig. 8B), likely improving water uptake from nutrient solution. In addition, 3WD plants had higher root to shoot ratio (Fig. 8C), which indicates changes in carbon partitioning and investment in root structures. Besides those morphological changes supporting higher stomatal aperture, our data suggest that ABA has a role in stomatal conductance of sugarcane under water deficit. We noticed that 3WD plants had lower leaf ABA concentration than 1WD plants (Fig. 5A), as well as lower amount of DPA, a product of ABA oxidation (Fig. 5D). Thus, we could argue that the amount of DPA was higher in 1WD plants due to a large amount of ABA produced and oxidized (Fig. 5). According to Virlouvet and Fromm (2015), plants previously exposed to drought would have low stomatal conductance caused by ABA biosynthesis. Such association between ABA and stomatal conductance was found herein, with the lowest g_s values being found in plants with the highest concentrations of ABA and its derivatives (Fig. 5). Interestingly, sugarcane plants subjected to three cycles of water deficit did not present such high levels of ABA and DPA, which are likely consequence of better hydration and/or changes in ABA metabolism caused by repetitive cycles of water deficit.

Besides ABA action being related to transcriptional changes induced by stress-responsive genes (Ding et al., 2012), this hormone can also promote the production of protective osmolytes that maintain the membrane structure (Verslues et al., 2006), including the protection of the photosynthetic apparatus (Fleta-Soriano et al., 2015). Evidence of osmoregulation is given by the maintenance of RWC with reduction of leaf water potential in plants under stress (Fig. 4). Increases in leaf concentration of sucrose and proline, two

important osmolytes, were found in plants subjected to water deficit (Fig. 6A,E). Osmoprotective molecules such as proline ensure preservation of protein structure and function under low water availability (Wingler, 2002; Verslues et al., 2006). However, the number of times that plants were exposed to water deficit cycles did not affect differentially this proposed osmoregulation. The reduction on proline concentration may also indicate that 3WD plants were less stressed than the others subjected to drought, as proline is produced under stressful conditions (Szabados and Savoure, 2010).

Higher instantaneous carboxylation efficiency in 3WD plants suggests improvements in C4 photosynthetic enzymes, another factor leading to improved photosynthesis in those plants (Fig. 2A, C). Alternatively, photosynthesis could be stimulated by root growth (Fig. 8B), an active sink for photoassimilates in 3WD plants. In fact, we have found previously that sugarcane photosynthesis is very sensitive to changes in source-sink relationship (Ribeiro et al., 2017). Higher photosynthesis consumes more NADPH and ATP and then can stimulate photochemical activity and cause higher ETR (Fig. 2D). As leaf CO_2 assimilation decreases due to water deficit in 1WD and 2WD plants and light energy reaching leaves remains similar, plants face excess of energy and such conditions would lead to accumulation of ROS and consequent oxidative stress (Foyer and Shigeoka, 2011).

Antioxidant metabolism in leaves and roots as affected by cycles of water deficit

The enzymatic antioxidant metabolism is one of the mechanisms that plants have to avoid oxidative damage induced by ROS in cell structure and functioning. Herein, leaf H_2O_2 and MDA concentrations did not suggest any oxidative damage due to water deficit (Fig. 7B). Changes in SOD and APX activities were likely able to maintain the redox state in leaves of plants under water deficit (Fig. 7C,E). One important point is that the maintenance of photosynthetic rates in 3WD plants lead to less excess of energy, being the main sink of excitation energy at chloroplast level. Interestingly, roots of 3WD plants presented the highest concentrations of H_2O_2 and MDA as well as the highest CAT activity among treatments (Fig. 7A, B, D). Such findings suggest a controlled increase in H_2O_2 level as CAT activity, an important enzyme involved in its degradation, was increased. As root elongates, H_2O_2 is produced and MDA concentration increased (Hu et al., 2015), with increases in both variables in 3WD plants indicating higher cell membranes renovation. In fact, H_2O_2 may be an oxidant and also a secondary messenger in signal transduction due to its long half-life and relatively

high permeability in membranes (Cheeseman et al., 2005; Silva et al., 2015). The MDA and H_2O_2 concentrations in WD plants are similar to previous papers with sugarcane under water deficit (Marchiori, 2014; Silveira et al., 2017) indicating that the plants in this work were not under intense stress, even with 3WD, suggesting a good antioxidant metabolic control.

Stress memory in sugarcane and its implication for plant development and crop production

The concept of memory may be controversial but here it is associated with the plant capacity of storage and recall information. Herein, we used memory to emphasize that stress causes an imprint in plant that is stored and let it in a more permissive state, improving plant response to a stressful event (Bruce et al., 2007; Walter et al., 2013). Stored information regulates plant responses to environmental changes over time (Thellier and Lüttge, 2013), with plants showing stress signals. Among those signals, ABA (Ding et al., 2012; Fleta-Soriano et al., 2015), ROS (Foyer and Shigeoka, 2011) and electrical signaling (Brenner et al., 2006) can lead to improved performance and to epigenetic modifications. Although leaf ABA accumulation has been found in sugarcane plants subjected to three cycles of water deficit, it affected only the intrinsic water use efficiency through reduced stomatal conductance (Figs. 5A and 2B, E). Indirectly, one would expect benefits of such reduction in stomatal conductance for canopy photosynthesis as shoot water balance is improved and leaf turgor is likely maintained. Regarding another signal, we noticed a large and controlled increase in root H_2O_2 concentration with increasing plant exposure to water deficit (Fig. 7A), without any oxidative damage and with plants showing increasing root dry matter, root/shoot ratio and growth efficiency (Figs. 8 and 9). Our data revealed that improvements in plant performance under water deficit caused by previous exposure to drought were associated with biochemical signals, indicating stress memory. An essential issue is the evaluation of plant performance and physiological status after stressful events (Walter et al., 2011). As plants exposed to three cycles of water deficit presented higher growth efficiency and photosynthetic performance than ones exposed to one or two cycles of water deficit, we may argue that sugarcane plants stored the information and used it for their benefit.

Epigenetics plays an important role in storing information and helping plants to adjust their metabolism to environmental fluctuations. Epigenetics regulation may occur through DNA methylation and histone modifications (Chinnusamy and Zhu, 2009), changing chromatin structure and function (Grafi and Ohad, 2013). These epigenetic modifications lead

to an up or down regulation of gene expression due to changes in chromatin condensation in histones protein and also in gene transcription (van Zanten et al., 2013). Interestingly, environmental stimuli may induce epigenetic changes and improve plant acclimation under biotic or abiotic stresses (Hauser et al., 2011). As plants are able to store environmental cues through epigenetics, stress memory is a way of improving plant performance under varying environmental conditions. Herein, we have reasonable evidence to suggest stress memory in sugarcane plants. In fact, plants exposed to three cycles of water deficit exhibited morphological and physiological changes associated with increases in both photosynthesis and growth efficiency (Figs. 2, 8 and 9). From a practical point of view, our data indicate that sugarcane tolerance to water deficit may be improved under husbandry conditions while saving water and electrical energy through less frequent irrigation. Another interesting venue to be explored is the trangenerational stress memory (Hauser et al., 2011), as sugarcane is propagated vegetatively. Future studies should complement our findings and explain the epigenetic and molecular nature of stress memory in sugarcane.

Differential physiological responses when comparing plants previously exposed or not to cycles of water deficit are clear evidence that plants can access the stored information of past events and use them to improve growth. Our findings also revealed a new practical perspective for the use of the plant memory mechanism to induce drought tolerance and save water in agriculture. Concluding, we found that sugarcane plants are able to incorporate information from previous stressful events for improving photosynthesis, water use efficiency and growth efficiency under water deficit. As a chemical signal, our data revealed the controlled accumulation of H_2O_2 in roots, which was associated with increases in root growth.

Acknowledgments

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Figures

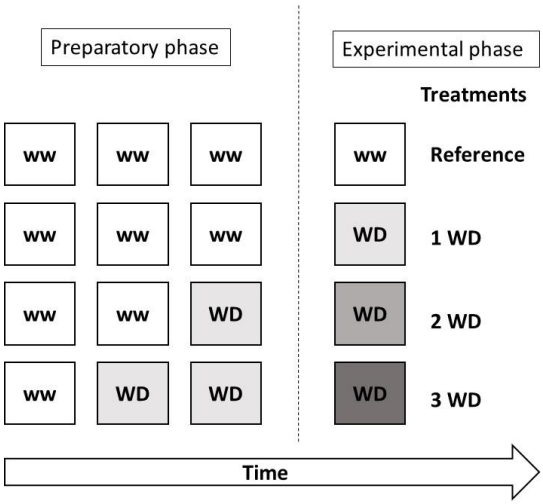

Fig. 1. Experimental strategy for evaluating stress memory in sugarcane plants. ww means well watered conditions and WD means water deficit cycle. Each water deficit cycle lasted eight days (five days under water deficit; and three days of recovery). In the experimental phase, reference indicates plants maintained always well-watered, whereas 1WD, 2WD and 3WD mean plants subjected to one, two and three cycles of water deficit, respectively.

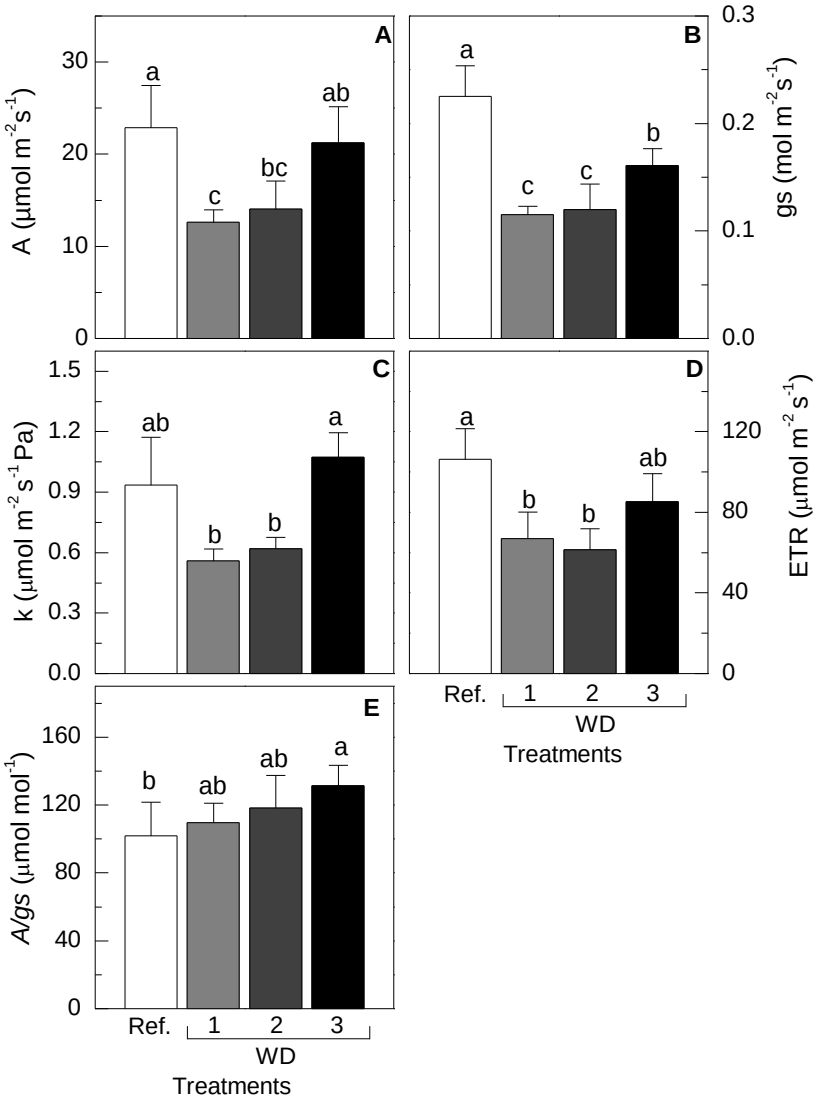

Fig. 2. Leaf CO_2 assimilation (A), stomatal conductance (B), instantaneous carboxylation efficiency (C), apparent electron transport rate (D) and *A/gs* (E) at the maximum water deficit in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments.

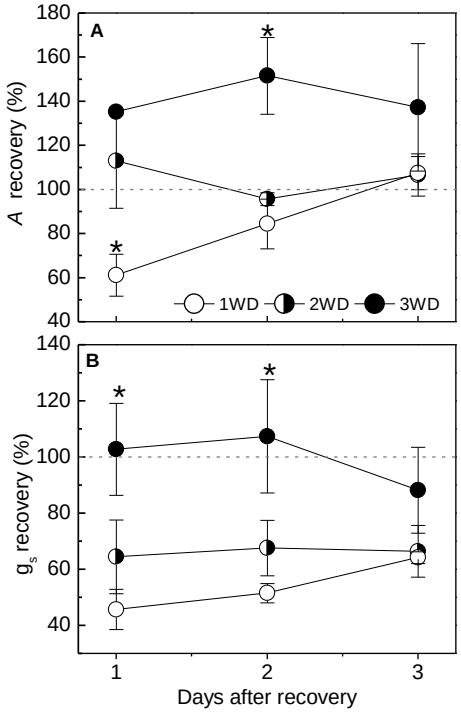

Fig. 3. Relative recovery of leaf CO_2 assimilation (A) and stomatal conductance (B) after rehydration of sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. Symbols represent the mean values ± SD (n=4). Asterisks indicate statistical difference ($p<0.05$) among treatments. Dotted lines at 100% indicate the A or gs values in reference conditions, i.e., well-watered plants.

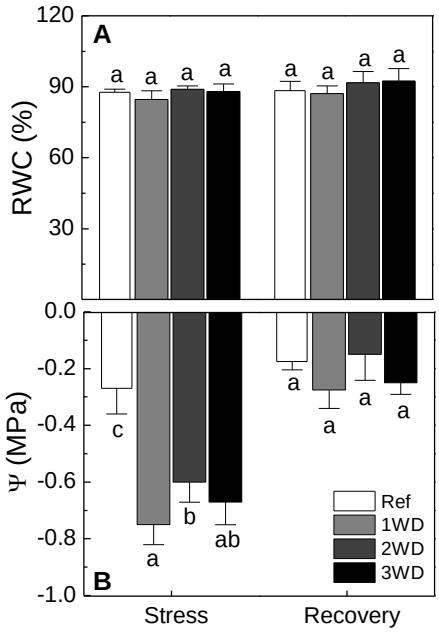

Fig. 4. Leaf relative water content (A) and pre-dawn leaf water potential (B) at the maximum water deficit (Stress) and after three days of rehydration (Recovery) in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments.

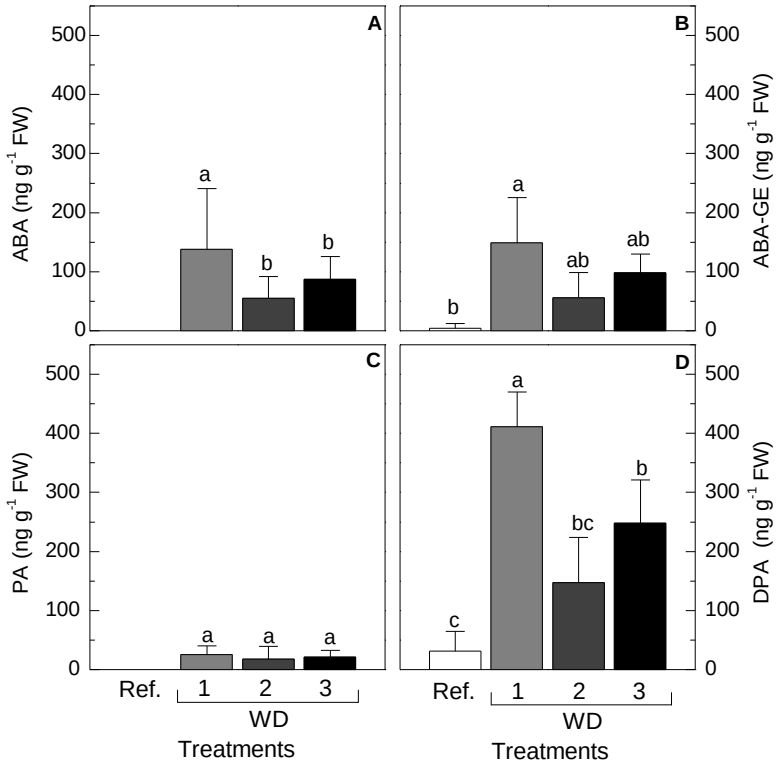

Fig. 5. Leaf concentration of abscisic acid (A), ABA-glucose ester (B), phaseic acid (C) and dihydrophaseic acid (D) at the maximum water deficit in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=5). Different letters indicate statistical difference ($p<0.05$) among treatments.

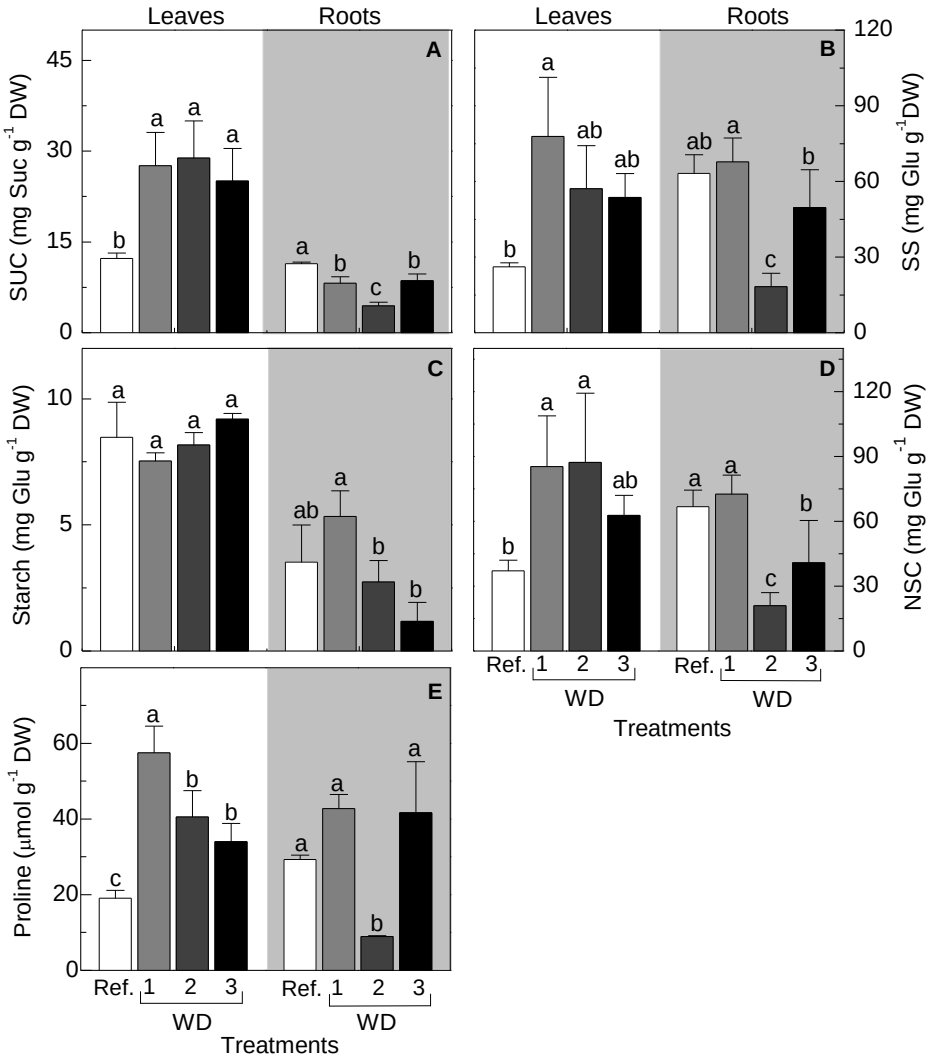

Fig. 6. Leaf and root concentration of sucrose (A), soluble sugars (B), starch (C), non-structural carbohydrates (D) and proline (E) at the maximum water deficit in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments.

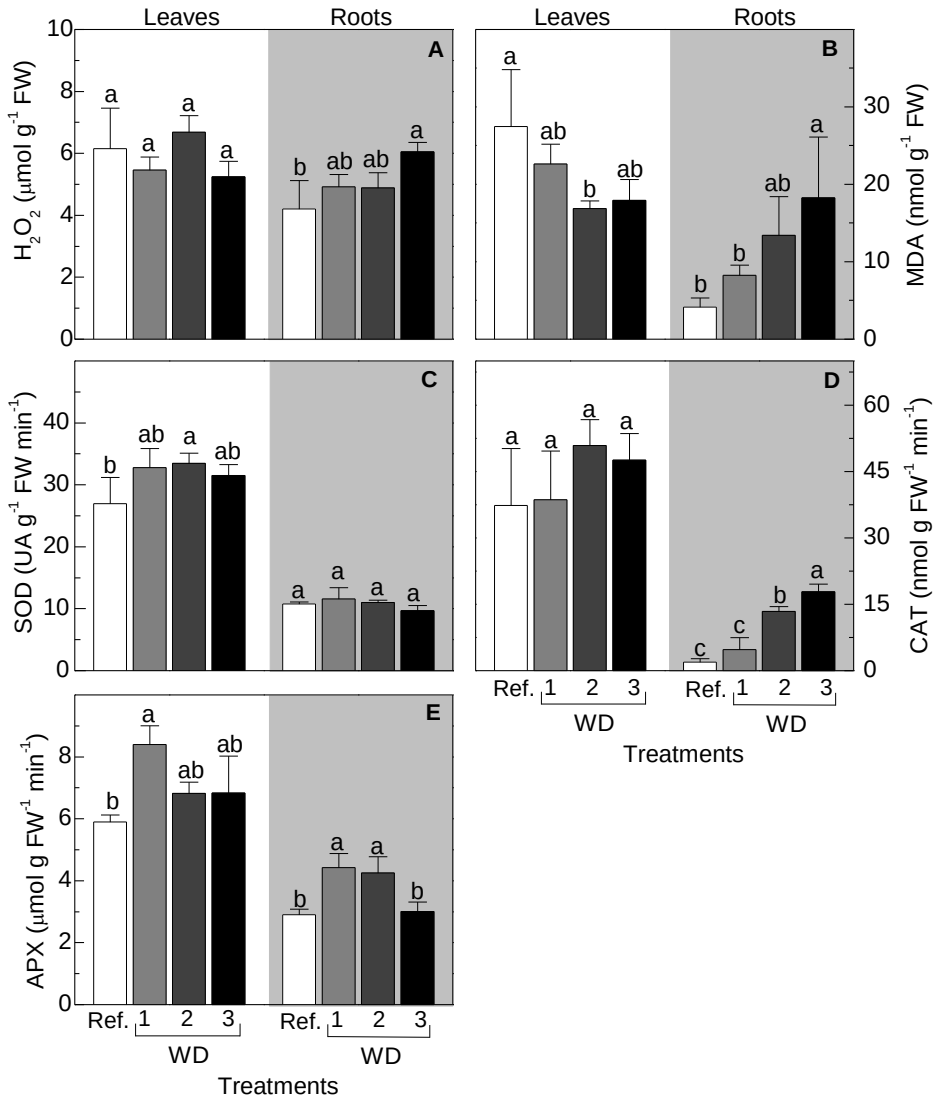

Fig. 7. Concentrations of H_2O_2 (A) and malondialdehyde (B) and activities of superoxide dismutase (C), catalase (D) and ascorbate peroxidase (E) at the maximum water deficit in leaves and roots of sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments.

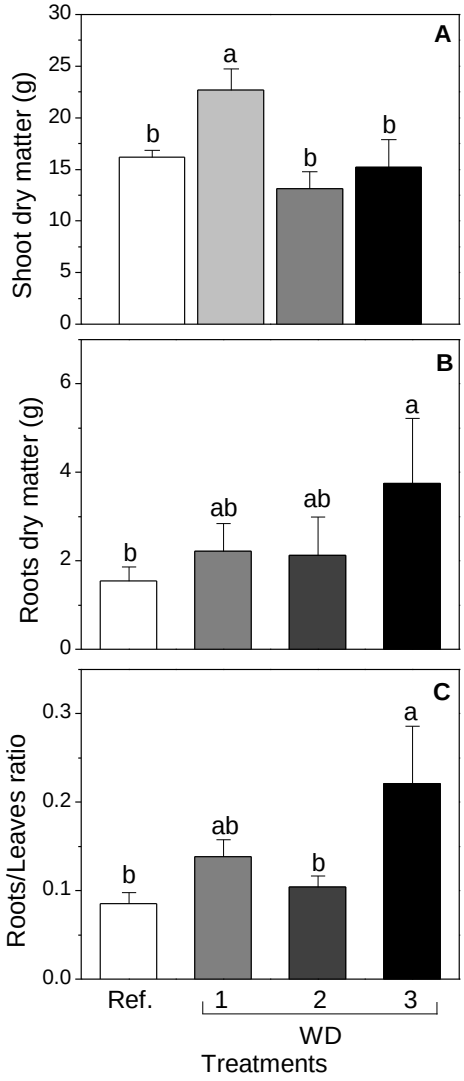

Fig. 8. Shoot dry matter (A), root dry matter (B) and root/shoot ratio (C) in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments. Measurements were taken at the end of the experimental period.

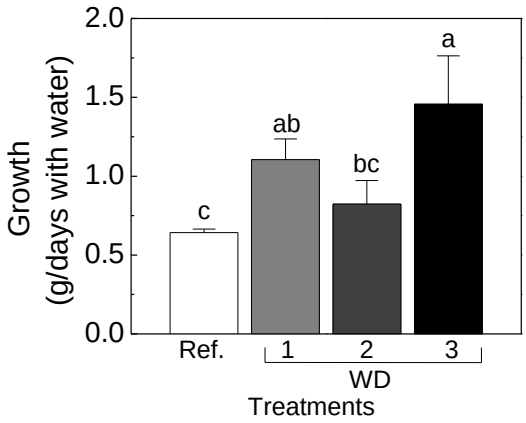

Fig. 9. Growth given by total biomass normalized by the number of days under well-watered conditions in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Histograms represent the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments. Measurements were taken at the end of the experimental period.

Supplemental material

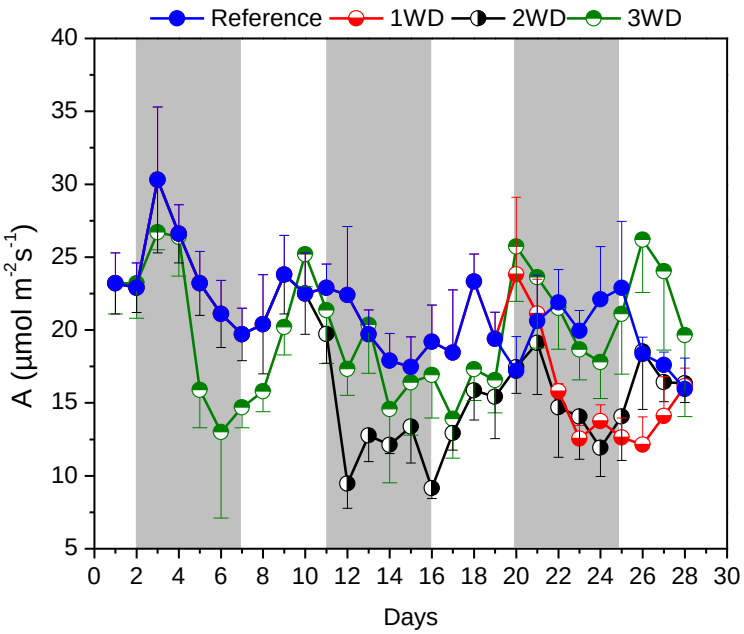

Fig. S1. Temporal dynamics of leaf CO_2 assimilation during the preparatory and experimental phases of sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference, plants were maintained well-watered. Each symbol represents the mean value ± SD (n=4). Gray areas represent periods of water deficit.

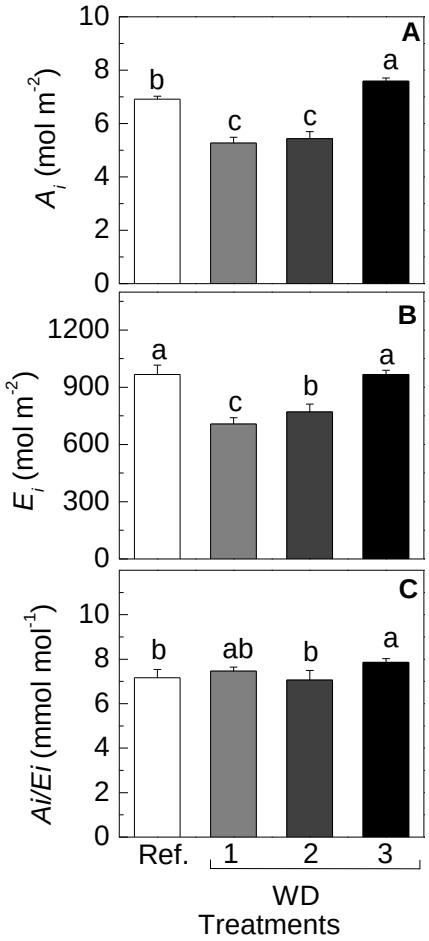

Fig. S2. Integrated leaf CO_2 assimilation (A), transpiration (B), and water use efficiency (C) during the experimental phase in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) cycles of water deficit. As reference (Ref.), plants were maintained well-watered. Each symbol represents the mean value ± SD (n=4). Different letters indicate statistical difference ($p<0.05$) among treatments.

Chapter II – Transgenerational drought memory in sugarcane plants

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Abstract

Drought stress can imprint marks in plants after a previous exposure, leading to a permissive state that could facilitate a quicker and more effective response to subsequent stress events. In crop species vegetative propagated, the stress imprint would benefit plants obtained from progenitors previously exposed to drought, this is called transgenerational memory. Herein, we tested the hypothesis that plants obtained from others previously exposed to water deficit will perform better under water deficit as compared to plants obtained from material that did not face stressful conditions. Mother-plants of sugarcane (*Saccharum* spp.) *cv*. IACSP94-2094 were grown well-hydrated and under greenhouse conditions until 6-month old, when one group of plants continued under daily irrigation (W) and another group was subjected to three cycles of water deficit (D) by water withholding. At this moment, there was a significant reduction in CO_2 assimilation after water withholding in all cycles of water deficit, as well the full recovery and the negative impact of water deficit was reduced from the first to the third cycle. Then, plants were produced through vegetative propagation from those plants that experienced or not cycles of water deficit. After sprouting, 1-month old plants were placed in nutrient solution and transferred to a growth chamber. Water deficit was imposed by adding PEG8000 in nutrient solution in one group of plants, then we had D plants subjected to a new water deficit (D/D), or kept well watered (D/W); well watered plants (W) subjected to a water deficit (W/D), or kept well watered (W/W). When these plants were exposed to water

withholding, there was a reduction in gas exchange regardless of the plant origin. Plants originated from mother-plants that experienced water deficit (D/D) presented a faster recovery of CO_2 assimilation and carboxylation efficiency as compared to W/D plants. Some plant metabolites had a different concentration related to mother-plants treatment leaf proline content was increased under water deficit, D/W plants had higher leaf sucrose content than W/W ones. As well as D/W plants had higher root H_2O_2 concentration and higher root CAT activity than W/W plants. The sucrose in leaves and H_2O_2 in roots were the chemical signals of these transgenerational stress memory in sugarcane under well-watered conditions. Our findings show that sugarcane growth is improved in plants obtained from mother-plants who had faced water deficit. It brings a new perspective to sugarcane production by favoring the expansion of cultivated areas, since transgenerational stress memory can improve plant performance under field conditions due to a large root system and faster recovery of photosynthesis after water deficit.

Key words: clonal plants, growth, photosynthesis, water deficit.

Introduction

As a semi-perennial species, sugarcane plants face seasonal drought under field conditions, where water deficit causes reduction in photosynthesis and accumulation of carbohydrates, changes in antioxidant metabolism, and finally impairment of plant growth and sucrose yield (Ribeiro et al., 2013; Sales et al., 2015). However, recurrent cycles of drought followed by rehydration are known to improve plant performance during a new stressful event (Bruce et al., 2007; Galle et al., 2011). Such phenomenon indicates that plants are able to change their metabolism and growth after external stimulus, improving recovery of photosynthesis, increasing intrinsic water use efficiency (Galle et al., 2011) and photoprotection (Walter et al., 2011) and reducing the negative impact of drought on yield (Izanloo et al., 2008).

Improved plant response induced by previous exposure to a limiting factor is an evidence of stress memory. The storage of information of stressful events by plants has been called stress memory and epigenetic changes as DNA methylation have been proposed as mechanism (Bruce et al., 2007; Hauser et al., 2011; Ding et al., 2012). In fact, such stress memory can assist plants in future stresses (Ding et al., 2012) and one important issue is the site in which information is stored within plants. Plants do not have a specific region to store

information and they can sense the environment with all their body and the intricate cell signaling system. Then, plants can perceive one stimulus in one site and the respective response be found in a different organ due to signaling (Thellier and Lüttge, 2012). One important requirement for retaining information is that stress-induced signals could not be reset when the stressor is no longer present (Chinnusamy and Zhu, 2009).

In nature, plant phenotype is also defined by transgenerational regulation, which occurs when internal changes persist in the next generation through epigenetic marks such as DNA methylation (Hauser et al., 2011). There is reasonable evidence for assuming that plants can sense changes in the environment during growth and modify the phenotype of their progeny to be more adapted to growing conditions (Boyko and Kovalchuk, 2011; Hauser et al., 2011). The stress-induced epigenetic information can be transferred to subsequent generations by seeds and through vegetative propagation (Dodd and Douhovnikoff, 2016). In fact, plant reproduction occurs not only by seed formation but also through vegetative propagation, which is based on meristem growth. In the first case, plants can pass epigenetic information through the meiosis process and produce seeds with stress memory (Boyko et al., 2010; Boyko and Kovalchuk, 2011). For instance, Boyko et al. (2010) showed that *Arabidopsis thaliana* exposed to cold, heat and flooding had increased global genome methylation and higher tolerance to stress as compared to progeny from plants not exposed to stressful conditions. However, stress-induced signals may be erased or diminished during meiosis, reducing stress memory stress. On the other hand, clonal plants produced by vegetative propagation have apparently better ability to recover signals acquired during stress events than non-clonal plants (Latzel et al., 2016).

Considering stress memory, plant propagation and drought-induced effects on plants, we hypothesized that plants obtained from others previously exposed to water deficit will perform better under water deficit as compared to plants obtained from material that did not face stressful conditions. Sugarcane is vegetative propagated and it is a good model to test our hypothesis as memory marks can be stored in buds, which will sprout and produce new plants; and buds are in the same plant axis and consequently face similar stressful conditions as compared to leaves and roots. Sugarcane is the main Brazilian crop for ethanol and bioenergy production, a clean alternative for energy production. Thus, the expansion of this crop to rainfed areas encourages the production of more resistant seedlings and induction of stress memory would be an important tool for improving crop establishment, growth and yield in such marginal areas.

Material and methods

Plant material and growth conditions

Plants of sugarcane (*Saccharum* spp.) cv. IACSP94-2094 were produced from mini-stalks containing one bud and grown in plastic pots (0.5 L) containing commercial substrate composed of sphagnum peat, expanded vermiculite, limestone dolomite, agricultural gypsum and NPK fertilizer - traces (Carolina Soil®, Vera Crus RS, Brazil). Thirty-four days after planting, plants were transferred to larger pots (20 L) containing typical red-yellow Latosoil (Dos Santos et al., 2013). The soil was fertilized with urea (6.7 g/pot, equivalent to 300 kg N ha^{-1}), superphosphate (16.7 g/pot, equivalent to 300 kg P_2O_5 ha^{-1}) and potassium chloride (4.3 g/pot, equivalent to 260 kg K_2O ha^{-1}), according to Dias and Rossetto (2006). During the experiment, three fertilizations were performed with the same amount of urea, superphosphate and potassium chloride as the first fertilization. The plants were grown under greenhouse conditions, where the average air temperature was 24.4±6.6 °C, relative humidity was 76±17% and the maximum photosynthetic photon flux density (PPFD) was approximately 1,200 µmol m^{-2} s^{-1}. Plants were irrigated daily and grown under well-hydrated conditions until they were 6-month old.

Inducing stress memory

When plants were 6-month old, one group of plants continued under daily irrigation (W) and another group was subjected to three cycles of water deficit (D) by water withholding. Each cycle of water deficit lasted nine days and soil moisture was monitored with soil moisture-sensors model Water Scout SM100 (Yara ZimTechnology, Berlin, Germany). While soil volumetric water content (VWC) reached 20% during cycles of water deficit, it was higher than 60% in well-watered pots. After nine days of water deficit, plants were irrigated and maintained under well-watered conditions for six days before the new cycle of water deficit. Leaf gas exchange was measured daily, whereas leaf water potential and leaf relative water content were measured at the 9^{th} day of water deficit and also after six days of recovery in each cycle of water deficit. After three cycles of water deficit, we evaluated the number of tillers, number of green and senescent leaves, total leaf area and dry matter of leaves, stems and roots. Then, plants were produced through vegetative propagation

from those plants that experienced or not cycles of water deficit, as cited in *Plant material and growth conditions*.

Testing plants for stress memory

After sprouting in commercial substrate (Carolina Soil®, Vera Crus RS, Brazil), 1-month old plants were placed in plastic boxes (12 L) with nutrient solution and transferred to a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) under air temperature of 30/20 °C (day/night), with 12 h photoperiod, relative humidity of 80% and PPFD of 800 µmol m^{-2} s^{-1}. Only the root system was immersed in modified Sarruge (1975) nutrient solution (15 mmol L^{-1} N (7% as NH_4^+); 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 µmol L^{-1} B; 54.0 µmol L^{-1} Fe; 5.5 µmol L^{-1} Mn; 2.1 µmol L^{-1} Zn; 1.1 µmol L^{-1} Cu and 0.01 µmol L^{-1} Mo). Nutrient solution was renewed in week intervals and the pH was maintained at 5.8±0.2 and electrical conductivity at 1.72±0.18 mS cm^{-1}. The osmotic potential of nutrient solution was -0.12 MPa. Two boxes containing plants obtained from irrigated mother-plants and two boxes containing plants from those subjected to three cycles of water deficit were prepared.

Forty-eight days after transferring plants to the hydroponic system, one group of plants was subjected to water deficit by adding PEG-8000 (CarbowaxTM PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution for 9 days. We added PEG-8000 gradually to prevent osmotic shock. Then, the osmotic potential of nutrient solution was reduced to -0.27, -0.57 and -0.77 MPa in three consecutive days. After these 9 days, the plants were recovered by a nutrient solution with osmotic potential of -0.12 MPa (control condition) for 5 days. At the end, four treatments were defined taking into account the material from which plants were obtained and also the water regime they were facing: plants obtained from mother-plants grown under well-watered conditions and then maintained under well-watered conditions (W/W); plants obtained from mother-plants grown under well-watered conditions and then subjected to water (W/D); plants obtained from mother-plants that experienced water deficit and then maintained under well-watered conditions (D/W); plants obtained from mother-plants that faced water deficit and then subjected to water (D/D).

Leaf gas exchange and photochemistry

Leaf gas exchange was measured daily with an infrared gas analyzer (LI-6400, LICOR, Lincoln NE, USA) attached to a modulated fluorometer (6400-40 LCF, LICOR, Lincoln NE, USA). The measurements were performed between 10:00 and 13:00 h under PPFD of 2,000 μmol m^{-2} s^{-1} and air CO_2 concentration of 380 μmol mol^{-1}. CO_2 assimilation (A), stomatal conductance (g_S), intercellular CO_2 concentration (C_i), transpiration (E), intrinsic water use efficiency (A/g_S), and the instantaneous carboxylation efficiency ($k=A/C_i$) were evaluated in fully expanded leaves. A and E values were integrated throughout the experimental period to estimate the total CO_2 gain (A_i) and the total H_2O_v loss (E_i), with the integrated water use efficiency (WUE=A_i/E_i) being estimated. The integrated values were estimated assuming that the values measured between 10:00 and 13:00 h were constant during the 12 hours of photoperiod. Chlorophyll fluorescence was measured simultaneously to leaf gas exchange and the apparent electron transport rate (ETR) was estimated as ETR=$\phi_{PSII}\times$ PPFD × 0.85 × 0.4, in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light absorption and 0.4 is the fraction of light energy partitioned to PSII (Edwards and Baker, 1993; Baker, 2008). Additionally, the non-photochemical quenching of fluorescence (NPQ) was evaluated and ETR/A calculated. In leaf tissues adapted to darkness (30 min), the potential quantum efficiency of photosystem II (F_V/F_M) was estimated according to Rohácek (2002).

Leaf water potential and relative water content

Leaf water potential (ψ) was evaluated at the predawn with a pressure chamber (model 3005, Soilmoisture Equipment Corp., Santa Barbara CA, USA). The leaf relative water content RWC) was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Weatherley (1950): RWC=100×[(FW−DW)/(TW−DW)]. Both variables were measured at the maximum stress condition and recovery period.

Carbohydrates and proline

The extraction of total soluble carbohydrates (SS) was done with methanol:chloroform:water solution (Bieleski and Turner, 1966) and quantified by the phenol–sulfuric acid method (Dubois et al., 1956). Sucrose content was quantified following

van Handel (1968) and starch (Sta) was determined by the enzymatic method proposed by Amaral et al. (2007). The concentration of nonstructural carbohydrates (NSC) in leaves and roots was calculated as NSC=SS+Sta. Total NSC was calculated considering the dry matter of each plant (mg pl^{-1}). Leaf proline content was determined in test tubes by the reaction with the sample, ninhydrin reagent (ninhydrin, acetic acid and orthophosphoric acid) glycine and acetic acid for 35 minutes at 100°C, and the reaction terminates in an ice bath. The reaction mixture was extracted with toluene and the proline concentration was determined from a standard curve (Rena and Masciotti, 1976). Plant nonstructural carbohydrates were calculated by the sum of leaf and root carbohydrates and carbohydrate partitioning among sugar types was also evaluated in both organs.

Hydrogen peroxide

Evaluation of hydrogen peroxide (H_2O_2) was performed in 0.16 g fresh tissue (leaves and roots) ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v) (Alexieva et al., 2001). The extract was centrifuged at 12,000 *g*, 4°C for 15 min. The crude extract was added to the reaction medium (1.2 mL of KI 1 mol L^{-1}, potassium phosphate buffer pH 7.5 and 0.1 mol L^{-1}) in microtubes and incubated on ice under dark for 1 h. After this period, the absorbance was evaluated at 390 nm. The calibration curve was done with H_2O_2 and the results were expressed as µmol H_2O_2 g^{-1} FW.

Antioxidant enzymes: extraction and activity assays

Enzymes were extracted from 0.2 g of fresh tissues of leaves and roots grounded in liquid nitrogen, with 1% of PVPP and 2 mL of extraction medium composed by 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 0.1 mmol L^{-1} ethylenediaminetetraacetic (EDTA) and 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF). This homogenate was centrifuged at 15,000 *g* for 15 min and 4°C and the supernatant was collected and preserved on ice. Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated in a reaction medium with 3 mL of 100 mmol L^{-1} sodium phosphate buffer (pH 7.8), 50 mmol L^{-1} methionine, 5 mmol L^{-1} EDTA, deionized water, crude extract, 100 µmol L^{-1} riboflavin and 1 mmol L^{-1} nitro blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp of 30 W) for 15 min, and another group remained in darkness. The absorbance was measured at 560

nm and one unit of SOD is the amount of enzyme required to inhibit the NBT photoreduction in 50% (Giannopolitis and Ries, 1977). SOD was expressed as U min^{-1} mg^{-1} of protein. Catalase (CAT, EC 1.11.1.6) activity was assayed in a reaction medium of 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.8), deionized water, 125 mmol L^{-1} H_2O_2 and crude extract. The decrease in absorbance at 240 nm was measured and CAT activity was estimated using a molar extinction coefficient of 36 M^{-1} cm^{-1} and expressed as nmol g^{-1} FW min^{-1} (Havir and McHale, 1987). For ascorbate peroxidase (APX, EC 1.11.1.11) activity, the reaction medium was composed by 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.0), deionized water, 10 mmol L^{-1} ascorbic acid, 10 mmol L^{-1} H_2O_2 and crude extract. The decrease in absorbance at 290 nm was measure and we used a molar extinction coefficient of 2.8 M^{-1} cm^{-1} to estimate APX in µmol g^{-1} FW min^{-1} (Nakano and Asada, 1981).

Biometry

The total leaf area was measured using a LI-3000 leaf area meter (LICOR, Lincoln NE, USA), and shoot and root dry matter were evaluated after drying samples in a forced air oven at 65 °C. Measurements were taken at the end of the experimental period.

Statistical analysis

The experimental design was in randomized blocks and the causes of variation were water conditions (two levels) and material origin (two levels). The data were subjected to ANOVA procedure and the mean values (n=4) were compared by the Tukey test at 5% probability level.

Results

Mother-plants under water deficit

Herein, mother-plants are defined as those ones that provided vegetative material for propagation, i.e., small stalks segments with buds. Mother-plants were subjected to three cycles of water deficit and leaf gas exchange was measured during dehydration and rehydration stages (Supplementary Material Figure S1). There was a significant reduction in CO_2 assimilation after four days of water withholding in all cycles of water deficit, with

photosynthetic rates reaching null values or even negative ones (respiration). Full recovery of CO_2 assimilation was noticed in all cycles and the negative impact of water deficit was reduced from the first to the third cycle (Supplementary Material Figure S1). After three cycles of water deficit, there was a significant inhibition of biomass production (Supplementary Material Figure S2), with reductions in the number, dry matter and area of green leaves as well as decreases in root and stem dry matter (Supplementary Material Table S1).

Then, small stalk segments with one bud were obtained from mother-plants and planted in individual recipients to produce new plants. Buds from mother-plants subjected to water deficit had higher germination (~95%) than buds from mother-plants maintained under well-watered conditions (~74%). Thirty days after planting, plants were placed in plastic boxes with nutrient solution and four treatments were formed: plants from mother-plants grown under well-watered conditions were maintained well-watered (W/W) or subjected to water deficit (W/D); and plants from mother-plants grown under cycles of water deficit were maintained well-watered (D/W) or subjected to water deficit (D/D).

Stress memory: photosynthesis and leaf water status under water deficit

Water deficit reduced leaf CO_2 assimilation, stomatal conductance and the instantaneous carboxylation efficiency, regardless of the plant origin (Fig. 1). Interestingly, plants originated from mother-plants that experienced water deficit (D/D) presented a faster recovery of CO_2 assimilation and carboxylation efficiency as compared to W/D plants (Fig. 1A,C). Integrated CO_2 assimilation and transpiration were reduced by water deficit in a similar way when comparing W/D and D/D treatments (Fig. 2A,B). However, recovery of photosynthesis was favored in D/D plants and then integrated water use efficiency was improved in plants under water deficit (Fig. 2C).

After 9 days of water deficit, pre-dawn leaf water potential was reduced and D/D plants showed the lowest values (Fig. 3A). Regarding the leaf relative water content, there was a similar response to water deficit and both W/D and D/D plants exhibited the lowest values (Fig. 3B). While the pre-dawn leaf water potential was fully recovered, leaf relative water content was partially recovered after 4 days of plant rehydration (Fig. 3).

Water deficit caused decreases in F_v/F_m and ETR of W/D and D/D plants (Fig. 4A,B). Although D/D plants had shown the lowest ETR values, the ratio ETR/*A* was similar between W/D and D/D plants, increasing in more than three times due to water deficit (Fig. 4C). Non-

photochemical quenching was also increased by water deficit only in W/D plants (Fig. 4D). All photochemical indexes were recovered after plant rehydration, with W/W *vs.* W/D and D/W *vs.* D/D showing similar values.

Stress memory: proline and carbohydrates under water deficit

Leaf proline content was increased under water deficit and D/D plants presented the highest values. After the recovery period, W/D plants presented higher proline content than D/D plants (Fig. 5).

Leaf sucrose content was increased by water deficit in plants originated from mother-plants maintained under well-watered conditions, i.e. W/W *vs.* W/D (Fig. 6A). Curiously, D/W plants had higher leaf sucrose content than W/W ones, suggesting an influence of mother-plants. Such influence was also found in roots, with D/W plants presenting lower sucrose, soluble total sugars and total non-structural carbohydrates than W/W plants (Fig. 6). Reductions in root concentrations of sucrose, soluble total sugars and total non-structural carbohydrates due to water deficit were found only in plants obtained from those ones that did not face drought events (Fig. 6E-H). When considering the total amount of non-structural carbohydrates in plants (Fig. 6I), D/W plants had higher values than W/W plants and the partitioning between leaves (86% to 91%) and roots (9% to 15%) was similar among treatments (Fig. 6J).

Stress memory: antioxidant metabolism under water deficit

Leaf SOD and CAT activities were not affected either by water regime or plant origin (Fig. 7A,D), but leaf H_2O_2 concentration and APX activity increased due to water deficit (Fig. 7B,C). The highest APX activity was found in W/D plants (Fig. 7C). In roots, non-significant changes were found for SOD and APX activities (Fig. 7E,G). Root H_2O_2 concentration and CAT activity increased due to water deficit in plants originated from mothers maintained well-watered (Fig. 7F,H). On the other hand, root H_2O_2 concentration was reduced and CAT activity did not change under water deficit when considering plants originated from other ones grown under cycles of water deficit (Fig. 7F,H). One interesting finding is that D/W plants had higher root H_2O_2 concentration and higher root CAT activity than W/W plants (Fig. 7F,H).

Stress memory: plant growth under water deficit

Regardless plant origin, water deficit reduced shoot biomass production, but D/D plants had higher shoot biomass than W/D plants (Fig. 8A). While plants obtained from mother-plants grown under well-watered conditions presented increases in root biomass under water deficit, the opposite was found in plants from mothers that experienced cycles of water deficit (Fig. 8B). In general, root biomass of D/W plants was about four times higher than one of W/W plants, with D/D plants showing similar root biomass as compared to W/D plants. Leaf area was also reduced by water deficit, regardless plant origin (Fig. 8C). However, plants from mothers subjected to water deficit had higher leaf area than ones obtained from well-watered mothers, despite the water regime.

Discussion

Transgenerational memory: morpho-physiological aspects

When exposing mother-plants to water deficit, stress memory was induced and the information likely stored in bud meristems, as suggested by improved performance of plant obtained from vegetative propagation. Besides causing decreases in photosynthesis (Fig. S1) and biomass production (Fig. S2; Table S1), cycles of dehydration and rehydration are able to create a number of chemical signals, such as increases in concentration of abscisic acid (ABA), a hormone that alter the expression pattern of many genes linked to drought response (Fleta-Soriano et al., 2015). Such changes in gene expression patterns can be stored by epigenetics through DNA methylation and acetylation and induce stress memory (Avramova, 2015). In spite of a large decrease in biomass production of mother-plants due to water deficit (Fig. S2; Table S1), plants originated from vegetative propagation had faster germination and were bigger than those ones obtained from mother-plants maintained well-hydrated, regardless water regime (Fig. 8). Such improved plant growth due to transgenerational stress memory was reported previously and it is likely linked to changes in DNA methylation (Hauser et al., 2011).

The ability of clone plants in recovering the stored environmental information (Latzel et al., 2016) can explain both morphological and physiological responses of D/D plants. D/D plants exhibited higher photosynthesis than W/D plants at recovery and this was caused by higher instantaneous carboxylation efficiency (Fig. 1A, C). Regarding primary

photochemistry, non-photochemical quenching was lower in D/D plants than in W/D plants, indicating less excess of energy at PSII level in D/D plants (Fig. 4D). Improved water use efficiency is a good parameter to suggest drought stress memory as it indicates optimization of CO_2 assimilation per unit of H_2O_v transpired under water limiting conditions (Fleta-Soriano et al., 2015). In fact, D/D plants had also higher integrated water use efficiency (Fig. 2C) and higher integrated photosynthesis during recovery (Fig. 2A) when compared to W/D plants.

It is interesting to point that D/D plants were able to maintain metabolic activity and produce more biomass than W/D plants (Fig. 8) even presenting lower leaf water potential (Fig. 3A). As RWC was similar between W/D and D/D plants (Fig. 3), our data suggest the occurrence of more intense osmotic adjustment in D/D plants. This can be explained by higher concentration of proline in leaves (Fig. 4), an osmotic and osmoprotectant molecule (Szabados and Savouré, 2010). During stressful conditions, high proline levels in D/D plants suggest that these plants have synthesized this osmolyte for adjusting the osmotic equilibrium and cell homeostasis, one form of memory according to Ding et al (2013). After rehydration, there was a large degradation of proline in D/D plants, suggesting remobilization of nitrogen to assimilatory pathways for resuming plant growth. A transgenerational stress memory was also noticed at the last day of rehydration, when D/D plants had higher photosynthesis (25.7±2.7 *vs.* 15.7±3.8 μmol m^{-2} s^{-1}) and integrated water use efficiency (7.2±0.3 vs. 6.3±0.4 μmol mol^{-1})) than W/D plants.

Drought memory & antioxidant and carbon metabolism

Plants respond to abiotic stresses by altering their metabolism and accumulating substances such as sugars, amino acids and other metabolites with important role in stress tolerance (Verslues et al., 2006). Maintenance of high sucrose concentration even under well-watered conditions may be another evidence of stress memory (Crisp et al., 2016), as found in D/W plants (Fig. 6A). In addition, plants obtained from mother-plants that faced drought did not present any change in both leaf and root sucrose concentrations under water deficit (Fig. 6E-H). In fact, sucrose accumulation may help plants under water deficit by improving osmoregulation, protecting proteins and maintaining photosynthesis under low water availability. Alternatively, Hu et al. (2015) have suggested that low concentrations of ROS in plants previously exposed to stressful conditions could be an indication of stress memory.

However, our data indicate that exposure of mother-plants to water deficit caused higher root H_2O_2 concentration in plants maintained well-watered (Fig. 7F).

In addition to its role in plant signaling (Foyer and Noctor, 2005), ROS accumulation is associated with modifications in DNA methylation pattern (Peng and Zang 2009), an epigenetic change that would store information and induce faster stress response. According to Hu et al. (2015), the presence of ROS in controlled amounts is important for plant growth, with plants showing higher H_2O_2 concentration in the region of root elongation. In this way, high root H_2O_2 concentration in D/W plants (Fig. 7F) explains high root biomass of these plants (Figs. 7F and 8B). In fact, H_2O_2 is produced by mitochondria during the synthesis of NADH and ATP for supplying plant metabolism in active growing regions (Gill and Tuteja, 2010).

Stress memory for improving drought tolerance of sugarcane plants

Epigenetic changes caused by varying environmental conditions allow clone plants to adapt and have advantageous growth and acclimation strategies that favor them in unstable environments (Dodd and Douhovnikoff, 2016). Although sugarcane propagation does not involve meiotic recombination, mitotic alterations during vegetative propagation may also produce a source of variation that helps plants to persist and succeed in environmental colonization (Dood and Douhovnikoff, 2016). Epigenetic changes may manifest in the future generation, a transgenerational effect (Boyko et al., 2010). Herein, we induced transgenerational stress memory through vegetative propagation of sugarcane by inducing cycles of water deficit in mother-plants. Such finding indicates that propagules obtained from plants growing in areas with low water availability would be more tolerant to drought as compared to propagules of the same genotype grown under irrigation of in areas without occurrence of water deficit. Interestingly, sugarcane plants obtained from mother-plants that faced water deficit produced more biomass than ones from mother-plants maintained well-watered, regardless water regime (Fig. 8). This suggest that plants have increased their efficiency in using natural resources such as water and sunlight through the transgenerational stress memory.

Conclusion

Our findings clearly show that sugarcane growth is improved in plants obtained from mother-plants who had faced water deficit. The bases of such transgenerational stress memory should be further studied taking into account epigenetic markers. Our data also revealed that bud meristems are able to store information acquired from previous stressful events in sugarcane. Accumulation of sucrose in leaves and H_2O_2 in roots are chemical signals of the transgenerational stress memory in sugarcane under well-watered conditions. Benefits of such stress memory in leaf gas exchange were noticed during the rehydration, an important issue to be considered when studying in studies of plant responses to water deficit. Finally, our results bring a new perspective to the production of sugarcane plants for expanding cultivated areas. Through the transgenerational stress memory, plant performance can be improved under field conditions due to a large root system and faster recovery of photosynthesis after water deficit.

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Figures

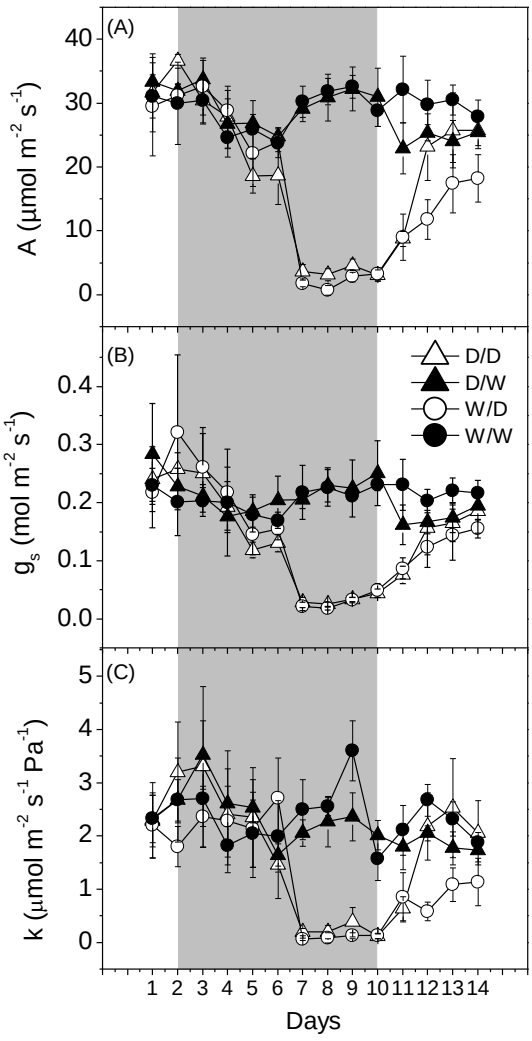

Fig 1. Leaf CO_2 assimilation (A), stomatal conductance (B), and instantaneous carboxylation efficiency (C) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). The gray area indicates water deficit period induced by water withholding. Each symbol represents the mean values ± s.d. (n = 4).

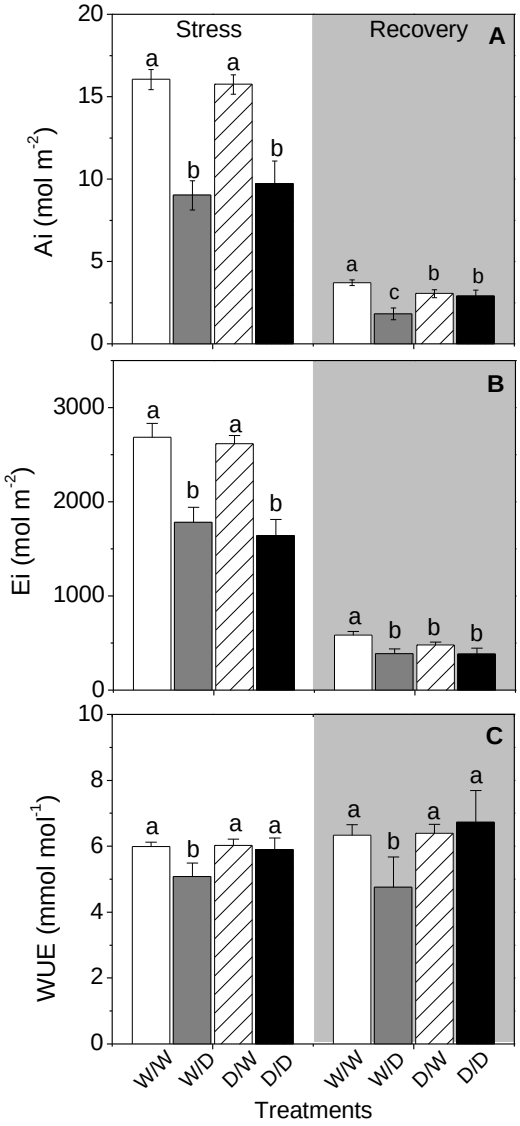

Fig 2. Integrated CO_2 assimilation (A), transpiration (B) and water use efficiency (C) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Integration was done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig. 1. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

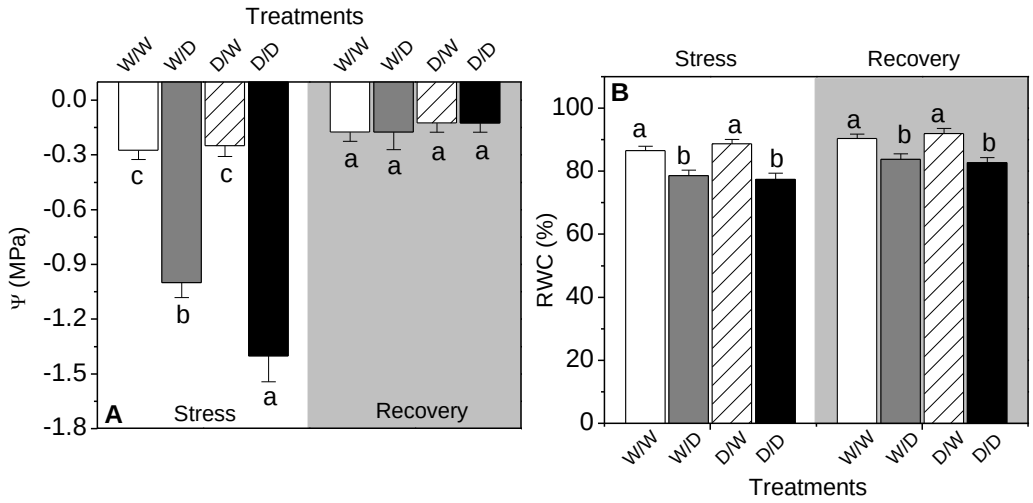

Fig. 3. Predawn leaf water potential (A) and relative water content (B) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig. 1. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

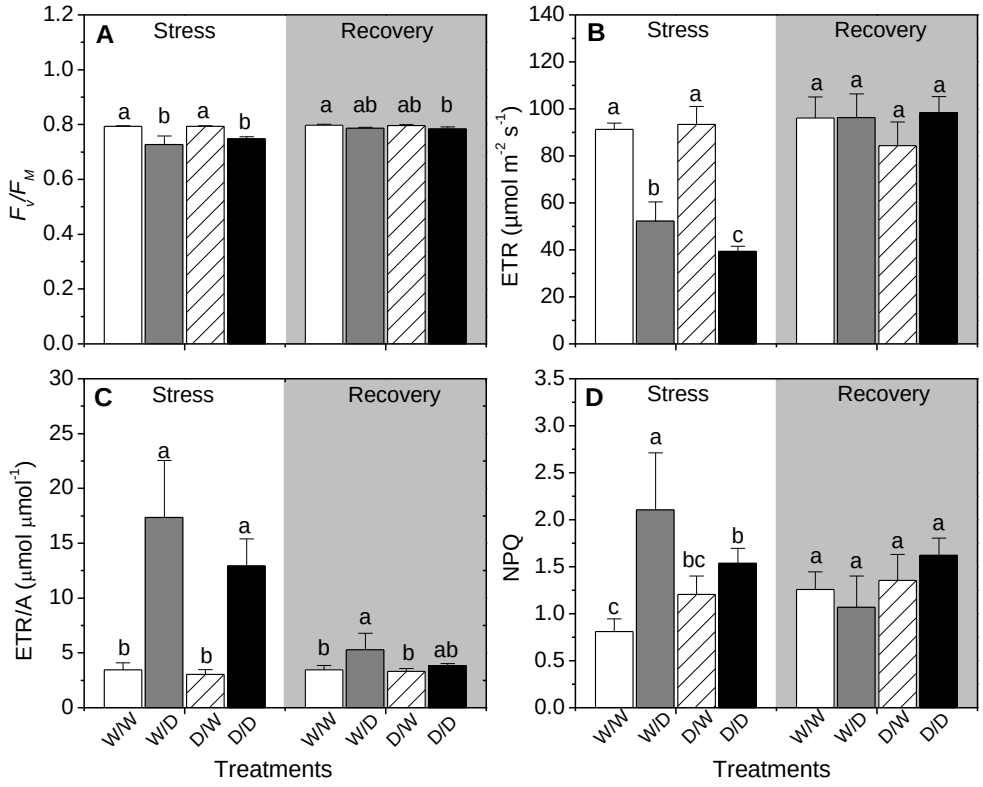

Fig. 4. Potential quantum efficiency of photosystem II (A), the apparent electron transport rate estimated (B), ETR/A ratio (C), and the non-photochemical quenching of fluorescence (D) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were done during the water deficit (stress) and recovery (gray area) periods, as shown in Fig. 1. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

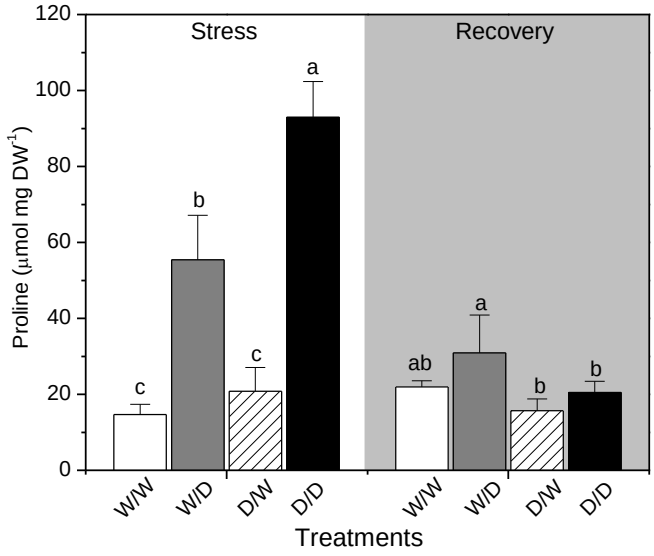

Fig. 5. Leaf proline content in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were taken during the water deficit (stress) and recovery (gray area) periods, as shown in Fig. 1. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

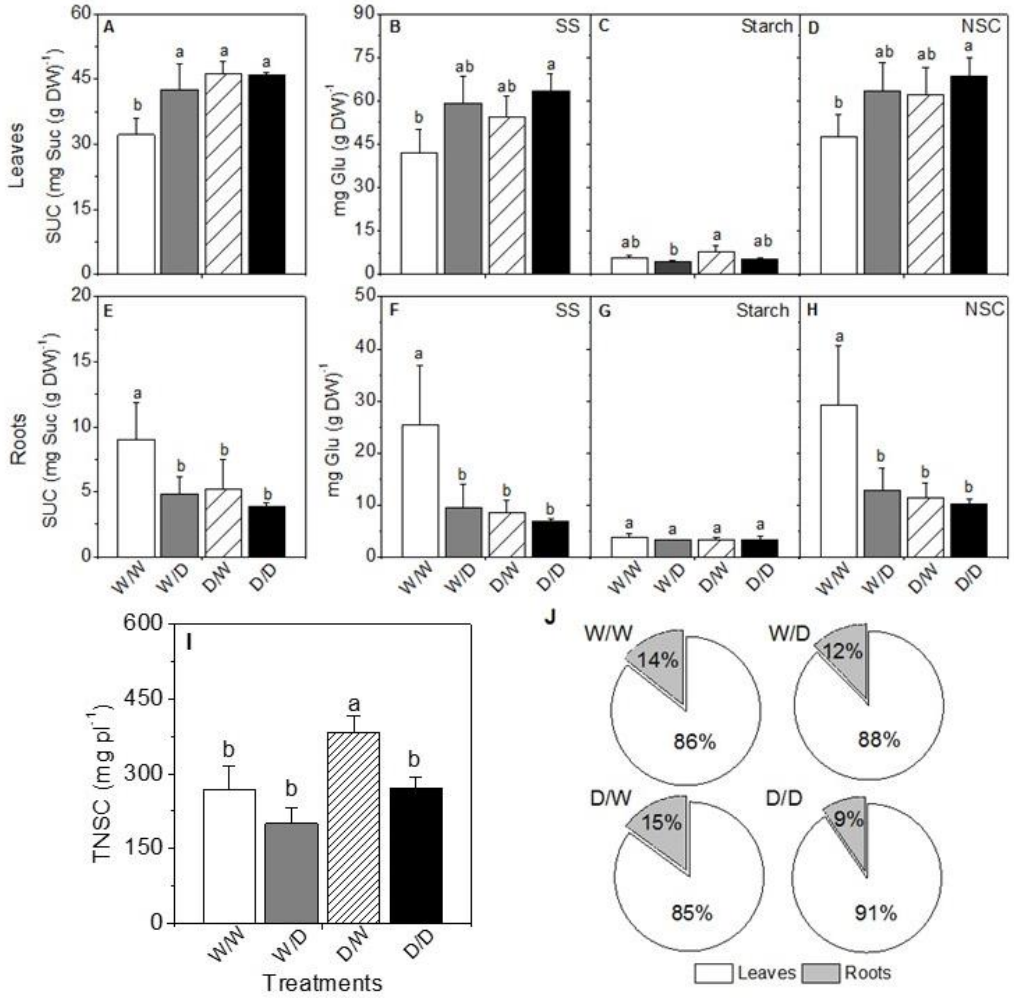

Fig. 6. Sucrose (A, E) soluble sugars (B, F), starch (C, G) and non-structural carbohydrates (D, H) in leaves (A-D) and roots (E-H), amount of total non-structural carbohydrates in the entire plant (I) and their partitioning among plant organs (J) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were taken after 10 days of water deficit. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

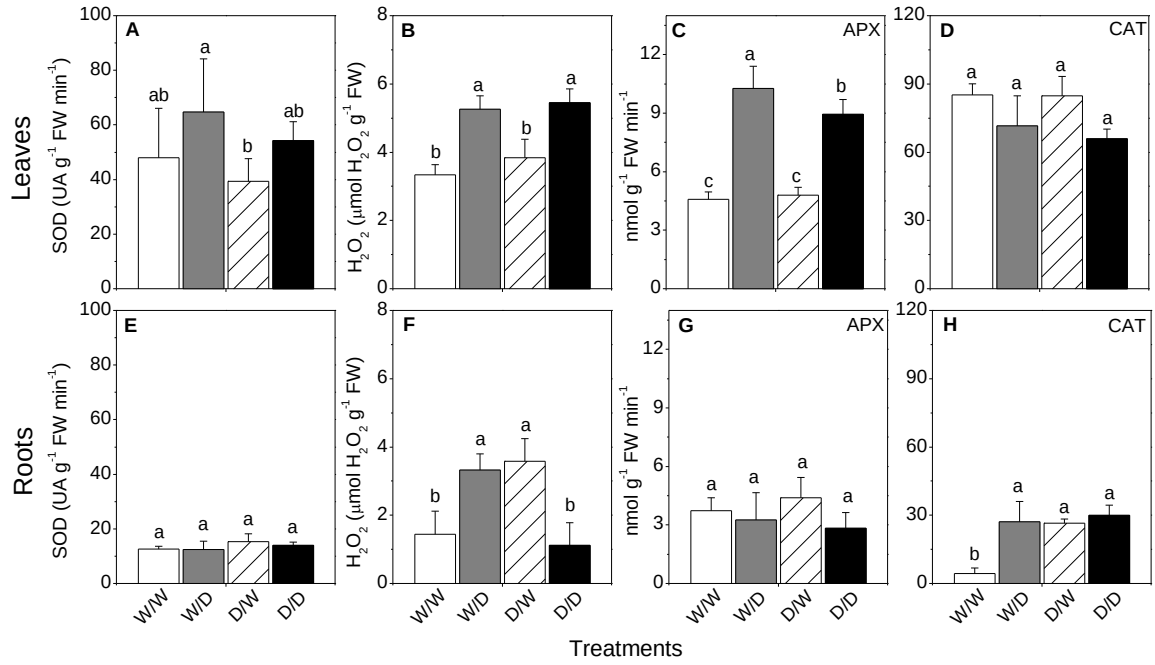

Fig. 7. Activities of SOD (A, E), APX (C, G), CAT (D, H) and H_2O_2 concentration (B, F) in leaves (A-D) and roots (E-H) of sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were taken after 10 days of water deficit. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

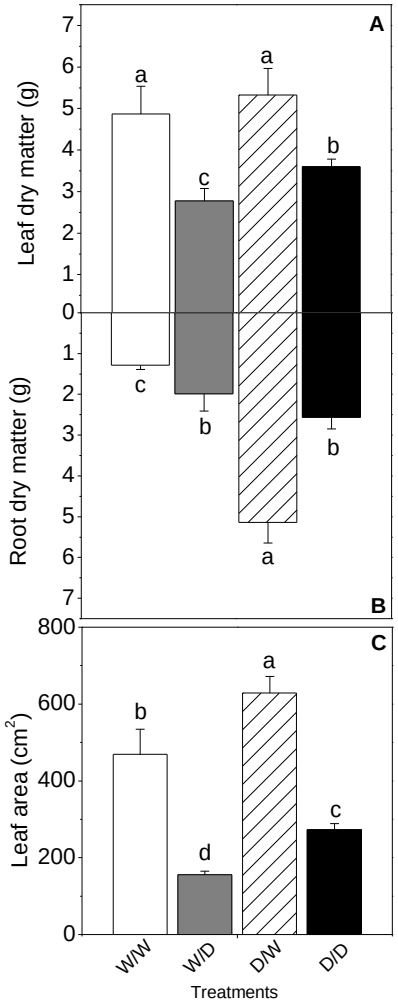

Fig. 8. Leaf (A) and root (B) dry matter and leaf area (C) in sugarcane plants maintained well-watered (W/W and D/W) and subjected to water deficit (W/D and D/D). Plants were obtained from mother-plants previously exposed to cycles of water deficit (D/W and D/D) or grown under well-watered conditions (W/W and W/D). Measurements were taken at the end of experiment. Each histogram represents the mean values ± s.d. (n = 4). Different letters means statistical differences among treatments (Tukey, $p<0.05$).

Supplemental Material

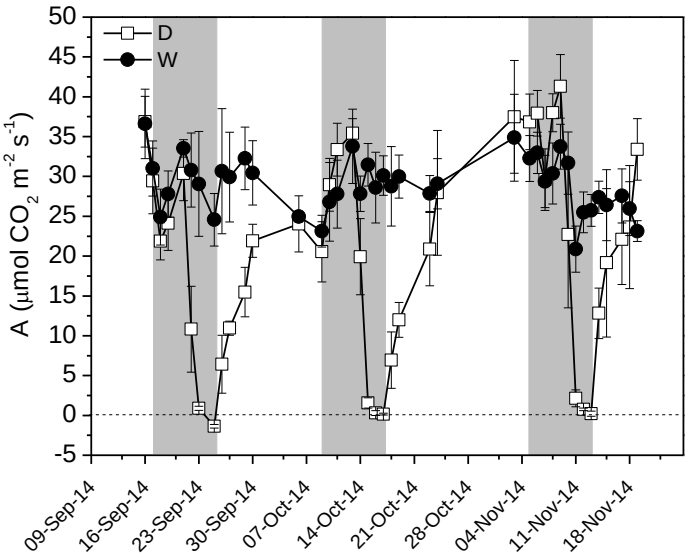

Fig. S1. Leaf CO_2 assimilation of mother-plants maintained well-watered (closed symbols) and subjected to three cycles of water deficit (open symbols). The grey area represents water withholding (nine days) and the dotted line indicates null photosynthesis. Each symbol represents the mean values ± s.d. (n = 4).

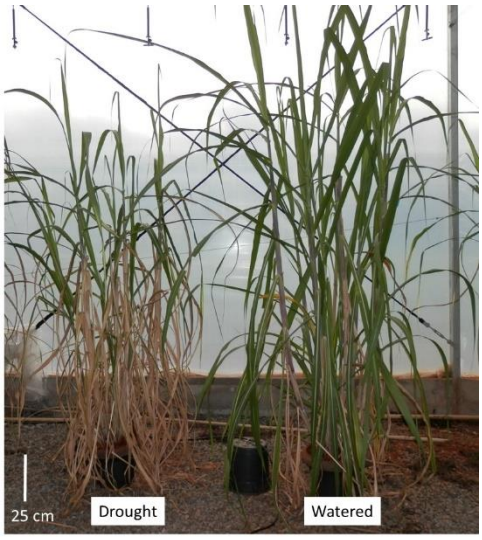

Fig. S2. Visual aspect of mother-plants grown under cycles of water deficit (left) or well-watered conditions (right).

Table S1. Biometry of mother-plants grown well-watered (reference) conditions or cycles of water deficit. Measurements were taken after 80 days of treatment. Letters indicate statistical differences between treatments by Tukey ($p<0.05$).

Variables	**Treatments**	
	Reference	Water deficit
Number of green leaves (units)	118 ± 13 a	38 ± 10 b
Number of dry leaves (units)	44 ± 9 a	131 ± 20 b
Leaf area (m^2)	4.7 ± 0.3 a	1.2 ± 0.4 b
Leaf dry matter (g)	405 ± 42 a	64 ± 20 b
Root dry matter (g)	759 ± 263 a	353 ± 33 b
Stem dry matter (g)	1370 ± 116 a	500 ± 50 b

Considerações finais

As estratégias das plantas para superar períodos de baixa disponibilidade hídrica podem envolver alterações morfológicas e fisiológicas, que podem inclusive levar à memória do estresse. Como consequência, as plantas poderão responder de modo mais rápido, ou melhor, aumentando a tolerância a um evento estressante subsequente. No entanto, não existem muitos trabalhos com espécies cultivadas e bastante complexas como a cana-de-açúcar. Neste sentido, este estudo pode ser considerado como uma importante contribuição para o entendimento de como as plantas são afetadas por eventos recorrentes de déficit hídrico, assunto ainda pouco estudado.

O trabalho indica que ciclos de déficit hídrico e reidratação melhoram a performance das plantas, tanto do ponto de vista fisiológico como morfológico, indicando a ocorrência de memória não apenas na geração submetida ao déficit hídrico mas também na geração seguinte obtida por propagação vegetativa. Sugere-se que as marcas provocadas pelo estresse são capazes de permanecer armazenadas nas gemas e podem ser recuperadas pelas novas plantas, que são maiores e se recuperaram do estresse de forma mais rápida. Os aspectos moleculares da memória à seca em cana-de-açúcar devem ser explorados em futuros estudos para revelar as bases genéticas da memória ao estresse e os potenciais marcadores moleculares dessa memória, podendo beneficiar programas de melhoramento genético.

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada " ***Memória de plantas de cana-de-açúcar à seca***", desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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