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Drought tolerance of sugarcane 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 lifespan. In this study, we hypothesized that sugarcane plants previously exposed to cycles of water deficits will perform better than plants that have never faced water deficits when both are subjected to low water availability. Sugarcane plants were grown in a nutrient solution and exposed to one (1WD), two (2WD) or three (3WD) water deficit cycles. As the reference, plants were grown in a nutrient solution without adding polyethylene glycol. Under water deficits, leaf gas exchange was significantly reduced in 1WD and 2WD plants. However, 3WD plants showed similar CO_2 assimilation and lower stomatal conductance compared to the reference plants, with increases in intrinsic water-use efficiency. Abscisic acid concentrations were lower in 3WD plants than in 1WD plants. Our data revealed root H_2O_2 concentration as an important chemical signal, with the highest root H_2O_2 concentrations found in 3WD plants. These plants presented higher root dry matter and root:shoot ratios compared to the reference plants, as well as higher biomass production when water was available. Our data suggest that sugarcane plants were able to store information from previous stressful events, with plant performance improving under water deficits. In addition, our findings provide a new perspective for increasing drought tolerance in sugarcane plants under nursery conditions.

1. Introduction

Plants close stomata to avoid losing water through transpiration under water limiting conditions (Chaves, 1991), a physiological response that is related to either hydraulic or chemical signals (Davies and Zhang, 1991; Christmann et al., 2007). Plant acclimation to water deficits involves morphological changes that 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 deficits, allowing the maintenance of water content and protecting cellular structures (Verlues et al., 2006). 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). However, 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 in the leaves. Therefore, production of reactive oxygen species (ROS) is enhanced under drought conditions, and plants should be able to control such deleterious molecules through their antioxidant system. This 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).

These 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 the consequences of such repetitive

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drought events are less understood (Walter et al., 2011). Plants can acclimate to varying water conditions through morphological and physiological changes, which favor the maintenance of plant growth or survival under stressful conditions (Chaves et al., 2002). Some changes during an acclimation period can allow faster responses and enhanced plant performances during the next stressful event. In fact, an experimental design with repeated cycles of droughts is a more realistic approach when considering plants in their natural environment, with improved plant performances under limiting conditions being found in several species when there was 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* that had previously been subjected to osmotic stress experienced increased 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 these studies compared plants of differing ages under varying stress intensities and environmental conditions, which makes the study of stress memory difficult.

As a semi-perennial crop grown in rainfed areas, sugarcane may experience seasonal variations in water availability and 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 deficits. We hypothesized that sugarcane plants subjected to previous droughts will exhibit improved performance, which would be achieved through changes in sugarcane physiology, biochemistry and morphology, under water deficit.

2. Materials and methods

2.1. Plant material and growth conditions

Sugarcane (*Saccharum* spp.) variety IACSP94-2094 was used in this study due to its reasonable yield under low water availability and its drought tolerance (Machado et al., 2009; Ribeiro et al., 2013). Plants were propagated using mini-stalks (with one bud) obtained from adult plants, which were planted in trays containing a commercial substrate composed of sphagnum peat, expanded vermiculite, limestone dolomite, gypsum and NPK fertilizer (Carolina Soil of Brazil, Vera Cruz RS, Brazil). Thirty-five days after planting (DAP), the 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, the ionic force was increased to 50% in the second week and to 100% in the following week. The electrical conductivity of the nutrient solution was monitored with a conductivity probe (Tec-4MPp, Tecnopon, Piracicaba SP, Brazil) and maintained at approximately 1.5 mS cm^{-1} by replacing the solution once a week. The pH of the nutrient solution was 5.4 ± 0.6 and it was monitored with a pHmeter (Tec-3MPp, Tecnopon, Piracicaba SP, Brazil). The osmotic potential of the 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). The nutrient solution with a 100% ionic force presented an osmotic potential of −0.12 MPa. After being moved to the nutrient solution, plants were placed in a growth chamber (PGR14, Conviron, Winnipeg MB, Canada) under 30/20 °C (day/night), 80% air relative humidity, a 12-h photoperiod (7:00 to 19:00 h) and a photosynthetic photon flux density (PPFD) of 800 µmol m^{-2} s^{-1}.

2.2. Water deficit treatments

Fifty-five day-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 the first day and −0.56 MPa the second day. These values were based on previous experiments with sugarcane (Silveira et al., 2016, 2017). Then, an osmotic potential of −0.56 MPa was maintained by replacing the solution with 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. not 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 three times (3WD). The water deficit cycles were similar in intensity and duration, and plants were the same age at the end of the experiment, as shown in Supplementary Material Fig. S1. Each water deficit cycle was five days in the nutrient solution with −0.56 MPa and other three days of recovery in nutrient solution with −0.12 MPa. During the experimental phase (Fig. S1), 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. This procedure was done on the fifth day of the water deficit cycle, i.e., the maximum water deficit.

2.3. 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) throughout the experimental period. The measurements were performed between 10:00 and 13:00 h under PPFD of 2000 µmol m^{-2} s^{-1} and an 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 ($k = A/C_i$) calculated according to Machado et al. (2009). The chlorophyll fluorescence was measured simultaneously with the 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/E_i) 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 h of the photoperiod. In the experimental phase (Fig. S1) and after plant rehydration, the relative recoveries of A and g_S were evaluated daily, considering the values of the reference plants as 100%.

2.4. Leaf water potential and relative water content

In the experimental phase, predawn 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 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 on the fifth day of the water deficit cycle and at the third day of recovery.

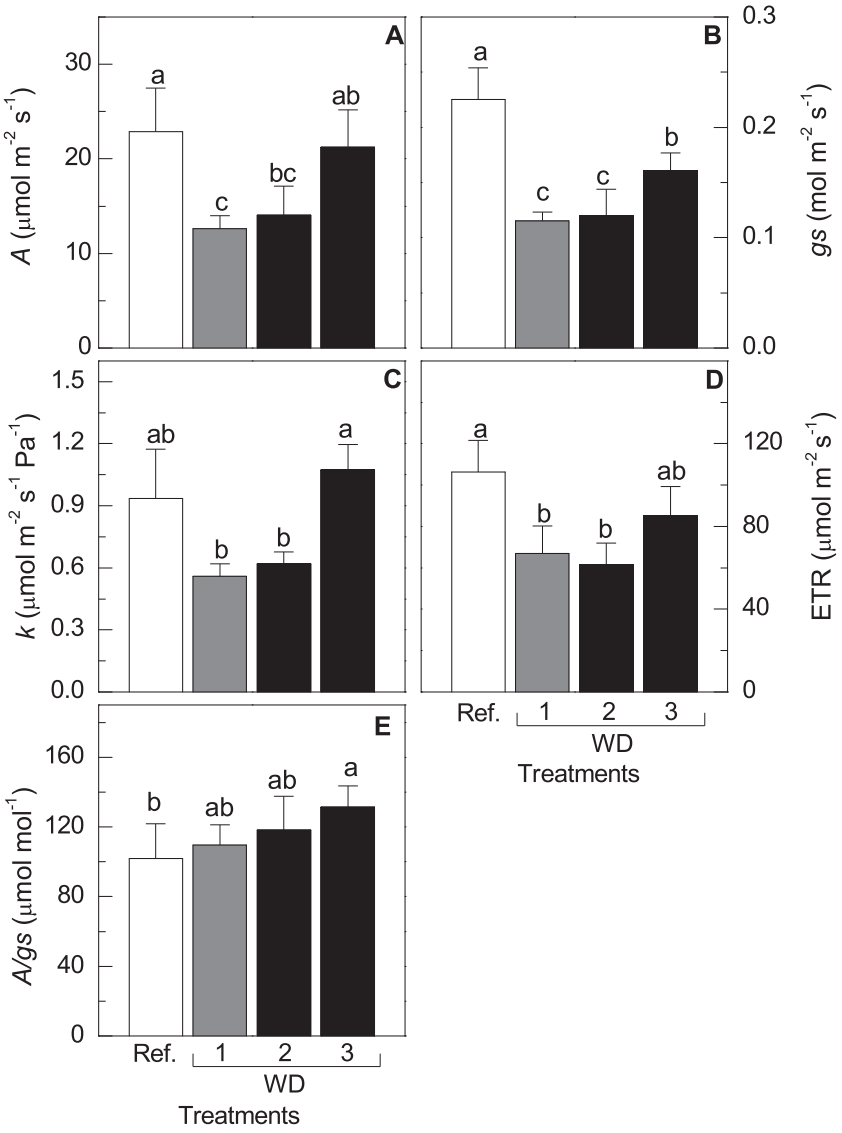

Fig. 1. Leaf CO_2 assimilation (A), stomatal conductance (B), instantaneous carboxylation efficiency (C), apparent electron transport rate (D) and instantaneous water-use efficiency (E) at the maximum water deficit level in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments.

2.5. Carbohydrates and proline

In the leaf and root samples, the extraction of total soluble carbohydrates (SS) was conducted 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 the 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 a reaction with a ninhydrin reagent (ninhydrin, acetic acid and orthophosphoric acid), glycine and acetic acid for 35 min at 100 °C. The reaction was stopped in an ice bath, and the reaction mixture was extracted with toluene. Then, the proline concentration was determined as conducted by Rena and Masciotti (1976).

2.6. Hydrogen peroxide and lipid peroxidation

The hydrogen peroxide (H_2O_2) content in the leaves and roots was quantified in 0.16 g of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and a 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 into 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 conditions for 1 h. After this period, the absorbance was read at 390 nm. The calibration curve was conducted with H_2O_2, and the results were expressed as μmol H_2O_2 g^{-1} FW. The malondialdehyde (MDA) concentrations in the leaf and root samples were measured and used as a parameter to evaluate lipid peroxidation. Plant tissue (0.16 g) was macerated in 1.5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 15 min. One aliquot of 0.5 mL of the supernatant was incubated with a 0.5% thiobarbituric acid solution in a 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 was 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.

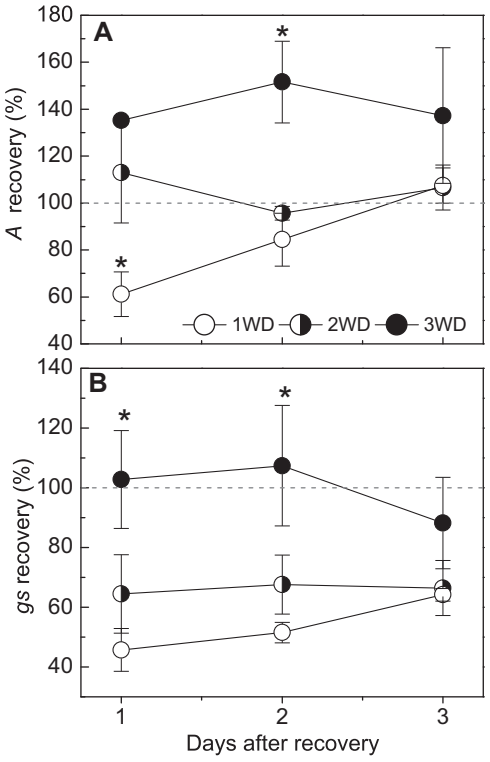

Fig. 2. Relative recovery of leaf CO_2 assimilation (A) and stomatal conductance (B) after rehydration of sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. Symbols represent the mean values ± SD (n = 4). Asterisks indicate significant differences ($p < .05$) among the treatments. Dotted lines at 100% indicate the *A* or *gs* values in the reference conditions, i.e., well-watered plants.

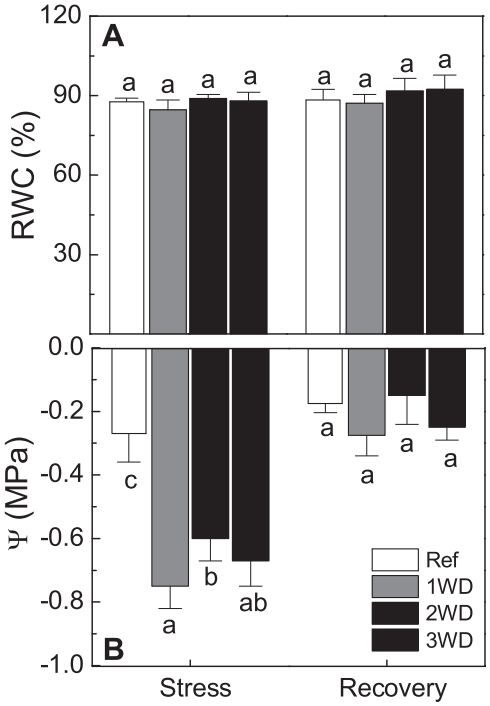

Fig. 3. Leaf relative water content (A) and predawn leaf water potential (B) at the maximum water deficit (Stress) and after three days of rehydration (Recovery) in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments.

2.7. Antioxidant enzymes: extraction and activity assays

The enzymatic extract was prepared with 0.2 g of fresh tissue (leaf or root) ground in liquid nitrogen, with 1% PVPP and 2 mL of the extraction medium composed of 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 carried out 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%, 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 measured to determine 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).

2.8. Abscisic acid (ABA) and its metabolites

Fresh leaf tissue samples were ground using liquid nitrogen, weighed (0.2 g) and placed in a capped plastic tube. The samples were extracted with 1.0 mL of methanol:water:acetic acid (10:89:1 v/v) overnight on a shaker at 4 °C under darkness (Silva et al., 2012). Then, the samples were centrifuged for 10 min at 12,000*g*, and the supernatant was dried in a N_2 stream. The dry extracts were suspended in 200 µL of methanol prior to the analysis. A chromatographic analysis was carried out using a UPLC Acquity chromatographer (Waters, Milford CT, USA) coupled with a TQD mass spectrometer (Micromass-Waters, Manchester, UK) with ESI source. We used a Waters Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm) equipped with a VanGuard BEH C18 precolumn (5 mm × 2.1 mm i.d., 1.7 µm) with both columns kept at 30 °C and a 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 a constant flow rate of 0.2 mL min^{-1}. Source and desolvation temperatures were set to 150 °C and 350 °C, respectively. The 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).

2.9. Biomass

Shoot and root dry matter were evaluated after drying the samples in a forced air oven at 65 °C, and the root/shoot ratio was estimated. We also calculated the growth in each treatment, using the total dry matter divided by the number of days in which the plants remained under well-

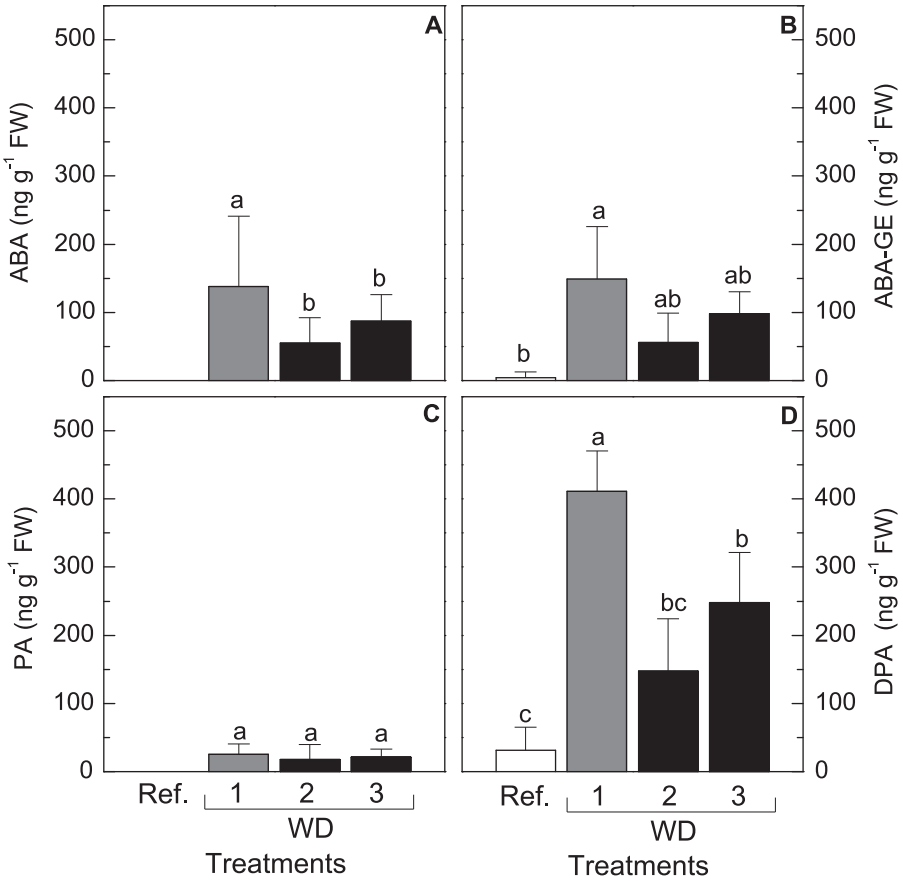

Fig. 4. Leaf concentration of abscisic acid (A), ABA-glucose ester (B), phaseic acid (C) and dihydrophaseic acid (D) at the maximum water deficit in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 5). Different letters indicate significant differences ($p < .05$) among the treatments.

watered conditions (i.e., nutrient solution with an osmotic potential of −0.12 MPa). Biometric evaluations were done at the end of the experimental period.

2.10. 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, and 3WD). Data were subjected to ANOVA procedure, and the mean values (n = 4 to 5) were compared by the Tukey test ($p < .05$) when significance was detected.

3. Results

3.1. Leaf gas exchange and photochemistry

Leaf gas exchange was evaluated every day for 28 days, including both the preparatory and experimental phases. The 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 of the reference plants, which did not experience any drought event (Supplementary Material Fig. S2). When considering the experimental phase, plants subjected to three cycles of water deficit showed photosynthesis similar to one of the reference plants after five days of water deficit (Fig. 1A). However, the stomatal conductance in plants that experienced three water deficit cycles was lower than that in the reference plants and higher than that in the plants exposed to one or two water deficit cycles (Fig. 1B). Plants exposed to three water deficit cycles also showed carboxylation efficiency similar to one of the reference plants and a higher carboxylation efficiency than that in the 1WD and 2WD treatments (Fig. 1C). Regarding the photochemistry, the apparent electron transport rate was similar among the reference plants and the plants subjected to three water deficit cycles (Fig. 1D). The intrinsic water-use efficiency increased as the number of drought events increased, with plants exposed to three water deficit cycles showing higher A/g_S than the reference plants (Fig. 1E). The ratio between the apparent electron transport rate and CO_2 assimilation revealed no differences among the treatments, varying between 5.3 μmol μmol^{-1} (1WD plants) and 4.0 μmol μmol^{-1} (3WD plants).

Photosynthesis recovery was also improved by previous exposure to water deficit, with the photosynthesis of plants exposed to three water deficit cycles exceeding the values found for the reference plants by 35% on the first day of recovery (Fig. 2A). While plants subjected to two water deficit cycles also reached full recovery of photosynthesis on the first day of rehydration, plants facing water deficit for the first time showed a full recovery of photosynthesis only after the third day of rehydration (Fig. 2A). Only plants subjected to three water deficit cycles presented stomatal conductance similar to that found in the reference plants during the first two days of rehydration (Fig. 2B).

By integrating A and E during the experimental phase, we verified that plants exposed to three water deficit cycles had a higher A_i than the reference plants, which presented higher values than the plants exposed to one or two water deficit cycles (Supplementary Material Fig. S3A). E_i was reduced by water deficit, but it increased with the number of drought events (Fig. S3B). As a result of the changes in A_i and E_i, plants exposed to three water deficit cycles had a higher integrated water-use efficiency than the reference plants (Fig. S3C).

3.2. Leaf water status

While the leaf relative water content was not affected by water regimes (Fig. 3A), the leaf water potential was reduced by water deficit (Fig. 3B). During the recovery period, there were no differences among the treatments for both variables (Fig. 3).

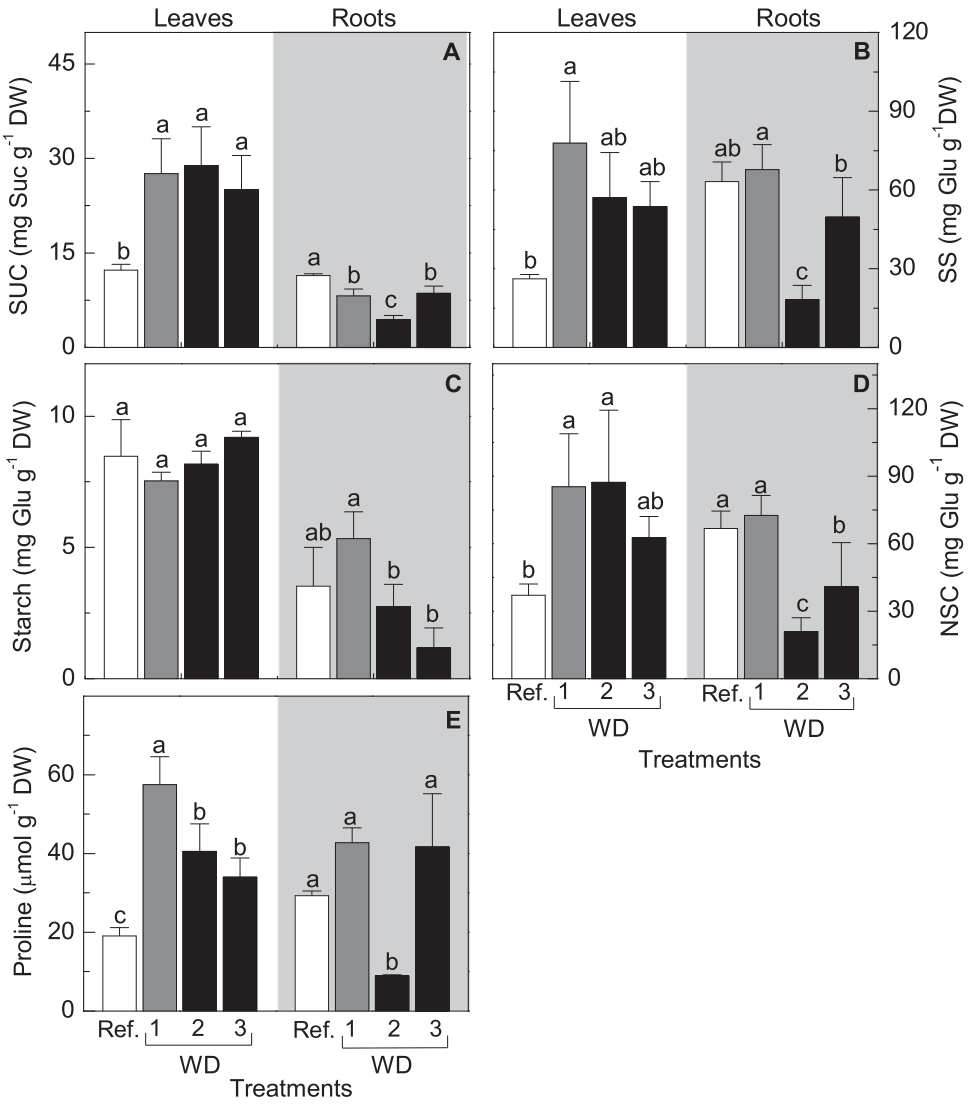

Fig. 5. Leaf and root concentrations of sucrose (A), soluble sugars (B), starch (C), non-structural carbohydrates (D) and proline (E) at the maximum water deficit in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments.

3.3. ABA and its derivatives

The 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. 4). In general, plants exposed to water deficit had higher levels of ABA, ABA-GE, PA, and DPA than the reference plants (Fig. 4). However, plants exposed to two or three water deficit cycles exhibited lower concentrations of ABA and DPA compared to the plants facing a water deficit for the first time (Fig. 4A and D).

3.4. Leaf and root carbohydrates and proline

Plants exposed to one, two or three water deficit cycles showed higher leaf sucrose concentrations than the reference plants (Fig. 5A). On the other hand, root sucrose concentrations were decreased by water deficit, and the lowest concentrations were found in plants exposed to two water deficit cycles (Fig. 5A). Plants exposed to one water deficit cycle also presented higher leaf content of soluble sugars than the reference plants (Fig. 5B). Only plants exposed to two water deficit cycles presented reductions in root soluble sugar concentrations (Fig. 5B). Leaf starch concentrations were not changed by the treatments; however, plants exposed to one water deficit cycle showed higher root starch concentrations than the plants exposed to two or three water deficit cycles (Fig. 5C). Leaf concentrations of non-structural carbohydrates increased in plants exposed to one or two water deficit cycles, whereas only plants exposed to two or three water deficit cycles presented reductions in root concentrations of non-structural carbohydrates (Fig. 5D). Leaf proline concentrations were increased by water deficit and the highest values were found in plants exposed to one water deficit cycle (Fig. 5E). In the roots, proline concentrations were reduced only in plants that were exposed to two water deficit cycles (Fig. 5E).

3.5. Antioxidant activity

Leaf H_2O_2 concentrations were not affected by the treatments, while plants exposed to three water deficit cycles presented higher root H_2O_2 concentrations than the reference plants (Fig. 6A). Leaf MDA concentrations were reduced by water deficit only in plants exposed to two water deficit cycles. Root MDA concentrations followed the same pattern of root H_2O_2 concentrations, i.e., the highest values were found in plants exposed to three water deficit cycles (Fig. 6B). Leaf SOD activity increased in plants exposed to two water deficit cycles, whereas the highest leaf APX activity was found in plants facing a water deficit for

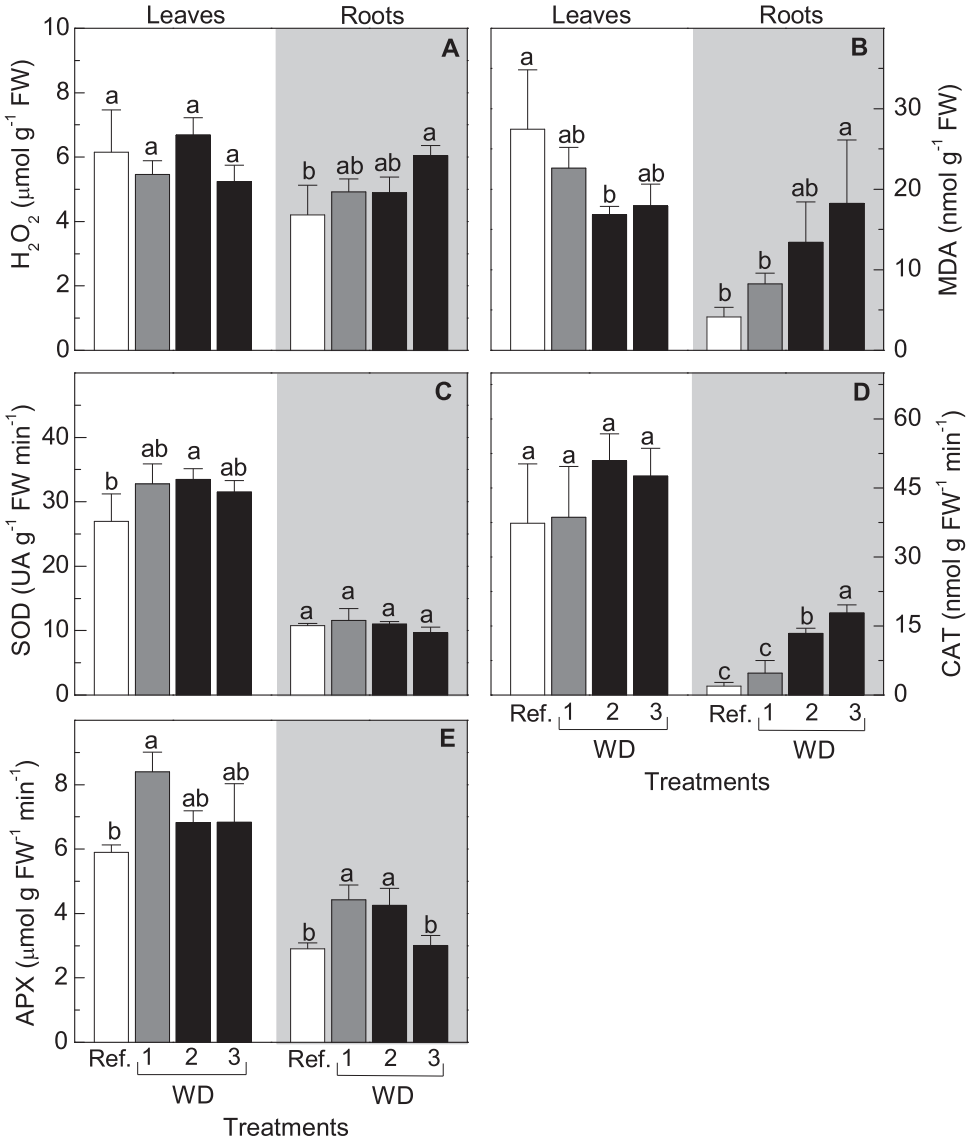

Fig. 6. Concentrations of H_2O_2 (A) and malondialdehyde (B) and activities of superoxide dismutase (C), catalase (D) and ascorbate peroxidase (E) at the maximum water deficit in leaves and roots of sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments.

the first time (Fig. 6C and E). Root SOD activity was not affected by the treatments and root APX activity was increased in plants exposed to one or two water deficit cycles (Fig. 6C and E). Leaf CAT activity was not changed by the treatments (Fig. 6D). In terms of roots, there was a significant increase in CAT activity, and the highest values were found in plants exposed to three water deficit cycles, which was 9 times higher than that in the reference plants (Fig. 6D).

3.6. Plant biomass and growth

At the end of the experimental phase, shoot dry matter was similar in the reference plants, 2WD plants, and 3WD plants, with 1WD plants showing higher shoot dry matter (Fig. 7A). Root dry matter and root:shoot ratio increased in plants exposed to three water deficit cycles compared to the reference plants (Fig. 7B and C). By considering the time spent under well-watered conditions (i.e., 28, 22, 16 and 10 days for the reference and 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 water deficit cycles presented the highest growth (Fig. 8), i.e., they were more efficient in using water resources.

4. Discussion

4.1. Sugarcane photosynthesis is benefited by repetitive cycles of drought/rehydration

Photosynthetic rates of plants exposed to three water deficit cycles were similar to the rates found in the reference plants (Fig. 1A), and the rates were even higher when considering the integrated CO_2 gain during the experimental period (Fig. S3A). In fact, a better performance was the result of higher photosynthetic rates during the recovery period (Fig. 2A). Under water deficit, both photosynthesis and stomatal conductance of plants exposed to three water deficit cycles were higher than that found in plants subjected to one or two water deficit cycles (Fig. 1A, B), suggesting that stomatal aperture was one factor leading to the better photosynthetic performance of 3WD plants under low water availability.

The higher stomatal conductance of 3WD plants was associated with increases in root biomass (Fig. 7B), which likely improved water uptake from the nutrient solution. In addition, 3WD plants had higher root to shoot ratios (Fig. 7C), indicating changes in carbon partitioning and investment in root structure. Besides these morphological changes that

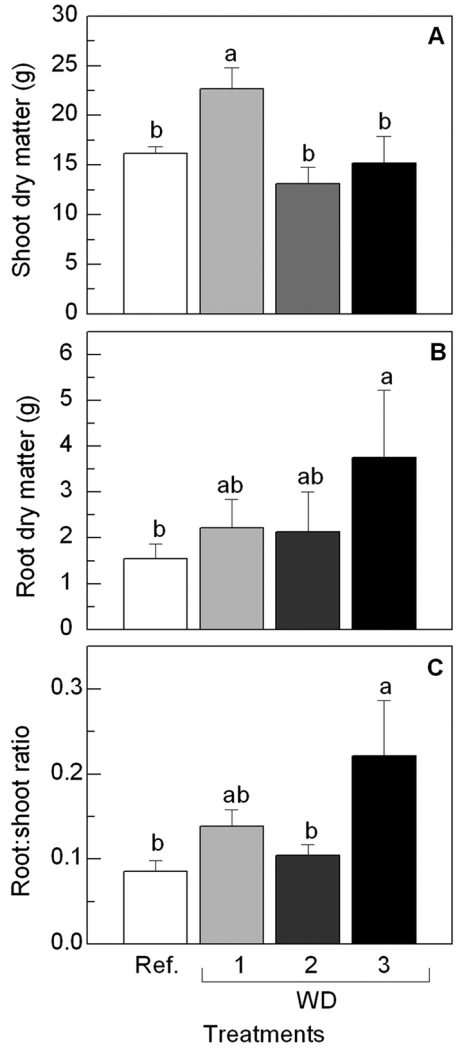

Fig. 7. Shoot dry matter (A), root dry matter (B) and root/shoot ratio (C) in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments. Measurements were taken at the end of the experimental period.

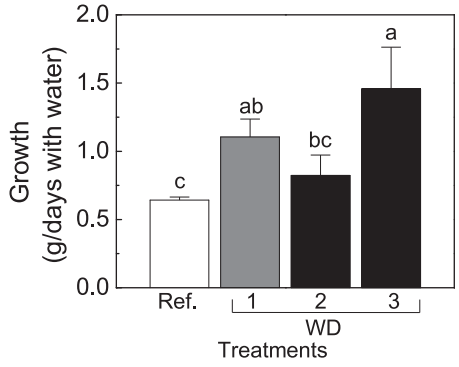

Fig. 8. Growth given by the total biomass normalized by the number of days under well-watered conditions in sugarcane plants subjected to one (1WD), two (2WD) and three (3WD) water deficit cycles. The reference (Ref.) plants were maintained under well-watered conditions. Histograms represent the mean value ± SD (n = 4). Different letters indicate significant differences ($p < .05$) among the treatments. Measurements were taken at the end of the experimental period.

support a higher stomatal aperture, our data suggest that ABA has a role in stomatal conductance of sugarcane plants under water deficit. We noticed that 3WD plants had lower leaf ABA concentrations than 1WD plants (Fig. 4A), as well as a lower amount of DPA, a product of ABA oxidation (Fig. 4D). Thus, we can argue that the amount of DPA was higher in 1WD plants due to a large amount of ABA being produced and oxidized (Fig. 4). Based on Virlouvet and Fromm (2015), plants previously exposed to drought have low stomatal conductance caused by ABA biosynthesis. Such associations between ABA and stomatal conductance were found in this study, with the lowest g_s values found in plants with the highest concentrations of ABA and its derivatives (Fig. 4). Interestingly, sugarcane plants subjected to three water deficit cycles did not present such high levels of ABA and DPA, which are a likely consequence of better hydration and/or changes in ABA metabolism caused by repetitive cycles of water deficits.

In addition to ABA actions 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 membrane structure (Verlues 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 the reduction in leaf water potential in plants under stress (Fig. 3). Increases in leaf concentrations of sucrose and proline, two important osmolytes, were found in plants subjected to water deficit (Fig. 5A and E). Osmoprotective molecules such as proline ensure the preservation of protein structures and functions under low water availability (Wingler, 2002; Verlues et al., 2006). However, the number of times that plants were exposed to water deficit cycles did not differentially affect this proposed osmoregulation. As proline is produced under stressful conditions (Szabados and Savouré, 2010), low proline concentration in the 3WD plants may also indicate that they were less stressed compared to the 1WD plants.

Higher instantaneous carboxylation efficiency in the 3WD plants suggests improvements in PEPC activity, another factor leading to improved photosynthesis in these plants (Fig. 1A and C). Alternatively, photosynthesis could be stimulated by root growth (Fig. 7B), an active sink for photoassimilates in 3WD plants. In fact, we previously found that sugarcane photosynthesis is very sensitive to changes in source-sink relationship (Ribeiro et al., 2017). Higher photosynthesis consumes more NADPH and ATP stimulating photochemical activity and causing higher ETR (Fig. 1D). As leaf CO_2 assimilation decreased due to water deficit in 1WD and 2WD plants and light energy that reached the leaves remained similar, plants faced excess energy, and these conditions lead to the accumulation of ROS and consequent oxidative stress (Foyer and Shigeoka, 2011).

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

Enzymatic antioxidant metabolism is one of the mechanisms that plants have to avoid oxidative damage in cell structure and functioning induced by ROS. In this study, leaf H_2O_2 and MDA concentrations did not suggest any oxidative damage due to water deficit (Fig. 6B). Changes in SOD and APX activities were likely able to maintain the redox states in the leaves of the plants under water deficit (Fig. 6C and E). One important issue is that the maintenance of photosynthetic rates in 3WD plants led to less excess energy, being the main sink of excitation energy at the 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 the treatments (Fig. 6A, B, D). These findings suggest a controlled increase in H_2O_2 levels as CAT activity, an important enzyme involved in its degradation, increased. As roots elongate, H_2O_2 was produced and MDA concentrations increased (Hu et al., 2015) and then, our data indicate that 3WD plants had high cell membrane turnover. In fact, H_2O_2 may be an oxidant and a secondary messenger in signal transduction due to its long half-life and relatively

high permeability through membranes (Cheeseman, 2007; Silva et al., 2015). The MDA and H_2O_2 concentrations in the plants under water deficit are similar to those found in previous experiments with sugarcane (Silveira et al., 2017), indicating that plants were not under severe water deficit.

4.3. Improved drought tolerance and chemical signals

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) and ROS (Foyer and Shigeoka, 2011) can lead to improved performances. Although leaf ABA accumulation has been found in sugarcane plants subjected to three water deficit cycles, ABA accumulation affected only the intrinsic water-use efficiency through reduced stomatal conductance (Figs. Fig. 11B,E and Fig. 44A). Indirectly, one would expect benefits of such a 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 concentrations with increasing plant exposure to water deficit (Fig. 6A), without any oxidative damage and with plants showing increases in root dry matter, root/shoot ratio and growth (Figs. 7 and 8). Our data revealed that improvements in plant performance under water deficit caused by previous exposure to water deficit were associated with biochemical signals. An essential issue is the evaluation of plant performance and physiological status after stressful events (Walter et al., 2011). As plants previously exposed to three water deficit cycles presented higher growth and higher photosynthetic performance under well-watered conditions (Figs. Fig. 22A and Fig. 88) than ones exposed to one or two water deficit cycles, we may argue that sugarcane plants stored this information and used it to their benefit. Currently, we know that epigenetics plays an important role in storing information and helping plants to adjust their metabolism to environmental fluctuations (Hauser et al., 2011; Grafi and Ohad, 2013), an issue that should be further investigated in sugarcane plants.

In this study, plants exposed to three water deficit cycles exhibited morphological and physiological changes associated with increases in both photosynthesis and growth (Figs. 1, 7 and 8). From a practical perspective, our data indicate that sugarcane tolerance to water deficit may be improved under husbandry conditions while saving water and energy through less frequent irrigation. Finally, we found that sugarcane plants can incorporate information from previous stressful events to improve their performance under low water availability. As a chemical signal, our data revealed the controlled accumulation of H_2O_2 in the roots, which was associated with increases in root growth.

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Authors' contribution

Conception and design of this study (FCCM, ECM, MGAL and RVR); Collection of physiological data (FCCM, NMS and PERM) and ABA analysis (JBM and ACHFS); Analysis and interpretation of the data (FCCM, ECM, GMS and RVR); Drafting of the article (FCCM and RVR); Critical revision and final approval of the article (all authors).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jplph.2018.02.001.

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