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ORIGINAL ARTICLE

Physiological and biochemical responses of sugarcane to oxidative stress induced by water deficit and paraquat

Claudiana Moura dos Santos[1] · Marcelo de Almeida Silva[1]

Abstract Physiological and biochemical responses to oxidative stress induced by paraquat or water deficit were examined in this study with respect to photoinhibition, stomatal conductance, photosynthetic pigments, organic solutes, and antioxidant enzymes, aiming at a better understanding of the defense networks connected with antioxidant agents in sugarcane and also to identify cultivars more tolerant to such stress conditions. In the drought-induced experiment, the plants were exposed to 15 days without irrigation, and in the study with paraquat (PQ), 150 μM of the herbicide was applied to the plants 24 h before the evaluations, conducted simultaneously with the drought experiment. In the second part of the experiment leaf disks were cut from cultivars and immersed in a paraquat solution in Petri dishes and analyzed for chlorophyll degradation. In cultivars SP83-2847 and IAC91-5155, smaller reductions were observed in the maximum photochemical efficiency of photosystem II (F_v/F_m) and photosynthetic pigments, when submitted to water deficit and treated with paraquat, and greater reductions were found in cultivars RB92579 and SP81-3250 which had lower dry matter under drought stress. Paraquat produced a toxic effect because of degradation of proline and carbohydrates in cultivars. Both stressors led to an increase in the activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzymes in most of the cultivars, confirming the importance of interaction of these enzymes against oxidative stress damages.

Abbreviations

+D	Water-deficit treatment
Ψ_w	Leaf water potential
APX	Ascorbate peroxidase
C	Control treatment
Car	Carotenoids
CAT	Catalase
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
Chl *total*	*Total* Chlorophyll
DW	Dry weight
F_v/F_m	Maximum photochemical efficiency of photosystem II
FW	Fresh weight
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
MDHA	Monodehydroascorbate reductase
NBT	Nitro blue tetrazolium
PAR	Photosynthetically active radiation
POX	Non-specific peroxidase
PQ	Paraquat
ROS	Reactive oxygen species
RDM	Root dry mass
RWC	Leaf relative water content
SDM	Shoot dry mass
SPAD	Estimated chlorophyll content
SOD	Superoxide dismutase
gs	Stomatal conductance

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✉ Marcelo de Almeida Silva
marcelosilva@fca.unesp.br

Claudiana Moura dos Santos
claudianabio@hotmail.com

[1] Department of Crop Production and Breeding, College of Agricultural Sciences, São Paulo State University (UNESP), PO Box 237, Botucatu, SP 18603-970, Brazil

SW   Saturated weight
TDM   Total dry mass

Introduction

Crops are often exposed to various environmental stress conditions, such as cold, intense light, drought, air pollution, herbicides, and pathogenic infection, many of which are associated with an excessive growth of reactive oxygen species (ROS) (Apel and Hirt 2004; Darkó et al. 2009). Under severe stress, over production of ROS causes oxidative stress on plants, resulting in increased damages, especially in cell components such as chlorophylls, lipids, proteins, and nucleic acids, and most likely to reduced yield or cell death (Mittler 2002). As a consequence, oxidative damages translate into significant changes affecting growth, development, and productivity of important sugarcane cultures.

Sugarcane, in particular, is an economically important crop because of its world-wide use in the food industry as well as a valuable source for the production of renewable biofuels. It is grown in tropical and subtropical regions in over 90 countries, and Brazil is the world's leading sugarcane producer (Waclawovsky et al. 2010).

Among the environmental stresses, drought or water deficit is one of the biggest problems of present times, affecting a great part of the areas of sugarcane plantations across the world, and is the main crop yield-limiting factor (Jangpromma et al. 2010a; Silva et al. 2014). A consequence of water stress is the limited photosynthesis that leads to the plant exposure to the excess of the energy photosynthesis, which if not dissipated safely may cause overexcitement of the reaction centers of photosystems II and I, raising ROS production in chloroplasts and causing a phytotoxicity effect in plants (Carvalho 2008).

Phytotoxic effects in chloroplasts may also be caused by stress induced by the herbicide paraquat, which, in contact with the plant, acts by accepting electrons from the primary acceptor of photosystem I. Interception of electrons in photosystem I stops the ferredoxin reduction, thus interfering with the reduction of $NADP^+$ to NADPH in the stroma, which would be used in the Calvin cycle to reduce CO_2 (Ekmekci and Terzioglu 2005; Taiz and Zeiger 2010). When paraquat is photoreduced in photosystem I, it exerts its phytotoxic effects by catalyzing the transfer of electrons to photosystem II from photosystem I and is then oxidized again by the transfer of electrons to O_2 in the presence of light. Upon receiving electrons, paraquat reacts with molecular oxygen, producing superoxide radical (O_2^-), resulting in the accumulation of O_2^- in chloroplasts, which causes oxidative stress in plants (Iturbe-Ormaetxe et al. 1998; Sood et al. 2011).

To protect against the harmful effects of ROS produced during oxidative stress, plants have a complex antioxidant defense system encompassing low-molecular-mass antioxidants, such as hydrophilic molecules, represented by ascorbic acid and reduced glutathione, lipophilic molecules, such as α-tocopherol and carotenoids, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), non-specific peroxidase (POX), glutathione reductase (GR), and monodehydroascorbate reductase (MDHA), which act in the reduction of ROS, mitigating the oxidative damages in the plant cell (Mittler 2002; Sharma et al. 2012).

Paraquat besides being widely used as a herbicide desiccant, has been a very useful tool in the research of basic biology of plants, in the simulations of oxidative stress, with successful reported results in maize (Darkó et al. 2009; Shahrtash et al. 2011), wheat (Sedigheh et al. 2011), rice (Guo et al. 2007), in addition to vegetable species such as cucumber (Lin et al. 2011) and pumpkin (Yoon et al. 2011). These studies reported oxidative damages such as photoinhibition, loss of photosynthetic activity, proteins breakdown, reduction of photosynthetic pigments, and chlorosis associated with the activities of the antioxidant enzymes, enabling a better understanding of many protective mechanisms against oxidative stress.

Currently, considerable attention has been given to the selection of sugarcane varieties tolerant to biotic and abiotic stresses, based on the optimum performance of the plant in the antioxidant activity against oxidative stress, as found in studies on water deficit (Patade et al. 2011; Cia et al. 2012), salinization (Gomathi and Rakkiyapan 2011), temperature rise (Srivastava et al. 2012), and flood (Gomathi et al. 2012). However, comparative studies on drought- and paraquat-induced stress involving physiological responses and biochemical damages in sugarcane plants are still scarce. Thus, our objective was to compare the physiological and biochemical responses of sugarcane grown under water stress with plants submitted to oxidative stress. The herbicide paraquat was used to induce oxidative stress. The assays can help to better understand the sugarcane antioxidant network and to provide a basis for selection of varieties with improved oxidative stress tolerance.

Materials and methods

Experimental procedure and plant material

The experiment was conducted in greenhouse. Cultivars were selected from the most representative species grown

in the Center-South region of Brazil, according to Chapola et al. (2009). Four sugarcane cultivars were used: RB92579, SP83-2847, SP81-3250, and IAC91-5155.

The plants were obtained from shoot buds from seed-pieces of the same age. The seedpieces were planted in 22-L pots and filled with Plantmax® substrate (sterile product prepared with expanded vermiculite and organic material). During the experiment, the average air temperature was 27.4 ± 5 °C and the daily average relative humidity was 62.8 %, while the daily average of photosynthetically active radiation (PAR) was 800 μmol m^{-2} s^{-1}.

Eighty-five days after planting, the cultivars were submitted to three experiments: appropriate water regime (control, C), water deficit (+D), and induced stress by herbicide paraquat (+PQ). In the control experiment (C), the plants were irrigated as necessary to keep the optimum moisture content in the substrate, i.e., around 22 % (corresponding to 100 % of the field capacity), and in the experiment with water deficit (+D) watering was interrupted for 15 days until moisture content in the substrate reached 10 %. To monitor the moisture in the pots, ECH2O moisture sensor (Decagon, Washington, USA) was used, which is considered an effective device for such control (Silva et al. 2013).

The experiment with paraquat (+PQ) occurred 85 days after planting, simultaneously with the beginning of the experiment with water deficit. The plant of each pot was entirely sprayed with paraquat at a concentration of 150 μM, and the effect was evaluated 24 h after application.

Before this experiment, preliminary studies were conducted in leaf disks (0.69 cm^2), were floated in PQ solutions (0, 50, 100, 150, 200 μM) for different durations (0, 24, 48, 72, and 96 h), and the whole experiment was conducted under the constant light exposure of 120 μmol m^{-2} s^{-1}. Chlorophyll degradation (a measure of PQ resistance) was monitored using reading spectrophotometer. The 150 μM PQ solution was found to be the most optimum screening concentration since it triggered quantifiable changes in chlorophyll content within 24 h.

The experimental design used was totally randomized in 4×3 factorial design, the factors being: four cultivars and three conditions of stress (control = C, water deficit = +D, and paraquat +PQ), with four replicates. The physiological evaluations and collection of samples for biochemical analyses were performed in the +D experiment 15 days after the induced stress, and in the +PQ experiment 24 h after application of the herbicide.

A second stage of the experiment, in the same period of time, was conducted with leaf segments to evaluate the photosynthetic pigment degradation after the application of paraquat, compared with the plants submitted to water deficit (+D) for 15 days. For this purpose, leaves of the plants were cut into disks (0.69 cm^2), and half of these leaf disks were immersed in a solution of paraquat for 24 h in Petri dishes with 150 μM dissolved in 30 mL of deionized water, and the other half was immersed in deionized water only. Then, all samples were kept in a growth chamber at a temperature of 27 °C and continuous light intensity of 120 μmol m^{-2} s^{-1} for 24 h.

Physiological trait evaluations

For leaf relative water content (RWC), we determined fresh weight (FW), saturated weight (SW), and dry weight (DW) in leaf disks extracted from the +2 leaves. RWC was estimated according to the following equation: RWC = $[(FW - DW)/(SW - DW)] \times 100$. Leaf water potential ($\Psi_w$) was evaluated between 10 a.m. and noon using a Scholander pressure chamber (SoilMoisture Equipment, Santa Barbara, CA, USA). Plants were collected and separated into shoot and roots. Shoot dry mass (SDM), root dry mass (RDM), and total dry mass (TDM) were determined after drying the plant tissues in an oven at 65 °C for 6 days.

The maximum photochemical efficiency of photosystem II (F_v/F_m) was determined by means of a portable fluorometer (Opti-Sciences, Inc., Hudson, NH, USA) after adapting the leaves in darkness for 30 min using leaf clipping. Readings were taken with saturating light pulses of 1 s to close the reaction centers of PSII, according to the method described by Maxwell and Johnson (2000). Stomatal conductance, gs (mmol m^{-2} s^{-1}) was determined by means of a porometer (Decagon Devices Inc., Pullman, WA, USA). Readings were taken at the middle region of a fully expanded leaf +1 at the abaxial side. Estimated chlorophyll contents via SPAD reading (SPAD index) were determined using a SPAD-502 chlorophyll meter (Minolta Corp., Ramsey, NJ, USA). Readings were taken at the middle region of a fully expanded leaf +1 in 10–12 h in the control and drought experiments and 24 h following the application of paraquat, measuring one leaf of each plant.

Proline, carbohydrate, and pigment measurement

The free proline in the lyophilized leaf tissue was measured by a reaction with ninhydrin according to the method of Bates et al. (1973), with modifications. Approximately 100 mg, dry weight, of tissue was macerated in liquid nitrogen and homogenized in 8 mL of 118 mM sulfosalicylic acid. The homogenate was then centrifuged at 3000g for 10 min at 25 °C, and the supernatant was used for the quantitative analysis. Readings of absorbance was taken at 520 nm. Only toluene was used as blank and pure proline as standard.

Total soluble carbohydrates were determined according to the method described by Dubois et al. (1956) using 100 mg of dried leaf material. 500 μL of extract was combined with 1 mL 0.56 M phenol and 5 mL of concentrated sulfuric acid. The carbohydrates were quantified by measuring the absorbance at 490 nm, using D-(+)-glucose as a standard.

The analysis of chlorophylls *a, b, total* and carotenoids was according to the method described by Lichtenthaler (1987), where the extract was obtained using leaf disks with 0.69 cm^2 immersed in 2 mL of dimethylformamide solvent, and subsequent reading of absorbance was taken by a spectrophotometer at 480, 647, and 664 nm wavelengths.

Assay of enzymatic antioxidants

The superoxide dismutase (SOD, E.C.1.15.1.1) activity was determined according to the method described by Beauchamp and Fridovich (1973). The extract was obtained from 300 mg of fresh leaf tissue, and 100 μL was taken from the vegetable extract and put into the reaction medium, and then the homogenate was centrifuged at 14,000g for 15 min at 4 °C. This medium was prepared with 50 mM potassium phosphate buffer (pH 7.8), 100 nM EDTA, 75 μM nitro blue tetrazolium (NBT), 13 mM methionine and 2 μM riboflavin, and final volume of 3 mL distilled water. Then, readings were taken by a spectrophotometer at 560 nm.

The catalase (CAT, EC 1.11.1.6) activity was determined according to Havir and Mchale (1987) with some changes. The extract was obtained following the method described by Kar and Mishra (1976), and from 400 mg of leaf material 150 μL was taken from the extract and put in the reaction medium with 50 mM potassium phosphate buffer (pH 7.0) and 50 mM H_2O_2, and final volume of 3 mL distilled water. Once homogenized, the extract was centrifuged at 14,000g for 15 min at 4 °C, and the supernatant was used for the analysis. Readings of absorbance were taken at 240 nm for 1 min and the decrease of H_2O_2 concentration was observed. An H_2O_2 extinction coefficient of 36 mM^{-1} cm^{-1} at 240 nm was adopted.

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method described by Nakano and Asada (1981) with some changes. The extract was obtained from 20 mg of fresh leaves, and 30 μL of the extract was taken and put into the reaction medium, and then the homogenate was centrifuged at 14,000g for 15 min at 4 °C. This medium was prepared with 50 mM potassium phosphate buffer (pH 7.5), 0.05 mM H_2O_2, and 0.5 mM sodium ascorbate, and the volume was completed with 3 mL of distilled water. Readings of absorbance were taken at 290 nm for 1 min, observing the decrease of

ascorbate concentration. An ascorbate extinction coefficient of 2.8 mM^{-1} cm^{-1} at 290 nm was used. Soluble proteins were quantitatively determined following the method of Bradford (1976).

Statistical analysis

The experimental data were submitted to the analysis of variance (F test) and means were compared by the Tukey test at 5 % probability. Pearson's (r) correlation was used to verify the relation between the physiological variables and the biochemical components in the studied period.

Results

Responses of physiological trait to stress induced by water stress and paraquat

After 15 days of water suppression, the lowest leaf relative water content (RWC) was observed in cultivar RB92579, with a mean RWC of 74 %, followed by SP81-3250 with a RWC of 77.3 % and SP83-2847 with a RWC of 84.5 % (Fig. 1a). There was a marked decrease in leaf water potential (Ψ_w) under water deficit in cultivar SP81-3250 (−2.3 MPa), followed by SP83-2847 (−1.8 MPa), RB92579 (−1.7 MPa), and IAC91-5155 (−1.7 MPa) (Fig. 1b).

Under the action of paraquat (+PQ), smaller reductions of the maximum photochemical efficiency of photosystem II (F_v/F_m) were observed in cultivars SP83-2847 and IAC91-5155, reaching values of 0.77 and 0.71, respectively. Lower values of the F_v/F_m ratio were observed in cultivars RB92579 (0.64) and SP81-3250 (0.63), corresponding to reductions of 19 and 20.2 %, respectively (Fig. 2a). Under water deficit (+D) for 15 days, similar mean values of F_v/F_m were found for the cultivars SP81-3250 (0.63) and RB92579 (0.66), indicating a greater photoinhibition and sensitivity of these cultivars to both stress conditions, while cultivars IAC91-5155 and SP83-2847 presented F_v/F_m means of 0.74 and 0.76, respectively, i.e., close to the control (Fig. 2a).

With respect to stomatal conductance, a decrease of gs values in the plants submitted to paraquat stress (+PQ) and water deficit (+D) was found (Fig. 2b). Results indicate greater decreases of gs in cultivars SP81-3250 and RB92579, of 84.6 and 68.6 %, respectively, when treated with +PQ. But reductions of *gs* were also observed in cultivars SP83-2847 (36.8 %) and IAC91-5155 (47.8 %) under +PQ-induced stress (Fig. 2b). In the same period, under water stress, the reduction of stomatal conductance was found in all cultivars, IAC91-5155 (73.8 %), SP83-
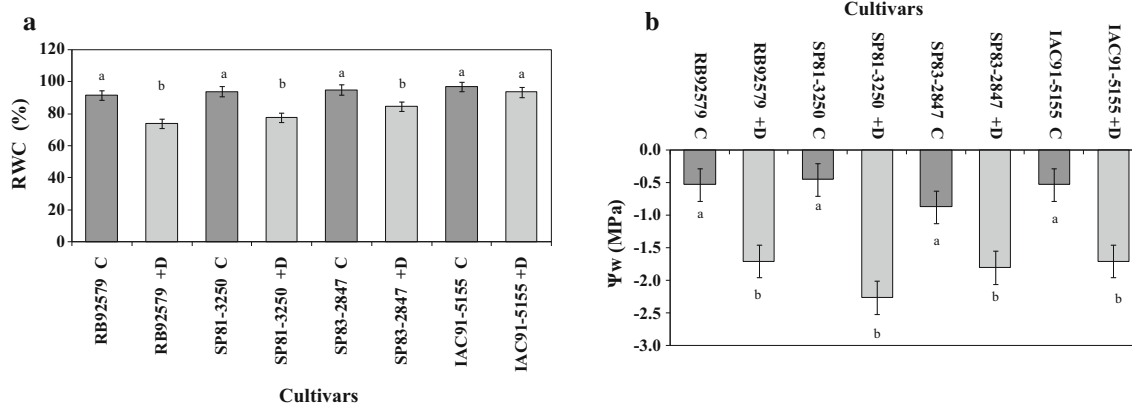
Fig. 1 Leaf relative water content, RWC (**a**) and leaf water potential, Ψ_w (**b**), in cultivars RB92579, SP81-3250, SP83-2847, and IAC91-5155 subjected to adequate water regimens (control, C) and water deficit (+D) for 15 days. Means followed by the *same letter* do not differ at 5 % probability by Tukey test
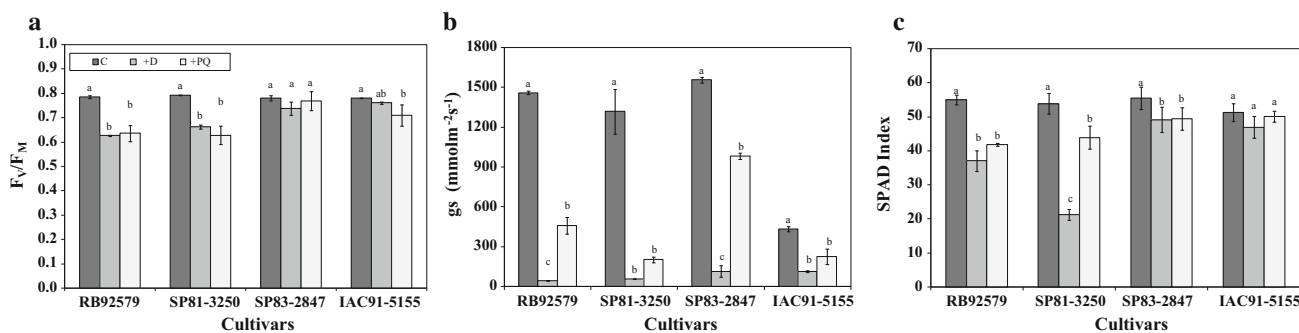
Fig. 2 Maximum photochemical efficiency of photosystem II, F_v/F_m (**a**), stomatal conductance, gs (**b**), and chlorophyll estimated content, SPAD index (**c**) in cultivars RB92579, SP81-3250, SP83-2847, and IAC91-5155 submitted to appropriate water regime (control, C), water deficit (+D), and concentration of 150 μM of paraquat (+PQ) for 24 h. Means followed by the *same letter* do not differ at 5 % probability by Tukey test

2847 (92.6 %), RB92579 (97 %), and SP81-3250 (96 %) (Fig. 2b).

It can be seen that gs presented positive correlation with F_v/F_m (0.64**) and SOD enzyme (0.60*) under +PQ-induced stress, and under water deficit gs correlated positively with F_v/F_m (0.64**), SPAD (0.57*), *total* chlorophyll (0.67**), and antioxidant enzymes SOD (0.70*), CAT (0.56*), and APX (0.56**) (Table 1), indicating that the reduction of stomatal conductance is associated with the oxidative damages found in both stress conditions.

Regarding the estimated chlorophyll content no difference was found between experiments +D and +PQ in cultivar IAC91-5155. Cultivar SP83-2847, despite a difference, maintained mean values of ~50 for SPAD index (Fig. 2c). Cultivars RB92579 and SP81-3250 showed reductions of 24 and 18.5 %, respectively, of SPAD index in the experiment with paraquat, and of 33 and 60.6 %, respectively, under water deficit (Fig. 2c), which indicates a more significant sensitivity of these cultivars to water stress with regard to this physiological attribute.

Under water deficit, it was found a positive correlation between the SPAD index and F_v/F_m (0.64*), *total* chlorophyll (0.97**), carotenoids (0.77**), and CAT enzyme (0.59*). When treated with +PQ, the SPAD correlated positively with F_v/F_m (0.60**) and with the antioxidant enzymes SOD (0.70**), CAT (0.70**), and APX (0.56**), and inversely with carotenoids (−0.51*) (Table 1). Such positive correlations suggest that the reduced photosynthetic pigments are associated with the damages caused by photoinhibition and inactivation of antioxidant enzymes during the oxidative stress in this study.

Effects of water stress and paraquat-induced stress on photosynthetic pigments

The decreased chlorophyll contents were more pronounced under paraquat-induced stress in cultivar SP81-3250, with a reduction of 65 % for Chl *a*, 55.4 % for Chl *b*, and 62.4 % for Chl *total*, and in cultivar RB92579, 55.4 % for Chl *a*, 50 % for Chl *b*, and 60 % Chl *total* (Fig. 3a, b and

Table 1 Pearson correlation coefficients between the physiological and biochemical variables of sugarcane submitted to appropriate water regime (C), water deficit (+D), and oxidative stress induced by paraquat (+PQ)

Variables	SPAD	F_V/F_M	Chl_{total}	C_{ar}	Prol	SOD	CAT	APX
Control								
gs	0.62*	0.14	0.67*	0.85**	−0.81**	−0.19	−0.60	−0.67*
SPAD		0.08	0.70**	0.65*	−0.55	−0.06	−0.43	−0.28
F_V/F_M			0.24	0.48	0.24	−0.63*	−0.08	−0.39
Chl_{total}				0.85**	−0.67*	−0.47	0.54	0.30
C_{ar}					−0.64*	−0.50	−0.44	−0.59*
Prol						0.29	0.28	−0.01
SOD							−0.42	0.14
CAT								0.66**
Drought stress								
gs	0.57*	0.64*	0.67**	0.85**	−0.61*	0.70*	0.56*	0.56**
SPAD		0.64*	0.97**	0.77**	−0.53	0.30	0.59*	0.31
F_V/F_M			0.64*	0.54*	−0.64*	0.80**	0.77**	0.77**
Chl_{total}				0.74**	−0.50	0.3	0.54*	0.55*
C_{ar}					−0.72**	0.24	0.22	0.22
Prol						−0.60**	−0.19	−0.80**
SOD							0.68**	0.80**
CAT								0.62*
Paraquat stress								
gs	0.25	0.64**	0.35	0.19	0.50*	0.60*	0.02	0.05
SPAD		0.60**	0.23	−0.51*	0.24	0.70**	0.70**	0.56**
F_V/F_M			0.15	0.03	0.43	0.88**	0.56*	0.48
Chl_{total}				−0.05	0.01	0.04	0.06	−0.72*
C_{ar}					0.06	−0.13	−0.35	−0.28
Prol						0.46	0.57*	0.52*
SOD							0.72**	0.44
CAT								0.70**

Stomatal conductance (gs), maximum photochemical efficiency of photosystem II (F_v/F_m), chlorophyll estimated content (SPAD index), *total* chlorophyll (Chl *total*) and carotenoids (Car), proline (Prol), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX)

* Significant at $P < 0.05$ and ** significant at $P < 0.01$

c). Reduced pigments were also found in cultivar IAC91-5155, with decreased concentrations of Chl *a*, Chl *b*, and Chl *total* of 54, 7, 47.7 %, respectively, and in cultivar SP83-2847 of 51.7, 37.3, 42.1 %, respectively (Fig. 3), but cultivars IAC91-5155 and SP83-2847 had lower reductions when compared to other cultivars.

Cultivars IAC91-5155 and SP83-2847 showed no differences in chlorophyll levels in the experiments with and without water deficit, suggesting a better capacity of these varieties to maintain chlorophyll levels during stress. But in cultivars RB92579 and SP81-3250 under water deficit, the reduced contents of Chl *a*, Chl *b*, and Chl *total* of 41.3, 22, 36.3, and 53.5, 46.8, 51.6 %, respectively (Fig. 3), indicate high chlorophyll degradation. In addition, an inverse correlation between *total* chlorophyll and APX enzyme (−0.72*) in the +PQ experiment (Table 1) was observed, suggesting that in the paraquat-induced stress there was

chlorophyll degradation due to the oxidative effect, which prompted an overproduction of APX to fight damages in chloroplasts.

Under water deficit, a positive correlation between Chl *total* and gs (0.67**), F_v/F_m (0.64*), CAT (0.54*), and APX (0.55*) (Table 1) was found, indicating that the increased photoinhibition and stomata closure contributed to chlorophyll degradation as a result of increased oxidative stress, also reducing the antioxidant enzymes activity in drought conditions.

Carotenoids had a different behavior under paraquat-induced stress and water deficit. Under paraquat (+PQ) there was an increased carotenoid production, of 55.6 % in RB92579, 53.6 % in SP81-3250, 31.4 % in SP83-2847, and 67.2 % in IAC91-5155 (Fig. 3d). On the other hand, the carotenoid values found during the 15 days under water stress were reduced by 27.4 and 29.6 % in cultivars
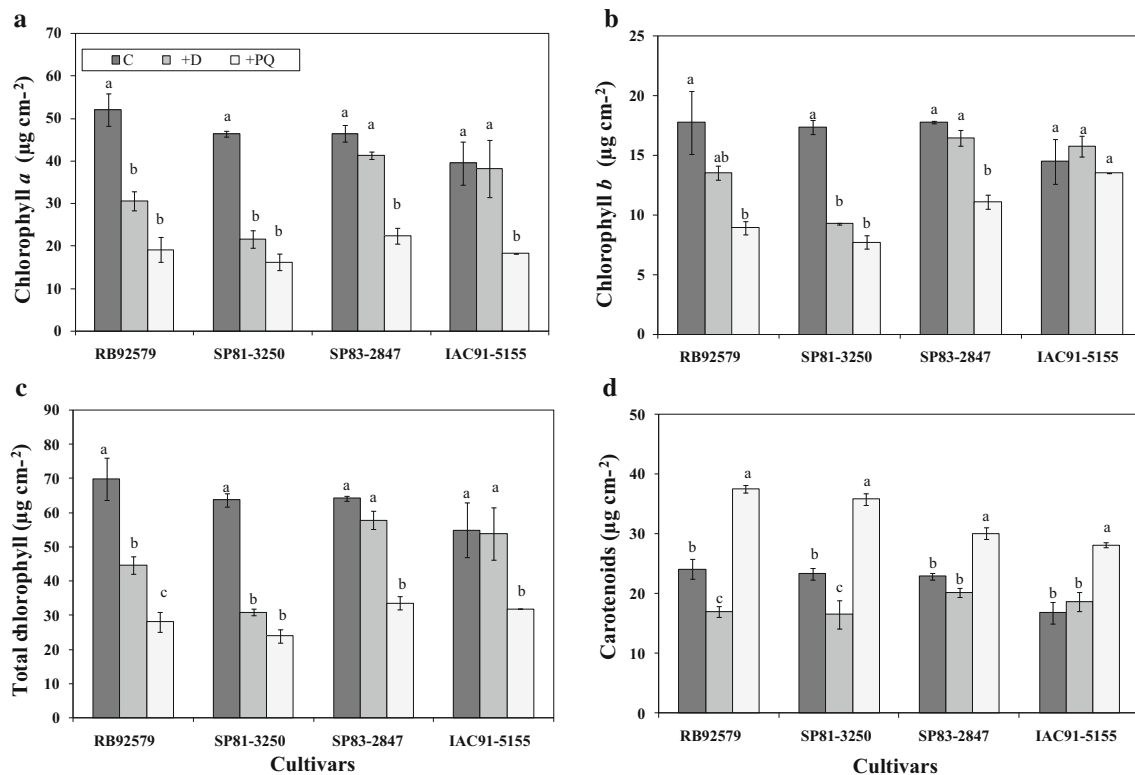
Fig. 3 Contents of chlorophyll *a* (**a**), chlorophyll *b* (**b**), *total* chlorophyll (**c**), and carotenoids (**d**) in cultivars RB92579, SP81-3250, SP83-2847, and IAC91-5155 submitted to appropriate water regime (control, C), water deficit (+D), and concentration of 150 μM of paraquat (+PQ) for 24 h using leaf disks. Means followed by the *same letter* do not differ at 5 % probability by the Tukey test

RB92579 and SP81-3250, respectively, while in cultivars IAC91-5155 and SP83-2847 the values were close to the control, but a small increase of 11 % in IAC91-5155 could be observed (Fig. 3d).

Effects of water stress and paraquat-induced stress on carbohydrate, proline, and protein concentrations

In general, +PQ-induced oxidative stress caused a reduction in total soluble carbohydrates in cultivars SP81-3250 (6 %), RB92579 (21.4 %), and SP83-2847 (11 %) (Fig. 4a). On the other hand, cultivar IAC91-5155 showed similar values to the control, and was not affected by +PQ-induced stress. Under water deficit, inverse responses were found, creating an increase of sugar content in cultivars RB92579 (48.1 %), SP83-2847 (18 %), and IAC91-5155 (25.3 %). But cultivar SP81-3250 presented a reduction of 14 % (Fig. 4a).

Different effects were also found for proline in +D and +PQ experiments. In the paraquat experiment, a reduction of proline occurred in cultivars RB92579 (26.7 %), SP81-3250 (10.8 %), SP83-2847 (53.8 %), and IAC-5155 (47 %) (Fig. 4b). In the period of 15 days under water deficit, cultivar IAC91-5155 showed proline concentration almost similar to the control. But the rates of proline increased significantly in cultivars RB92579 (102 %), SP81-3250 (20.1 %), and SP83-2847 (26 %) under drought (Fig. 4b).

In this study, a response pattern similar to the altered concentrations of soluble proteins in the four cultivars when submitted to the water deficit or +PQ experiments was observed (Fig. 4c). Water deficit caused a reduction in protein concentrations, of 36.8 % in SP81-3250, 33.4 % in RB92579, and 26.6 % in SP83-2847. In the +PQ experiment protein reductions were observed in SP81-3250 (52.4 %), RB92579 (40.5 %), and in SP83-2847 (22.2 %). But cultivar IAC91-5155 was not affected by +D or +PQ treatments, maintaining means close to the control (Fig. 4c).

Response of antioxidant enzymes to oxidative stress induced by water deficit or paraquat in sugarcane

Such different responses can also be observed in the correlations. Under water stress, proline was inversely correlated with gs (-0.61^*), F_v/F_m (-0.64^*), carotenoids (-0.72^{**}), antioxidant enzymes SOD (-0.60^{**}) and APX (-0.80^{**}) (Table 1), suggesting that this amino acid
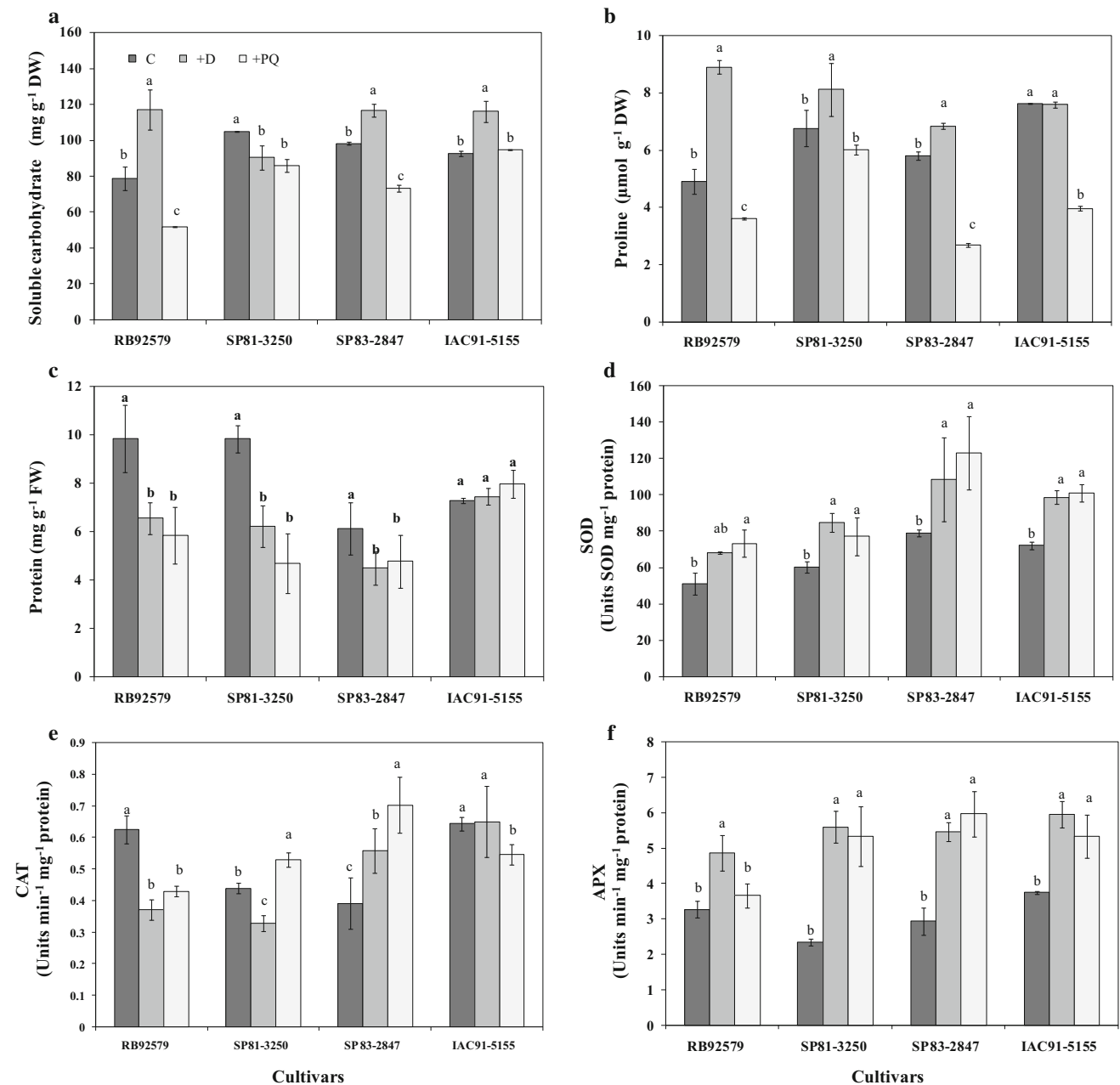
Fig. 4 Concentrations of soluble carbohydrate (**a**), proline (**b**), protein (**c**), and antioxidant enzymes, superoxide dismutase, SOD (**d**), catalase, CAT (**e**), and ascorbate peroxidase, APX (**f**) in cultivars RB92579, SP81-3250, SP83-2847, and IAC91-5155 submitted to appropriate water regime (control, C), water deficit (+D), and concentration of 150 μM of paraquat (+PQ) for 24 h. Means followed by the *same letter* do not differ at 5 % probability by Tukey test

played the role of antioxidant agent due to its increased production and a reduction of the stomatal conductance, F_v/F_m, and antioxidant enzymes caused by oxidative stress. When the plants were treated with +PQ, proline correlated positively with CAT (0.57*) and APX (0.52*) (Table 1), indicating reduction in these variables in response to more intense oxidative stress.

In all cultivars, an increased activity of SOD caused by oxidative stress induced by +PQ and +D was observed.

Increases in SOD activity in the +PQ experiment were of 43.5 % in cultivar RB92579, of 28 % in SP81-3250, 56 % in SP83-2847, and 40 % in IAC91-5155. In the drought-induced experiment increases in SOD levels were of 33.6 % for RB92579, 40.3 % for SP81-3250, 37.5 % for SP83-2847, and 36.6 % for IAC91-5155 (Fig. 4d), signaling oxidative stress in both experiments.

Such important role of SOD as indispensable for ROS detoxification during oxidative stress in sugarcane can be

demonstrated by the positive correlations of this enzyme with gs (0.60*), SPAD index (0.70**), F_v/F_m (0.80**), and CAT (0.72**) (Table 1) in plants under paraquat-induced stress. When submitted to water stress, SOD correlated positively with gs (0.70*), F_v/F_m (0.80**), CAT (0.68**), and APX enzymes (0.80**) (Table 1).

Concerning the catalase enzyme activity, the cultivars presented different behaviors in +D and +PQ experiments (Fig. 4e). Under oxidative stress induced by +PQ the CAT levels increased by 20.4 % in cultivar SP81-3250 and 79.5 % in SP83-2847. But reductions in the CAT activity were found in cultivars RB92579 (30.6 %) and IAC91-5155 (14 %). Under water stress, a reduction of the CAT activity was observed in cultivars SP81-3250 (25 %) and RB92579 (40.3 %). In cultivar SP83-2847 an increase in the CAT activity of 43.6 % occurred , and IAC91-5155 showed no alterations in CAT under water stress condition (Fig. 4e).

An increase in APX activity was observed in all cultivars under water stress or paraquat. In the +PQ experiment, the highest APX increase was in cultivar SP81-3250 (127 %), followed by SP83-2847 (104 %,) and IAC91-5155 (42.4 %), and the lowest increase was found in RB92579 (12 %) (Fig. 4e). Similar behaviors were found under water stress with APX increases of 138 % in cultivar SP81-3250, 86 % in SP83-2847, 58.6 % in IAC91-5155, and 48.6 % in RB92579 (Fig. 4e), indicating a good performance of this enzyme against oxidative stress in both conditions. In addition, the APX enzyme correlated positively with CAT of 0.70** and 0.62* in the plants treated with +PQ and under water stress, respectively (Table 1), which confirms the importance of the interaction of these enzymes against oxidative stress damages.

Dry mass production

Water stress reduced the dry matter in the four cultivars but lower values were observed, i.e., the greater plant reductions, in the shoot, root, and total dry matter of RB92579 (74, 61.6, and 72.7 %, respectively) and SP81-3250 (73.1, 69, and 72.6 %, respectively) compared to SP83-2847 (57.7, 45.6, and 58.7 %, respectively) and IAC91-5155 (52.1, 37.6, and 50.6 %, respectively) (Table 2).

Discussion

The normal RWC in the IAC91-5155 cultivar under water deficit is a good indicator of cell and leaf tissue hydration (Fig. 1), and suggests that the plant is under osmotic adjustment, which is a crucial factor for optimal physiological functions such as stomatal control (Gorai et al. 2010). The high RWC reduction in cultivars RB92579 and SP81-3250 is a strong indication of these plants' sensitivity to drought. A progressive decline in RWC (varying between 80 amd 60 %) in susceptible sugarcane cultivars subjected to water deficit has also been reported by Silva et al. (2011) and Cia et al. (2012). These studies have shown that high RWC values during water shortages are strongly indicative of tolerance in sugarcane cultivars.

In paraquat-induced stress, greater reductions of F_v/F_m in cultivars RB92579 and SP81-3250, with averages nearly 0.6 was observed (Fig. 2a), suggesting that these plants had severe photooxidative damages 24 h after the application of paraquat. Such stress conditions may have been highly detrimental to the chloroplast functional integrity, thus causing photoinhibition or photooxidation with destabilization of the reaction center of photosystem I (Maxwell and Johnson 2000; Sedigheh et al. 2011).

Chagas et al. (2008) studied paraquat phototoxicity in cultivar SP80-3280 and found that PQ concentrations above 2 mM for 18 h after the application of the herbicide had caused significant damage to the photosystems, with high photoinhibition, F_v/F_m close to 0.3. Reductions of F_v/F_m below 0.7 were also found in wheat leaves when submitted to paraquat-induced stress (Ekmekci and Terzioglu 2005; Sedigheh et al. 2011) and in maize (Darkó et al. 2009).

Cultivars IAC91-5155 and SP83-2847 showed F_v/F_m values higher than 0.7 when treated with paraquat and

Table 2 Dry mass production of four sugarcane cultivars submitted to appropriate water regime (control) and water deficit in greenhouse conditions

Cultivars	SDM (g)		RDM (g)		TDM (g)	
	Control	Water deficit	Control	Water deficit	Control	Water deficit
IAC91-5155	717.3[Ba]	343.0[Ab]	83.78[Ba]	52.24[Ab]	801.1[Ba]	395.2[Ab]
SP83-2847	763.3[ABa]	303.3[ABb]	90.7[ABa]	49.3[ABb]	854.1[ABa]	352.7[Ab]
RB92579	797.3[ABa]	207.6[Cb]	91.2[ABa]	35.05[BCb]	888.5[ABa]	242.7[Bb]
SP81-3250	825.6[Aa]	222.3[BCb]	103.9[Aa]	32.3[Cb]	929.5[Aa]	254.7[Bb]
CV (%)	7.0		9.9		6.4	

Identical letters, lower case among treatments in the row and upper case between cultivars in the column, do not differ at 5 % probability by Tukey test

SDM shoot dry mass, *RDM* root dry mass, *TDM* total dry mass

under water stress (Fig. 2a), suggesting that these cultivars tend to have minor photoinhibition damages caused by both stressors and may indicate adequacy or adaptation to stress conditions. This indicates that photoinhibition should not be seen as damage but rather as a protective mechanism that allows dissipate excess thermal energy (Long et al. 1994; Murchie and Niyogi 2011).

Greater reductions of F_v/F_m values under water deficit were also observed in cultivars RB92579 and SP81-3250, with mean values close to 0.6 (Fig. 2a). In other researches, sugarcane cultivars under water deficit with mean F_v/F_m values below 0.7 were considered sensitive to drought (Graça et al. 2010; Silva et al. 2014). This was also found in this study both under water deficit and paraquat-induced stress, which indicates that the decrease in the F_v/F_m ratio is a good indicator of photoinhibition damage when plants are submitted to both stressors.

The greater gs reductions in all cultivars, when treated with +PQ, indicate that the herbicide toxic effect induced oxidative stress, causing damage in the sub-stomatal cells and may have contributed to the stomatal closure or rupture in these cultivars. Behaviors like these were also reported in other vegetable species with a great reduction of gs in plants treated with paraquat, e.g., in wheat (Sedigheh et al. 2011) and Conyza bonariensis (Shaaltiel and Gressel 1987). These authors suggested that paraquat caused the closure of the stomata because of damage in the membrane and loss of water from the guard cells, hindering CO_2 fixation.

In fact, stomatal closure was the primary cause of the response to water stress in cultivars, particularly SP81-3250, with lower gs values (Fig. 2b), i.e., greater stomatal sensitivity. Similar findings in sugarcane were also reported by Graça et al. (2010) and Silva et al. (2012), who found greater stomatal closure in cultivars regarded as more sensitive to stress. On the other hand, the smaller gs reduction observed in SP83-2847, when treated with +D, suggest that these cultivars are more effective in stomatal control in water-stressed soils when compared with other cultivars. According to Lawlor and Tezara (2009), under water stress, plants keep their stomata closed as a way to minimize loss of water and maintain foliar turgor, an important feature for drought tolerance.

Under water deficit, the lowest SPAD values were observed in cultivars RB92579 and SP81-3250, with mean values below 40 (Fig. 2c), suggesting that these plants are more sensitive to drought. These results corroborate those found by Jangpromma et al. (2010a), Silva et al. (2012), and Silva et al. (2014), who reported SPAD values below 40 in sugarcane-sensitive cultivars when submitted to drought conditions. According to Silva et al. (2007), SPAD readings below 40 indicate the onset of chlorophyll deficit, which affects the photosynthetic process, a parameter

considered as a good indicator of disturbances caused by water stress in sugarcane. But such considerable reduction was not observed when cultivars were treated with paraquat, suggesting that the 24 h period after application may have been a short time, requiring a longer period to confirm the occurrence of chlorophyll damage in sugarcane leaves.

Regarding the contents of photosynthetic pigments, cultivars IAC91-5155 and SP83-2847 presented lower decreases when treated with +PQ (Fig. 3a–c), suggesting a greater ability to maintain chlorophyll levels under the phototoxicity of paraquat herbicide. According to Altinkut et al. (2001), reduced concentrations of chlorophylls may be associated with photooxidative processes or alteration in the organization of photosystems, so as to act as a mechanism of photoprotection to prevent severe photodestruction of the chloroplast. Chagas et al. (2008) also found a reduction in the total chlorophyll contents in sugarcane and Darkó et al. (2009) found it in maize when treated with paraquat.

It was found in cultivars SP81-3250 and RB92579, a high degradation of chlorophyll under water deficit and treated with +PQ (Fig. 3a–c). Chlorophyll degradation is one of the consequences of stress that may result from photoinhibition and reduced photosynthetic efficiency among other cellular processes (Long et al. 1994; Murchie and Niyogi 2011). According to Sood et al. (2011), paraquat causes oxidative stress in chloroplasts, which may have contributed to the increased production of ROS, resulting in damages of the photosynthetic apparatus such as chlorophyll pigment degradation.

Great reductions in chlorophyll levels were also found in other sugarcane cultivars considered susceptible as a result of water stress (Cha-Um and Kirdmanee 2009; Jangpromma et al. 2010b) and salt stress (Gomathi and Rakkiyapan 2011). Based on this, it can be assumed that, with respect to chlorophyll levels, the sensitivity of the cultivars in this study to drought and paraquat-induced stress indicates that the mechanisms of adaptation are similar to those to oxidative stress, which suggests that this parameter is considered a good indicator of disturbances caused by both stresses in plants.

The unaltered carotenoid levels in cultivars in SP83-2847 and IAC91-5155 under water stress (Fig. 3d) suggests that these cultivars have a greater protection against photooxidation during the oxidative stress, but the rise of the carotenoid levels in cultivars SP81-3250 and RB92579 treated with paraquat were not sufficient to prevent oxidative damages, as suggested by the high chlorophyll degradation rates and the great reduction of the maximum photochemical efficiency of photosystem II in these cultivars.

Present in chloroplasts, carotenoids have a non-enzymatic antioxidant photoprotective role in photosynthetic

tissue with the function of dissipating the excess energy of chlorophyll as heat, reducing the chlorophyll excited state. When this does not occur, reactive chlorophyll may react with molecular oxygen, forming singlet oxygen, superoxide anion, hydroxyl radicals, and hydrogen peroxide, which are detrimental to many cell components, especially lipids (Jaleel et al. 2009; Gomathi and Rakkiyapan 2011). Thus, these pigments are important in preventing induction of oxidative damage caused by stress (Wahid 2007).

The increased carotenoids concentration has been suggested as a mechanism of tolerance of sugarcane to oxidative stress, reported in various stress conditions in sugarcane, such as water deficit and salinization (Cha-Um and Kirdmanee 2009; Patade et al. 2011), as well as thermal stress (Wahid 2007). Few studies have reported the phytotoxicity effect of paraquat on the carotenoid levels, but some results were published on wheat leaves (Ekmekci and Terzioglu 2005) and maize leaves (Shahrtash et al. 2011), but nothing was found on sugarcane.

The plants exposure to the PQ herbicide was detrimental to the metabolism for protein production, causing its reduction in all cultivars. According to Sood et al. (2011) and Shahrtash et al. (2011), paraquat in plants induces oxidative stress, contributing to an increased production of ROS, which favors protein denaturation or degradation.

Under paraquat-induced stress, protein reductions in sugarcane cultivars (Chagas et al. 2008), maize (Shahrtash et al. 2011) and inferior *Azolla microphylla* Kaul (Sood et al. 2011) were also observed. According to these authors, protein denaturation and degradation as well as membrane damages are higher as the exposure time and paraquat dosage increase, which is due to the increased ROS levels induced by oxidative stress. The reduction in the soluble protein concentrations in all cultivars during the period of water stress also suggests that this was probably due to the interruption of protein synthesis (Patade et al. 2011).

Paraquat-induced oxidative stress caused reduction in total soluble carbohydrates for most of the cultivars (Fig. 4a). In the literature, no results addressing responses to paraquat-induced stress in the leaf contents of soluble carbohydrates were found. However, this study suggests that carbohydrate reduction was due to the paraquat toxic effect, causing damage to the chloroplast, such as degradation and destruction of amino molecules in plants.

Under water stress, such responses were inverse, with increased sugar concentrations in cultivars RB92579, SP83-2847, and IAC91-5155 (Fig. 4a), which indicates that the buildup of soluble sugar is probably associated with mechanisms that prevent water loss in plants, such as osmotic adjustment (Hayat et al. 2012). Sales et al. (2012), studying water-stressed sugarcane found that total soluble carbohydrates remained constant in the leaves and greater in the roots. According to these authors, soluble carbohydrates remain in the leaves and roots probably because of the degradation of starch reserves. The increased levels of total soluble carbohydrates were also observed in water- and salt-stressed sugarcane (Patade et al. 2011). These studies suggest that the accumulation of soluble carbohydrates is involved in the osmotic adjustment.

Paraquat-induced oxidative stress contributed to the reduction of proline in all cultivars (Fig. 4b) indicating that this herbicide was involved in biosynthesis inhibition and/or in proline destruction, an based on this it can be assumed that the PQ concentration of 150 μM for 24 h was highly toxic for proline and should have inhibited the restoration processes, which are linked to proline synthesis in chloroplast.

However, some studies demonstrated that proline is involved in the protective system against oxidative damages induced by paraquat stress (Molinari et al. 2007; Shevyakova et al. 2009; Shahrtash et al. 2011), a fact not found in this research. In a study using sugarcane leaf disks, Molinari et al. (2007) studied oxidative stress mediated by 5 μM of paraquat for 24 h, and found in transgenic plants some genes that stimulated the production of proline, and when treated with paraquat, leaves remained green and with higher content of chlorophyll. Such study demonstrated the protective effects of proline against damages caused by paraquat, confirming the hypothesis of its role as ROS antioxidant agent.

The increased accumulation of proline in cultivars under water stress suggests that such amino acid may be involved in the mechanism of osmoregulation and antioxidant defense, contributing to balanced ROS in leaves, as reported in previous studies on water-stressed sugarcane (Molinari et al. 2007; Cia et al. 2012; Hemaprabha et al. 2013). Thus, proline concentrations may be a good biochemical parameter as indicator of water stress, although they were not efficient in signaling paraquat mediated stress due to the great reduction caused by the herbicide in organic solute.

The increased SOD activity in both stress conditions suggests that this enzyme played a signaling role of ROS overproduction, especially superoxide radical ($O_2^{\cdot-}$), because SOD acts in chloroplasts removing and transforming $O_2^{\cdot-}$ to H_2O_2, and this is detoxified by another set of enzymes, CAT, GPX, and APX (Mittler 2002; Sharma et al. 2012).

The stress caused by paraquat induced the increase of the SOD enzyme in all cultivars (Fig. 4d), indicating that this herbicide is a powerful inducer of oxidative stress. About paraquat-induced stress, various studies report that the herbicide toxicity is mediated by the significantly increased SOD activation to balance the antioxidant system, as previously observed in sugarcane (Chagas et al.

2008), maize (Darkó et al. 2009), rice (Guo et al. 2007) and wheat (Shevyakova et al. 2009).

In 15 days under water stress, all cultivars also showed an increased SOD activity, a signal of oxidative stress condition in the plants. According to Cia et al. (2012), an unaltered or increased SOD activity in sugarcane under drought may be considered an evidence of tolerance, while a reduced activity may indicate sensitivity to drought. The association between the levels of SOD activity with sugarcane drought tolerance has been previously observed. For example, Jangpromma et al. (2010b) found a higher increase in SOD activity in drought-tolerant cultivars, and Gomathi and Rakkiyapan (2011), when studying sugarcane under salt-induced stress, found that the SOD activity tended to increase in the tolerant cultivar.

The increased activity of the CAT enzyme in cultivars treated with +PQ (Fig. 4e) suggests that the plants used this enzyme as a defense mechanism against the oxidative damages provoked by the herbicide. Various studies consider the increased CAT activity as an important antioxidant defense system against paraquat-induced oxidative stress, such as in pea leaves (Iturbe-Ormaetxe 1998), wheat (Ekmekci and Terzioglu 2005), and maize (Shahrtash et al. 2011). The high concentrations of the CAT enzyme are important to reduce H_2O_2 concentrations, so this enzyme plays a key role in the plants' final adaptation and survival during stress periods (Asada 1999; Gill and Tuteja 2010).

The reduction of the catalase activity in cultivars RB92579 and SP81-3250 under water deficit suggests more sensitivity of these plants when exposed to drought stress. The CAT is an enzyme considered very sensitive to abiotic stress, which may induce CAT inactivation in sugarcane (Srivastava et al. 2012), and so it is a good enzyme to signal drought stress in this culture. The maintenance of the CAT activity in cultivar IAC91-5155 and the increased CAT activity in SP83-2847 may be an indicative feature of these cultivars to respond to oxidative stress and perceived as having better leaf ability to decompose H_2O_2.

The CAT enzyme is the second prevention system of oxidative stress. This enzyme acts in the dismutation of hydrogen peroxide (H_2O_2) produced by SOD, the first prevention system, with oxygen and water (Mittler 2002; Sharma et al. 2012). Cia et al. (2012) found that the sensitive cultivar showed a decline in the CAT activity as water deficit increased. Srivastava et al. (2012), in sugarcane under thermal stress, found that high temperatures reduced CAT activity in tolerant cultivars.

In APX activity, it was observed an increased production of this enzyme in all cultivars under drought or paraquat-induced stress (Fig. 4f). This indicates the key role of APX in providing cultivars with protection against the potential increase of de H_2O_2 produced by drought and toxicity of the paraquat herbicide, which confirms that the increased APX activity may be closely related to tolerance to these stressors, due to its effective ability to eliminate H_2O_2 (Noctor and Foyer 1998; Sharma et al. 2012).

Other studies reported that the increased activity of the antioxidant enzymes SOD, CAT, and APX had a detoxification action, contributing to the decrease in lipid peroxidation, thus playing a key role in the mechanism of tolerance to PQ-induced oxidative stress in various cultures (Ekmekci and Terzioglu 2005; Sood et al. 2011; Yoon et al. 2011). Inverse responses were found by Chagas et al. (2008), who studied paraquat-induced stress in sugarcane and noticed a high APX reduction, suggesting that the oxidative damages in sugarcane leaves were associated with excess H_2O_2, probably in chloroplasts, caused by an unbalanced activity of SOD and APX, because while SOD increased, APX decreased.

The ability of cultivars IAC91-5155 and SP83-2847 to produce higher dry matter of shoots, roots and total under water stress can confer greater tolerance to drought conditions. According to Aguilera et al. (1999), the degree of limitation promoted by environmental stress upon the biomass varies among genotypes of the same species. Thus, the ability to maintain key physiological processes under water scarcity is a potential indicative of also maintaining the productivity.

Conclusion

The results discussed here are related to oxidative stress induced by paraquat and water deficit, mediated by physiological attributes and biochemical damages, aspects not available today in the literature on sugarcane crop. Thus, cultivars were exposed to two kinds of stress of economic interest to sustainable agriculture. Due to minor damage caused by drought in the physiology and biochemistry of cultivars SP83-2847 and IAC91-5155, these cultivars exhibited a greater potential for tolerating and acclimating to drought. Consequently, oxidative stress can be largely avoided in the chloroplasts of these cultivars by maintaining a balance of antioxidant enzyme activity, photosynthetic efficiency, stomatal control and water status. The lower toxicity degrees of paraquat in cultivars SP83-2847 and IAC91-5155 are related to the increase or at least the maintenance of the activity of SOD, CAT, and APX enzymes, which must have contributed to the reduction of oxidative stress, as evidenced by the lower reductions of F_v/F_m and chlorophylls. Therefore, antioxidant enzymes may be considered potential defense mechanisms for oxidative stress in sugarcane. The variables F_v/F_m, Ψ_w, SPAD index, photosynthetic pigments, and dry mass are potentially reliable physiological indicators for selecting sugarcane cultivars tolerant to water deficit, and F_v/F_m and photosynthetic pigments to oxidative stress induced by

paraquat. We recommend these measures for identifying tolerant genotypes in genetic breeding programs, thus providing ideal material for molecular studies on drought.

Authors contribution statement Claudiana M. dos Santos set up and carried out the experiments, analyzed and interpreted the data, and prepared the manuscript. Marcelo de A. Silva planned and wrote the proposal, designed and supervised the experiments, interpreted the data, and was responsible for the manuscript preparation.

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