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Short-term physiological changes in roots and leaves of sugarcane varieties exposed to H_2O_2 in root medium

Karina I. Silva[a,b], Cristina R.G. Sales[a,b], Paulo E.R. Marchiori[a,b], Neidiquele M. Silveira[a,b], Eduardo C. Machado[b], Rafael V. Ribeiro[c,*]

[a] Graduate Program in Tropical and Subtropical Agriculture, Agronomic Institute (IAC), Campinas, SP, Brazil
[b] Laboratory of Plant Physiology "Coaracy M. Franco", Center R&D in Ecophysiology and Biophysics, IAC, Campinas, SP, Brazil
[c] Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, SP, Brazil

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ABSTRACT

The aim of this study was to evaluate the differential sensitivity of sugarcane genotypes to H_2O_2 in root medium. As a hypothesis, the drought tolerant genotype would be able to minimize the oxidative damage and maintain the water transport from roots to shoots, reducing the negative effects on photosynthesis. The sugarcane genotypes IACSP94-2094 (drought tolerant) and IACSP94-2101 (drought sensitive) were grown in a growth chamber and exposed to three levels of H_2O_2 in nutrient solution: control; 3 mmol L^{-1} and 80 mmol L^{-1}. Leaf gas exchange, photochemical activity, root hydraulic conductance (L_r) and antioxidant metabolism in both roots and leaves were evaluated after 15 min of treatment with H_2O_2. Although, root hydraulic conductance, stomatal aperture, apparent electron transport rate and instantaneous carboxylation efficiency have been reduced by H_2O_2 in both genotypes, IACSP94-2094 presented higher values of those variables as compared to IACSP94-2101. There was a significant genotypic variation in relation to the physiological responses of sugarcane to increasing H_2O_2 in root tissues, being root changes associated with modifications in plant shoots. IACSP94-2094 presented a root antioxidant system more effective against H_2O_2 in root medium, regardless H_2O_2 concentration. Under low H_2O_2 concentration, water transport and leaf gas exchange of IACSP94-2094 were less affected as compared to IACSP94-2101. Under high H_2O_2 concentration, the lower sensitivity of IACSP94-2094 was associated with increases in superoxide dismutase activity in roots and leaves and increases in catalase activity in roots. In conclusion, we propose a general model of sugarcane reaction to H_2O_2, linking root and shoot physiological responses.

Introduction

Root system is the first plant organ affected by low soil water availability, with sugarcane plants showing reduction in shoot hydration and decreases in stomatal conductance, transpiration and CO_2 assimilation (Ribeiro et al., 2013; Sales et al., 2013). Regarding the causes of low photosynthesis, the following can be noted, low mesophyll CO_2 conductance, reductions in chlorophyll content and impairment of photochemical and biochemical reactions under severe water deficit (Ghannoum, 2009; Machado et al., 2009), when plants are likely under oxidative stress. The reduced consumption of photochemical products due to decreases in carboxylation reactions generates excess energy at photosystem level that can lead to photoinhibition and further decreases in quantum efficiency of photosynthesis (Chagas et al., 2008; Ribeiro et al., 2013).

Under cellular redox imbalance conditions due to excessive energetic pressure, reactive oxygen species (ROS) are formed, such as superoxide anion and hydrogen peroxide (H_2O_2). In C_4 plants, H_2O_2 is generated in chloroplasts and is harmful to cell organelles and membranes. It also acts as a secondary messenger in signal transduction due to its relative long half-life and high membrane permeability (Hung et al., 2005; Cheeseman, 2007; Quan et al., 2008). In addition, H_2O_2 is involved in the coordination of root-shoot metabolism and in stomatal control of leaf gas exchange

Abbreviations: A_N, leaf CO_2 assimilation; APX, ascorbate peroxidase; CAT, catalase; ETR, apparent electron transport rate; EXC, relative energy excess at PSII level; F_V/F_M, potential quantum efficiency of photosystem II; g_S, stomatal conductance; IWUE, intrinsic water use efficiency; k, instantaneous carboxylation efficiency; L_r, root hydraulic conductance; PSII, photosystem II; Q, photosynthetically active radiation; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author. Tel.: +55 1935216214.
E-mail address: rvr@unicamp.br (R.V. Ribeiro).

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(Desikan et al., 2004). This role is based on the inhibitory effect of H_2O_2 on aquaporin activity, affecting water transport (Boursiac et al., 2008). Under stressful conditions, H_2O_2 accumulation may decrease root hydraulic conductance (L_r) by forming an oxidative gate that closes aquaporin channels and reduces water transport through plant membranes (Ye and Steudle, 2006; Ehlert et al., 2009).

Plants have evolved an antioxidant system to avoid damaging effects of ROS, eliminating such molecules through reactions mediated mainly by superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Bienert et al., 2007; Gill and Tuteja, 2010). Genotypic variation is found when we consider the physiological responses of sugarcane to water deficit, with drought tolerance being associated with increased activity of antioxidant enzymes. While SOD is the main enzyme for $O_2^{\bullet-}$ degradation, CAT acts in reducing H_2O_2 concentration in sugarcane plants under stressful conditions (Mitler, 2002; Cia et al., 2012; Sales et al., 2013). On the other hand, drought-sensitive sugarcane genotypes present high lipid peroxidation and increased H_2O_2 concentration in leaves (Boaretto et al., 2014).

Studies relating the antioxidant system to water transport are scarce and they mostly focus only on leaves or roots separately without establishing a connection between both organs. This fact limits our overall understanding of how plants respond to stressful situations and interactions between water relations, antioxidant system and photosynthetic metabolism remain unknown. As plants face soil water deficiency, an increase in ROS production is expected in their roots (Hung et al., 2005). In such situation, the drought-tolerant genotype should present rapid antioxidant response in roots, preserving the water transport and the photosynthetic apparatus. Taking into account the genotypic variation found in sugarcane, this study aims to test the hypothesis that the drought-tolerant genotype would present a more effective root antioxidant system response against exogenous H_2O_2, thus, reducing the negative impact of H_2O_2 on root hydraulic conductance and leaf gas exchange when compared to the drought-sensitive genotype.

Materials and methods

Plant material and experimental conditions

We tested our hypothesis with sugarcane (*Saccharum* spp.) genotypes developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil). IACSP94-2094 is a drought-tolerant genotype, whereas IACSP94-2101 is sensitive to water deficit (Landell et al., 2005; Ribeiro et al., 2013). Thirty days-old plants grown in commercial substrate (Carolina Soil of Brazil, Vera Cruz SC, Brazil) with three to four leaves were transferred to a nutrient solution adapted from Sarruge (1975). Such solution was composed by potassium nitrate (0.31 g L^{-1}), calcium nitrate (1.20 g L^{-1}), magnesium sulfate (0.50 g L^{-1}), ammonium nitrate (0.08 g L^{-1}), di-hydrogen potassium phosphate (0.14 g L^{-1}), potassium chlorate (0.06 g L^{-1}), EDDHMA iron (0.07 g L^{-1}), boric acid (1.69 mg L^{-1}), zinc sulfate (1.10 mg L^{-1}), copper sulfate (0.16 mg L^{-1}), manganese sulfate (0.92 mg L^{-1}) and ammonium molybdate (2.32 mg L^{-1}).

The experiment was carried out in a growth chamber (model PGR15, Conviron, Winnipeg MB, Canada), with a 12-h photoperiod, air temperature of 30/20 °C (day/night), air relative humidity of 80% and photosynthetically active radiation (Q) of 800 μmol m^{-2} s^{-1}. In such environment, plants were subjected to two exogenous application of H_2O_2 in nutrient solution, which reached H_2O_2 concentrations of 3 and 80 mmol L^{-1}. Plants grown in nutrient solution without exogenous H_2O_2 application were considered as reference. The H_2O_2 treatments were chosen in previous experiments in which the H_2O_2 concentration in the nutrient solution varied from 0.25 to 80 mmol L^{-1}. 3 mmol L^{-1} was the lowest concentration, which caused changes in leaf gas exchange whereas 80 mmol L^{-1} was the highest concentration tested, inducing severe reduction in stomatal conductance. The H_2O_2 concentration in nutrient solution was determined according to Baccan et al. (2001).

Physiological evaluations and samplings were taken 15 min after H_2O_2 treatment, on the first fully expanded leaf with apparent ligule. The exposure time was based on previous experiments, in which we noticed significant changes in the leaf gas exchange just after 15 min of treatment with H_2O_2 in nutrient solution. The leaf and root samples were collected, immediately immersed in liquid nitrogen and then stored at −80 °C for further biochemical analyses.

Leaf gas exchange and photochemical activity

Leaf gas exchange was measured with an infrared gas analyzer (model Li-6400, Licor, Lincoln NE, USA), coupled to a modulated fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO_2 assimilation (A_N), stomatal conductance (g_S) and intercellular CO_2 concentration (C_I) were measured under Q of 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 380 μmol mol^{-1}. The vapor pressure difference between leaf and air (VPD_L) was 2.2 ± 0.3 kPa and leaf temperature (T_F) was 29.1 ± 0.3 °C during the evaluations. The intrinsic water use efficiency (IWUE = A_N/g_S) and the instantaneous carboxylation efficiency ($k = A_N/C_I$) were calculated according to Machado et al. (2009).

Simultaneous measurements of leaf gas exchange and chlorophyll fluorescence were taken and the apparent electron transport rate estimated as ETR = $\phi_{PSII} \times Q \times 0.85 \times 0.4$, 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 (Edwards and Baker, 1993; Baker, 2008). In leaf tissues adapted to darkness (30 min), the potential quantum efficiency of photosystem II (F_V/F_M) was estimated according to Rohácek (2002). The relative energy excess at PSII level was calculated as EXC = $[(F_V/F_M) - (\phi_{PSII})]/(F_V/F_M)$, as suggested by Bilger et al. (1995).

Root hydraulic conductance

Root hydraulic conductance (L_r) was estimated according to the procedure described by Aroca et al. (2001). The procedure was based on the shoot cutting at the plant base, with root system in nutrient solution being placed into a Scholander pressure chamber (model 3005, Soil Moisture Equipment Corp., Santa Barbara CA, USA). Steady-state exudation rates were measured sequentially under varying pressures (from 0.2 to 0.8 MPa) in periods that ranged from 8 to 12 min. Afterwards, roots were stored in 80% ethanol solution and root area was measured with the Safira software v. 1.1 (Stonway, São Carlos SP, Brazil). L_r was calculated as the slope of the relation between the exudation flow and pressure, corrected for the root area and it was expressed as mmol m^{-2} s^{-1} MPa^{-1}. Measurements were carried out around noon to avoid differences in L_r due to circadian rhythm (Hachez et al., 2012).

Hydrogen peroxide content and lipid peroxidation

The quantification of H_2O_2 in plant material was performed following the method of Alexieva et al. (2001). We collected the homogenate obtained from 0.1 g of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at 10,000 rpm and 4 °C for 15 min. The reaction medium consisted of 1.2 mL of KI 1 mol L^{-1}, potassium phosphate buffer (pH 7.5 and 0.1 mol L^{-1}) and crude extract. The microtubes were incubated on ice under dark for 1 h. After this period, the absorbance

reading was done at 390 nm. The calibration curve was done with H_2O_2 and the results were expressed as μmol H_2O_2 g^{-1} FW.

The lipid peroxidation was evaluated through production of malondialdehyde (MDA) according to Cakmak and Horst (1991). Samples of 0.16 g of fresh tissue were macerated in 0.1% TCA solution (w/v) in a mortar and centrifuged at 10,000 rpm and 4 °C for 15 min, with supernatant being collected. An extract aliquot of 0.5 mL was placed in a test-tube with 0.5% thiobarbituric acid solution and incubated in water bath at 90 °C for 20 min, while stirring. The reaction was stopped in an ice bath and the sample was centrifuged at 10,000 rpm for 10 min. After 30 min at room temperature, the sample absorbance was read at 532 nm and 600 nm and the non-specific absorbance at 600 nm discounted. To calculate the amount of MDA, we considered the MDA absorptivity coefficient of 155 mM^{-1} cm^{-1} and results were expressed as nmol MDA g^{-1} FW.

Enzyme extraction and activity assays

We used the extract obtained from the macerate of 0.2 g of fresh tissue with liquid nitrogen, 1% PVPP and 2 mL of extraction medium containing 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). After centrifugation of the homogenate at 15,000 g for 15 min and 4 °C, we collected the supernatant (crude extract) that was preserved in an ice bath.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The reaction medium consisted of 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 4 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the amount of enzyme required to inhibit the NBT photoreduction in 50%, being expressed as U min^{-1} mg^{-1} of protein.

The catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure described in Havir and McHale (1987). The reaction medium consisted 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 reaction was carried out in water bath at 25 °C for 2 min and we followed the decrease in absorbance at 240 nm. The CAT activity was calculated using the molar extinction coefficient of 36 M^{-1} cm^{-1} and expressed as μmol min^{-1} mg^{-1} of protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as done by Nakano and Asada (1981). The reaction medium was composed by 3 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.0), deionized water, 10 mmol L^{-1} ascorbic acid, 10 mmol L^{-1} H_2O_2 and crude extract. The reaction was carried out in a water bath at 25 °C for 2 min and we monitored the decrease in absorbance at 290 nm. We used the molar extinction coefficient of 2.8 M^{-1} cm^{-1} to measure the APX activity, which was expressed as μmol min^{-1} mg^{-1} of protein.

Superoxide dismutase zymogram

The identification of SOD isoforms was performed by electrophoresis in polyacrylamide gel (12.5%) in non-denaturing system (native-PAGE), at 4 °C under constant current of 30 mA in Tris–HCl (pH 8.3) and glycine buffer. The protein extract used was obtained after maceration of a composite leaf sample (0.4 g, composed by ~0.1 g per replicate) (Ferreira et al., 2002). SOD isoforms were identified through specific inhibition by H_2O_2 and KCN, with isoforms being classified as Mn-SOD (resistant to both inhibitors), Fe-SOD (resistant only to KCN) and Cu/Zn-SOD (inhibited by both inhibitors) (Azevedo et al., 1998). As significant changes due to H_2O_2 treatment were only found under 80 mmol L^{-1}, SOD isoforms were only identified in plants exposed to the highest H_2O_2 concentration and in the reference ones.

Protein content

The quantification of total soluble proteins followed the protocol of Bradford (1976). The reaction mixture was composed by crude extract, purified water and protein dye reagent (Bradford reagent Sigma, B6916—Coomassie brilliant blue, BG-250). The absorbance reading was done at 595 nm, with the standard curve being prepared with bovine serum albumin (BSA) and the results were expressed as mg g^{-1}.

Data analysis

The experimental design was in randomized blocks and we evaluated two causes of variation (factors): genotypes *vs.* H_2O_2 concentration. The data were subjected to the analysis of variance (ANOVA) and mean values ($n = 3$) were compared by the Tukey test when significance was detected ($p < 0.05$).

Results

After 15 min of H_2O_2 application in nutrient solution, only IACSP94-2094 had increased SOD activity in leaves (two-times) and roots (four-times) under 80 mmol L^{-1} (Fig. 1A and C). IACSP94-2101 presented higher (+60%) leaf SOD activity than IACSP94-2094 in reference plants (Fig. 1A). When considering leaf SOD isoforms, we were able to identify four isoforms in IACSP94-2101 and three in IACSP94-2094, with the genotypes differing in one isoform Cu/Zn-SOD (Fig. 2). In general, there were slight decreases in Cu/Zn-SOD activities when IACSP94-2101 was exposed to 80 mmol L^{-1} of H_2O_2. In this stressful condition, IACSP94-2094 presented an increase in Cu/Zn-SOD activity (Fig. 2).

The leaf H_2O_2 concentration was higher in plants subjected to 80 mmol L^{-1} when compared to reference plants and those treated with 3 mmol L^{-1} (Fig. 1B). In the roots, both genotypes showed

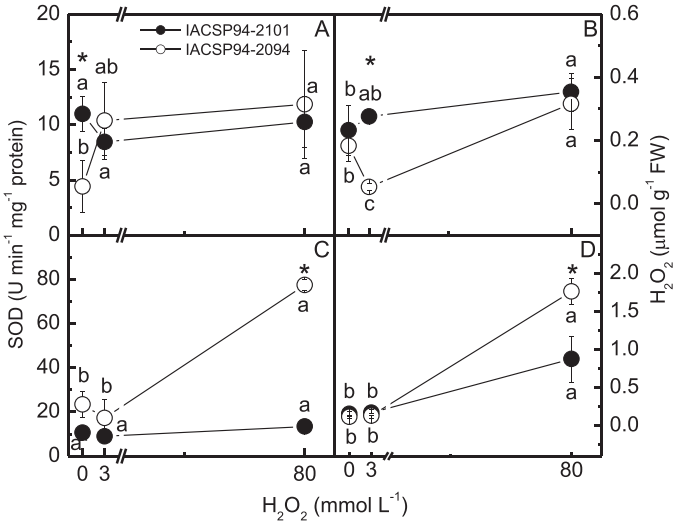

Fig. 1. Activity of SOD (A and C) and concentration of hydrogen peroxide (B and D) in leaves (A and B) and roots (C and D) of sugarcane genotypes IACSP94-2101 (●) and IACSP94-2094 (○) while affected by H_2O_2 concentration in nutrient solution. Measurements were taken 15 min after applying H_2O_2. Each symbol represents the mean value of three replications ± standard deviation. Different lowercase letters indicate statistical difference ($p < 0.05$) between H_2O_2 treatments, while asterisks indicate statistical difference between genotypes ($p < 0.05$).

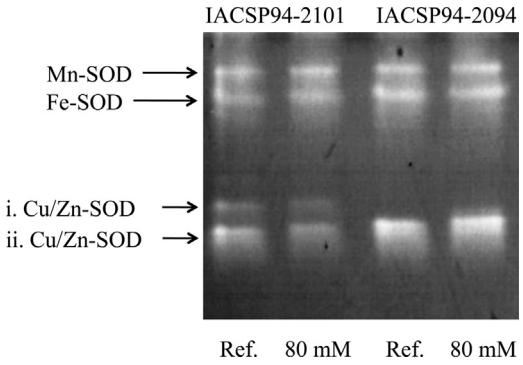

Fig. 2. Superoxide dismutase isoforms by non-denaturing polyacrylamide gel electrophoresis in leaves of sugarcane genotypes IACSP94-2101 and IACSP94-2094 as affected by 80 mmol L^{-1} H_2O_2 in nutrient solution. Samples were collected 15 min after applying H_2O_2.

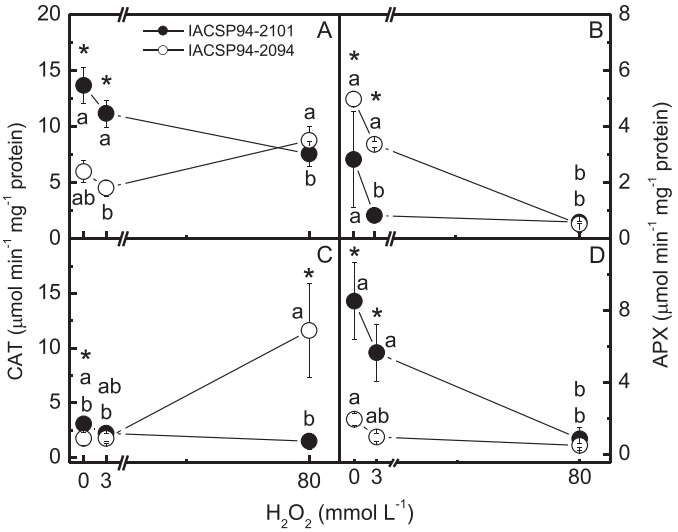

Fig. 3. Activities of CAT (A and C) and APX (B and D) in leaves (A and B) and roots (C and D) of sugarcane genotypes IACSP94-2101 (●) and IACSP94-2094 (○) as affected by H_2O_2 concentration in nutrient solution. Measurements were taken 15 min after applying H_2O_2. Each symbol represents the mean value of three replications ± standard deviation. Different lowercase letters indicate statistical difference ($p < 0.05$) between H_2O_2 treatments, while asterisks indicate statistical difference ($p < 0.05$) between genotypes.

significant increase in H_2O_2 concentration at 80 mmol L^{-1}, being the highest values found in IACSP94-2094 (Fig. 1D).

The highest leaf CAT activity was noticed in IACSP94-2101 plants under reference and 3 mmol L^{-1} conditions, with a reduction around 45% being found in 80 mmol L^{-1} (Fig. 3A). In this concentration, non-significant changes were found in leaf CAT activity of IACSP94-2094 as compared to the reference condition. While the root CAT activity decreased in IACSP94-2101, it increased around five times in IACSP94-2094 under 80 mmol L^{-1} (Fig. 3C).

High H_2O_2 concentration in nutrient solution caused significant reductions in leaf APX activity of both genotypes (Fig. 3B). However, IACSP94-2094 presented higher leaf APX activity than IACSP94-2101 under reference and 3 mmol L^{-1} conditions, with the latter genotype being sensitive to low H_2O_2 concentration (Fig. 3B). A similar response pattern was found in leaves and roots, but the highest root APX activities were measured in IACSP94-2101 under reference and 3 mmol L^{-1} conditions (Fig. 3D).

IACSP94-2101 and IACSP94-2094 showed similar protein content either in leaves (7.28 ± 0.45 and 6.27 ± 0.33 mg g^{-1}, respectively) or roots (2.27 ± 0.47 and 1.92 ± 0.42 mg g^{-1}, respectively) and no significant changes were observed in both organs after

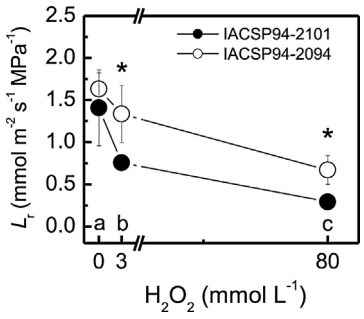

Fig. 4. Root hydraulic conductance in sugarcane genotypes IACSP94-2101 (●) and IACSP94-2094 (○) as affected by H_2O_2 concentration in nutrient solution. Measurements were taken 15 min after applying H_2O_2. Each symbol represents the mean value of three replications ± standard deviation. As the interaction genotype *vs.* H_2O_2 was non-significant, different letters indicate statistical difference ($p < 0.05$) between H_2O_2 treatments and asterisks compare genotypes ($p < 0.05$).

15 min of exposure to H_2O_2. Lipid peroxidation given by increases in MDA was not changed by the H_2O_2 exposure and IACSP94-2101 and IACSP94-2094 presented similar values in the leaves (20.8 ± 2.0 and 16.9 ± 2.6 nmol g^{-1}, respectively) and roots (9.9 ± 2.3 and 6.4 ± 1.1 nmol g^{-1}, respectively).

Both genotypes exhibited reductions in L_r with increasing H_2O_2 concentration in nutrient solution (Fig. 4). While IACSP94-2101 had decreases in L_r of 46% in 3 mmol L^{-1} and 85% in 80 mmol L^{-1}, IACSP94-2094 was less affected and its L_r was reduced in 27% in 3 mmol L^{-1} and 60% in 80 mmol L^{-1}. Regarding the water use efficiency, both genotypes showed an increase in intrinsic water use efficiency (IWUE) when treated with H_2O_2, with IACSP94-2094 showing higher values than IACSP94-2101 (Fig. 5E).

Although both genotypes have shown reductions in leaf CO_2 assimilation (A_N) with increasing H_2O_2 in nutrient solution, IACSP94-2094 was less affected when compared to IACSP94-2101. For instance, A_N was reduced in 4% and 30% in IACSP94-2094 and IACSP94-2101 exposed to 3 mmol L^{-1}, respectively (Fig. 5A). IACSP94-2094 also presented higher A_N than IACSP94-2101 when H_2O_2 was added to the nutrient solution. Accordingly, stomatal conductance (g_S) was also reduced due to H_2O_2 and while such inhibition in IACSP94-2404 ranged from 14% to 51%, it varied between 39% and 70% in IACSP94-2101 under H_2O_2 treatments (Fig. 5B). Changes in instantaneous carboxylation efficiency (k) were noticed only when plants faced high H_2O_2 concentration in nutrient solution (Fig. 5C). However, IACSP94-2094 had higher k than IACSP94-2101, regardless of root condition.

There was no decrease in potential quantum efficiency of photosystem II (F_V/F_M) when roots of both genotypes were exposed to H_2O_2 for 15 min, with mean values of 0.770 ± 0.004 in IACSP94-2094 and 0.766 ± 0.009 in IACSP94-2101. Under light conditions, photochemical impairment was noticed in both genotypes and the lowest apparent electron transport rate (ETR) was measured under 80 mmol L^{-1} (Fig. 5D). Exogenous H_2O_2 supplying also increased relative energy excess at PSII level (EXC) in both genotypes, with IACSP94-2101 showing higher values than IACSP94-2094 under 3 and 80 mmol L^{-1} (Fig. 5F). Regarding ETR and EXC, IACSP94-2094 was once again less sensitive to H_2O_2 as compared to IACSP94-2101.

Discussion

Our results revealed that H_2O_2 supplied in a nutrient solution significantly affected sugarcane physiology, with the effects being dose- and genotype-dependent and occurring in short-time (after 15 min). In general, we expected that the application of H_2O_2 in nutrient solution would generate an oxidative stress in the root

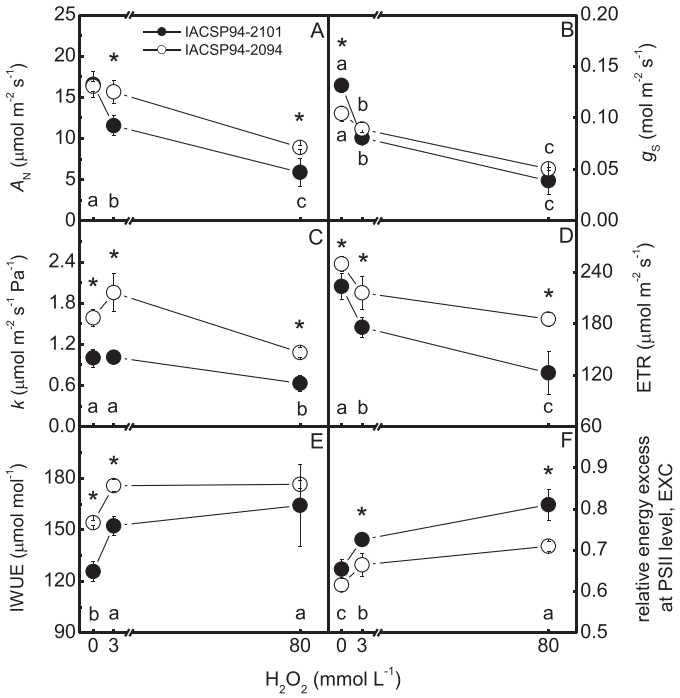

Fig. 5. Leaf CO_2 assimilation (A), stomatal conductance (B), instantaneous carboxylation efficiency (C), apparent electron transport rate (D), intrinsic water use efficiency (E) and relative energy excess at PSII level (F) in leaves of sugarcane genotypes IACSP94-2101 (●) and IACSP94-2094 (○) as affected by H_2O_2 concentration in nutrient solution. Measurements were taken 15 min after applying H_2O_2. Each symbol represents the mean value of three replications ± standard deviation. As the interaction genotype *vs.* H_2O_2 was non-significant in (A) and (C–F), different letters indicate statistical difference ($p < 0.05$) between H_2O_2 treatments and asterisks compare genotypes ($p < 0.05$). In (B), distinct lowercase letters indicate statistical difference ($p < 0.05$) between H_2O_2 treatments in a same genotype, while asterisks indicate statistical difference ($p < 0.05$) between genotypes in each H_2O_2 concentration.

system by inducing the accumulation of $O_2^{\bullet-}$ and production of other ROS (Bolwell et al., 2002). We already know that exogenous H_2O_2 can diffuse through membranes, without changing endogenous H_2O_2 concentration (Yu et al., 2003). This happened in the roots when the plants were subjected to 3 mmol L^{-1} H_2O_2 in nutrient solution (Fig. 1D). Significant increases in the root H_2O_2 concentration were found with 80 mmol L^{-1} (Fig. 1D), suggesting the root $O_2^{\bullet-}$ production. Under stressful conditions, increases in $O_2^{\bullet-}$ production are associated with ATPase inhibition or stimulation of electron flow through cytochrome *b* in mitochondrial metabolism (Hung et al., 2005). Root accumulation of ROS triggers an antioxidant response and the first enzyme to act is SOD, which converts $O_2^{\bullet-}$ into H_2O_2 (Bienert et al., 2006). Afterwards, H_2O_2 may be degraded by the action of CAT and APX enzymes, being the latter enzyme the most important mechanism of detoxification under low H_2O_2 concentration while CAT has a key role under high H_2O_2 concentrations (Mitler, 2002).

Herein, sugarcane plants treated with 3 mmol L^{-1} of H_2O_2 in nutrition solution did not face oxidative stress after 15 min of exposure, as suggested by the unchanged SOD activity (Fig. 1A and C), leaf and root H_2O_2 contents (Fig. 1B and D) and maintenance or even the reduction of CAT and APX activities in leaves and roots of both genotypes (Fig. 3). In fact, the reduction in the leaf H_2O_2 concentration found in IACSP94-2094 with 3 mmol L^{-1} of H_2O_2 may be a consequence of its degradation through the glutathione peroxidase cycle, as previously reported in sugarcane by Cia et al. (2012) and Boaretto et al. (2014). Under high H_2O_2 concentration (80 mmol L^{-1}), root SOD activity increased only in IACSP94-2094, which is an expected antioxidant response (Fig. 1C). As a consequence, IACSP94-2094 showed increases in its root H_2O_2 concentration and activation of the detoxification pathway mediated by CAT (Fig. 3C), which has as primary function to eliminate H_2O_2 (Mitler, 2002). Curiously, root H_2O_2 concentration also increased in IACSP94-2101 plants subjected to 80 mmol L^{-1} and this was associated with reductions in APX and CAT activities (Fig. 3C and D) rather than increase in root SOD activity (Fig. 1C and D). Reduced APX activity could be caused by the non-enzymatic oxidation of ascorbate, its substrate. In fact, H_2O_2 is also a product of ascorbate oxidation and this reaction may contribute to high root H_2O_2 concentration under 80 mmol L^{-1} (Debolt et al., 2007). Moreover, APX itself as well as other peroxidases transiently produce H_2O_2 in presence of iron III (Bindschedler et al., 2006). Regarding the inhibition of CAT, Polidoros and Scandalios (1999) reported that high H_2O_2 concentrations could lead to an oxidative burst, thus causing a systemic inhibition of the CAT gene expression.

Based on the lipid peroxidation and protein content, we can consider that the antioxidant system was effective in preventing damage in both genotypes after 15 min of exposure to high H_2O_2 concentration. However, H_2O_2 in the nutrient solution affected plant water transport. In this study, L_r was reduced in both genotypes with H_2O_2 supplying (Fig. 4), with consequent decreases in g_S (Fig. 5B). An interesting finding was that L_r reduced without increases in root H_2O_2 content in plants subjected to 3 mmol L^{-1} (Fig. 4), which suggests root sensitivity to external H_2O_2 availability, i.e., root medium. Increases in root H_2O_2 concentrations due to 80 mmol L^{-1} was associated with decreases in L_r of both genotypes (Figs. 1D and 4), suggesting the inhibition of aquaporins mediated by H_2O_2 in roots (Boursiac et al., 2008). Comparing genotypes, IACSP94-2101 presented higher L_r sensitivity to root H_2O_2 accumulation, even showing lower root H_2O_2 content than IACSP94-2094 (Fig. 1D). This disturbance in the water relations is probably associated with the inactivation of aquaporins (Ye and Steudle, 2006; Pou et al., 2013) induced by exogenous H_2O_2 application. At this time, we may argue that such differential sensitivity of root aquaporins to H_2O_2 is caused by abundance and/or activity of a particular aquaporin isoform, which remains to be evaluated in further studies. In addition, we have evidence that H_2O_2-induced stress in sugarcane roots is hydraulically and rapidly (15 min) signaled to shoots, through reductions in water transport and consequent stomatal closure.

As a consequence of reduced water transport, leaf responses in both sugarcane genotypes were similar to those found in plants under water deficit (Ribeiro et al., 2013; Sales et al., 2013). Under low water availability, the stomatal opening is reduced to avoid excessive loss of water vapor to the atmosphere. However, stomatal closure also causes low CO_2 availability, affecting photosynthesis. Besides g_S, reduction in A_N was also associated with decreases in ETR (Fig. 5A and D). Although carboxylation reactions of photosynthesis were not affected with 3 mmol L^{-1} of H_2O_2 (Fig. 5C), plants treated with 80 mmol L^{-1} of H_2O_2 presented a significant reduction of *k* (Fig. 5C), indicating impairment of carboxylation reactions. As F_V/F_M remained unchanged in both genotypes under H_2O_2 treatments, we can suggest that the down-regulation of ETR represents a physiological mechanism related to closure of photosystem II (PSII) reaction centers to avoid excessive energy pressure (Fig. 5D). However, both genotypes presented increases in EXC when supplied with H_2O_2 (Fig. 5F), indicating an unbalance between photochemical and biochemical reactions of photosynthesis (Cruz De Carvalho, 2008).

As reduced intermediates in thylakoidal electron transport chain accumulate, we expect increases in ROS production and the leaf antioxidant system must be able to protect tissues from oxidative damage (Cheeseman, 2007). Organisms less sensitive to drought probably have an antioxidant system effective in

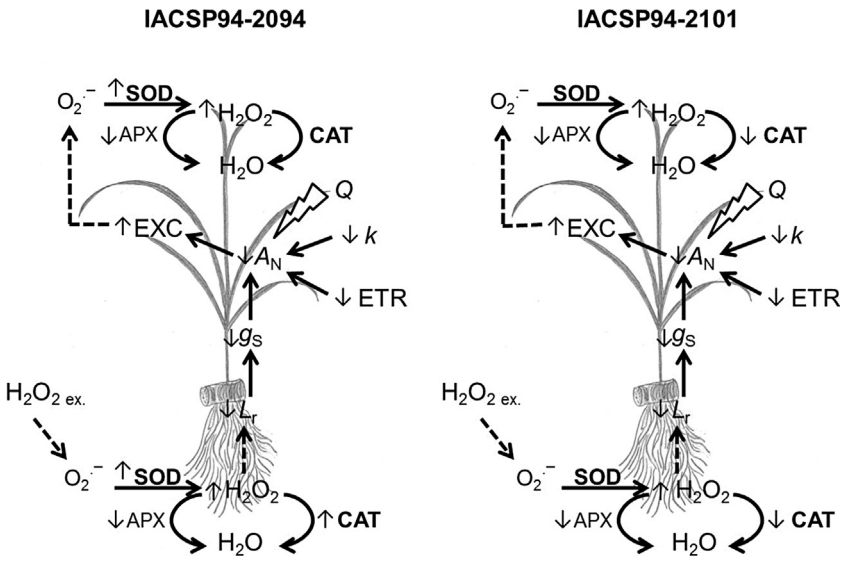

Fig. 6. General reaction of the antioxidant system, water relations and gas exchange in sugarcane genotypes IACSP94-2094 (drought-tolerant) and IACSP94-2101 (drought sensitive) to H_2O_2 supplying in nutrient solution (80 mmol L^{-1}). SOD: superoxide dismutase; APX: ascorbate peroxidase; CAT: catalase; $O_2^{\cdot-}$: superoxide anion; $H_2O_{2\ ex.}$: exogenous hydrogen peroxide; H_2O_2: hydrogen peroxide; EXC: relative energy excess at PSII level; Q: photosynthetically active radiation; H_2O: water; L_r: root hydraulic conductance; g_S: stomatal conductance; A_N: leaf CO_2 assimilation, k: instantaneous carboxylation efficiency. Arrows indicate increases or decreases in activities or processes. Bold abbreviations represent the main differences between genotypes. Dotted lines indicate an indirect connection.

preventing or minimizing oxidative stress (Mitler et al., 2004). In addition, such organisms should maintain water transport between roots and shoots, being the stomatal apparatus and CO_2 assimilation less affected (Machado et al., 2009). Our results revealed that the physiological responses presented by IACSP94-2094 are in accordance to its drought tolerance reported by Machado et al. (2009) and Ribeiro et al. (2013), and we propose a model of how both sugarcane genotypes react to high H_2O_2 concentration in root medium, considering the rapid physiological responses detected in roots and leaves under acute stress, i.e., 80 mmol L^{-1} of H_2O_2 (Fig. 6). This scheme reveals the linking between root and shoot metabolism and shows clearly differences in the antioxidant system of both genotypes.

SOD and CAT seem to play an important role in the redox homeostasis of both leaf and root cells, with higher activities in IACSP94-2094 than in IACSP94-2101 (Figs. 1A and C, 3A and C and 6). Accordingly, the high leaf SOD activity was associated with rapid recovery of photosynthesis in IACSP94-2094 after simultaneous drought and low temperature stresses (Sales et al., 2013). Our data revealed slight increases in the Cu/Zn-SOD isoform activity of IACSP94-2094 leaves (Fig. 2), which is in agreement with a slight change in its overall SOD activity (Fig. 1A). Our data also revealed that most of the SOD activity in IACSP94-2094 is due to one cytosolic Cu/Zn-SOD (Fig. 2).

Under acute stress as one imposed by 80 mmol L^{-1} of H_2O_2, both genotypes would have difficulties in maintaining water balance and photosynthesis for longer periods, showing limited growth as medium- to long-term responses. Although leaf and root protein contents and lipid peroxidation have not been affected after 15 min of H_2O_2 treatment, a later analysis of membrane integrity and protein amount would reveal cell damage, and thus the physiological responses reported herein represent the first physiological reactions to H_2O_2 in root medium, before the occurrence of severe oxidative stress.

When comparing sugarcane genotypes, IACSP94-2094 presented higher photosynthetic rates than IACSP94-2101, being that ability associated with a higher root hydraulic conductance, photochemistry and biochemical activity of photosynthesis under stressful conditions (Figs. 4 and 5C and D). Although Ribeiro et al. (2013) have reported previously the resistance of photosynthetic apparatus in IACSP94-2094 under water deficit, the connection between root and leaf metabolisms is for the first time established by evaluating antioxidant metabolism, water transport and photosynthesis. In conclusion, we have found a significant genotypic variation of sugarcane responses to increasing H_2O_2 concentration in root medium, with changes observed in roots being associated with changes in shoot physiology. The drought-tolerant genotype IACSP94-2094 presents an effective root antioxidant system under high H_2O_2 concentration, with water transport and leaf gas exchange being less sensitive to H_2O_2 as compared to IACSP94-2101. This lower sensitivity of IACSP94-2094 to the high H_2O_2 concentration was associated with increases in root and leaf SOD activities and root CAT activity.

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