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Abstract: Nitric oxide (NO) may be formed enzymatically and non-enzymatically and the main NO source is subject of much debate in plants. The aim of this study was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, in which the more drought-tolerant genotype presenting higher NO accumulation. The sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) were grown in growth chamber and submitted to water deficit by adding polyethylene glycol (PEG-8000) in nutrient solution to reduce the osmotic potential to -0.4 MPa. For evaluating short-time responses to water deficit, samples were taken after 24 h under water deficit. IACSP95-5000 presented higher root extracellular NO content, which was accompanied by higher root nitrate reductase (NR) activity as compared to IACSP97-7065 under water deficit. In addition, IACSP95-5000 had higher leaf intracellular NO content than IACSP97-7065. The drought-tolerant genotype exhibited decreases in root S-nitrosoglutathione reductase (GSNOR) activity under water deficit, suggesting that S-nitrosoglutathione (GSNO) is less degraded and IACSP95-5000 has a higher natural reservoir of NO than IACSP97-7065. Those differences in intracellular and extracellular NO contents and enzymatic activities were associated with higher leaf hydration in the drought-tolerant genotype as compared to the sensitive one under water deficit.

Cover Letter

Dear Prof. Mario De Tullio

Editor-in-Chief | Plant Physiology and Biochemistry

We would like to submit our paper entitled "Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane" for your appreciation. This paper provides new information and insights about the involvement of NO production and its metabolism on drought tolerance of sugarcane plants. Here, we present data about the intracellular and extracellular NO production and some related enzymes in two sugarcane genotypes differing in drought response, as evaluated by leaf relative water content. Our data indicate that NO metabolism is more active in IACSP95-5000 than in IACSP97-7065, with the drought-tolerant IACSP95-5000 presenting higher leaf intracellular NO content, higher root extracellular NO content, higher root NR activity and lower root GSNOR activity as compared to IACSP97-7065.

We look forward to hearing from you.

Yours sincerely,

Rafael V. Ribeiro
Corresponding author

Highlights

- NO production and metabolism were studied in two sugarcane genotypes under drought
- Higher root extracellular and leaf intracellular NO content were found in drought-tolerant genotype
- Drought-tolerant genotype exhibited higher root NR activity and lower root GSNOR activity

Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane

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Abbreviations: GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; GSSG, oxidized glutathione; NH_4^+, ammonium; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PEG, polyethylene glycol; PPFD, photosynthetic photon flux density; RSNO, *S*-nitrosothiol; RWC, relative water content; WD, water deficit.

Abstract

Nitric oxide (NO) may be formed enzymatically and non-enzymatically and the main NO source is subject of much debate in plants. The aim of this study was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, in which the more drought-tolerant genotype presenting higher NO accumulation. The sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) were grown in growth chamber and submitted to water deficit by adding polyethylene glycol (PEG-8000) in nutrient solution to reduce the osmotic potential to -0.4 MPa. For evaluating short-time responses to water deficit, samples were taken after 24 h under water deficit. IACSP95-5000 presented higher root extracellular NO content, which was accompanied by higher root nitrate reductase (NR) activity as compared to IACSP97-7065 under water deficit. In addition, the drought-tolerant genotype had higher leaf intracellular NO content than the drought-sensitive one. IACSP95-5000 exhibited decreases in root *S*-nitrosoglutathione reductase (GSNOR) activity under water deficit, suggesting that *S*-nitrosoglutathione (GSNO) is less degraded and that the drought-tolerant has a higher natural reservoir of NO than the drought-sensitive genotype. Those differences in intracellular and extracellular NO contents and enzymatic activities were associated with higher leaf hydration in the drought-tolerant genotype as compared to the sensitive one under water deficit.

Keywords: Nitrate reductase; *S*-nitrosoglutathione reductase; NO metabolism; genotype dependent.

1. Introduction

Despite evidence regarding the importance of nitric oxide (NO) in plant signaling, the mechanism responsible for NO synthesis is still controversial. It is now widely accepted that NO plays a key role in signaling among plant cells, however, it has been a challenge to determine the sources of NO in plants and there is considerable discussion of how exactly NO is formed in plant cells (Hancock, 2012; Salgado et al., 2013). In biological systems, NO can be formed both enzymatically and non-enzymatically. In mammals, the enzyme responsible for NO generation is NO synthase (NOS), with L-arginine being converted to citrulline, using NADPH as electron donor and O_2 as co-substrate and producing NO and water (Alderton et al., 2001). However, the existence of NOS remains questionable in plants. Although NO production is dependent on L-arginine and its production is sensitive to inhibitors of NOS (Moreau et al., 2010), a homologous gene for this protein has not been found in plants. A recent extensive survey of higher plant genomes failed to uncover the presence of a NOS encoding region in any species (Jeandroz et al., 2016).

The nitrate reductase (NR) enzyme is essential for nitrogen assimilation and also involved in NO production both *in vitro* (Rockel et al., 2002) and *in vivo* (Kaiser et al., 2002). As a secondary activity, NR reduces nitrite to NO using NADPH, being NO synthesis dependent on the nitrite and nitrate contents of plant tissues. The efficiency of this reaction for NO production is considered low and requires high concentrations of nitrite (Yamasaki and Sakihama, 2000; Rockel et al., 2002). Modolo et al. (2005) have suggested that the primary role of NR for NO production is as a pathway to provide nitrite. Electrons

required for the reduction of nitrite to NO can be provided by the mitochondrial respiratory chain (Planchet et al., 2005) or by the photosynthetic system (Jasid et al., 2006).

The NO bioavailability may be affected by glutathione (GSH), an antioxidant present at high intracellular concentrations. Spontaneous reaction of NO with the thiol grouping of GSH will form *S*-nitrosoglutathione (GSNO). The control of intracellular GSNO is partly regulated by degradation catalyzed by *S*-nitrosoglutathione reductase (GSNOR) (Frungillo et al., 2014). The GSNOR catabolizes GSNO to oxidized glutathione (GSSG) and ammonium (NH_4^+), resulting in depletion of intracellular levels of GSNO and reduction of *S*-nitrosothiol (RSNO) formation by transnitrosation processes. In fact, GSNO has an important role in *S*-nitrosation and also represents a natural intracellular reservoir of NO (Ji et al., 1999; Liu et al., 2001).

Recent studies have shown that NO plays an important role in plants under stressful conditions, such as drought (Santisree et al., 2015; Farnese et al., 2016; Silveira et al., 2016). For instance, Arasimowicz-Jeloneka et al. (2009) found that roots subjected to mild water deficit enhanced NO synthesis in root cells of *Cucumis sativus*, with an intense NO production in elongation zone. Although several reports have shown increased NO production under drought (Filippou et al., 2011; Fan and Liu, 2012; Xiong et al., 2012; Cai et al., 2015), there is no information about how plant species/varieties differ in NO production and how this differential NO production is related to drought tolerance. The aim of this work was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, with the more drought-tolerant genotype presenting higher NO accumulation.

2. Material and methods

2.1. *Plant material and growth conditions*

Two sugarcane genotypes (*Saccharum* spp.) developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) with differential biomass production and drought tolerance were studied: IACSP95-5000 is a drought-tolerant genotype (Marchiori, 2014), whereas IACSP97-7065 is sensitive to water deficit (Oliveira, 2012; Sales et al., 2013). The plants these two genotypes were obtained from mini-stalks taken from adult plants and planted in commercial substrate (Levington M2 Compost, Heerlen UK). After 50 days, plants with five to six leaves were transferred to modified Sarruge (1975) nutrient solution which is composed of 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; the pH of nutrient solution was kept between 5.5 and 6.0 and its electrical conductivity between 1.53 and 1.70 mS cm^{-1} by weekly monitoring and corrected when necessary. Plants were grown in growth chamber, with a 12-h photoperiod, air temperature of 30/20^{o}C (day/night), air relative humidity of 80% and the photosynthetic photon flux density (PPFD) about 700 µmol m^{-2} s^{-1}.

2.2. *Water deficit induced by PEG*

Sugarcane plants growing in nutrient solution were submitted to water deficit (WD) by adding polyethylene glycol (PEG-8000, Fisher Scientific, Leicestershire, UK) to the solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a

gradual decrease in its osmotic potential until -0.4 MPa. All evaluations were taken 24 hours after the solution reached the desired osmotic potential, being the short-term responses to water deficit evaluated. Leaf and root samples were collected, immediately immersed in liquid nitrogen and then stored at −80 ^{o}C for further enzymatic analyses.

2.3. *Relative water content*

The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Jamaux et al. (1997): RWC=100×[(FW−DW)/(TW−DW)].

2.4. *DAF2 fluorimetric assay for extracellular NO*

Leaf and root samples (100 mg) were incubated in 10 mM Tris, 50 mM KCl, pH 7.2 buffer in 1 mL microcentrifuge tubes for 40 min, before the addition of 5 µM 4,5-diaminofluorescein diacetate (DAF2). The sample was placed into a quartz cuvette and fluorescence measured for 30 min (Suppl. Fig. S1) using a fluorescence spectrophotometer (F-2500, Hitachi - Science & Technology, Berkshire, UK) with excitation and emission at 488 and 512 nm, respectively (Bright et al., 2009). For the negative control, samples were incubated in the absence of DAF2. Data are shown as average value (n=3) for each treatment and they represent the fluorescence signal after 30 min, considering the negative control (data shown = sample – negative control).

2.5. *DAF2-DA detection of intracellular NO*

Intracellular NO was visualized using the cell permeable NO-specific dye 4,5 diaminofluorescein-2 diacetate (DAF2-DA). Leaf and root segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM $CaCl_2$, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in solution of 10 µM DAF2-DA, mixing gently per 40 min in dark and at room temperature (Desikan et al., 2002; Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-DA and placed onto a glass slide and covered with a glass slip before observing fluorescence using laser-scanning microscopy with excitation at 488 nm and emission at 515 nm (Nikon PCM 2000, Nikon, Kingston-upon-Thames, UK). Photos were taken with a 10x magnification, 15 s exposure and 1x gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data are presented as mean pixel intensities.

2.6. *S-nitrosoglutathione reductase (GSNOR) activity*

Leaf and root GSNO reductase activity was estimated spectrophotometrically as the rate of NADH oxidation in presence of GSNO as described previously (Frungillo et al., 2014). Briefly, 0.1 g of fresh tissue was grounded with liquid nitrogen, resuspended in 20 mM HEPES buffer, pH 8.0, 0.5 mM EDTA, 0.5 mM PMSF and proteinase inhibitors (50 mg mL^{-1} TPCK and 50 mg mL^{-1} TLCK) and centrifuged for 10 min at 10,000 x*g* at 4 ^{o}C. The protein extract was then incubated with 20 mM HEPES buffer, pH 8.0, 350 µM NADH in the presence or not of 350 µM GSNO. GSNO reductase activity was estimated by subtracting the rate of NADH oxidation in the absence of GSNO from that in the presence

of GSNO by using the NADH molar extinction coefficient (6.22 M^{-1} cm^{-1}) and normalized by protein content.

2.7. *Nitrate reductase (NR) activity*

Actual NR activity was estimated as the rate of NO_2^- production as described before (Frungillo et al., 2014). Protein extract was obtained from the macerate of 0.1 g of fresh tissue with liquid nitrogen in 20 mM HEPES, pH 8.0, 0.5 mM EDTA, 10 mM FAD, 5 mM Na_2MoO_4, 6 mM $MgCl_2$, 0.5 mM PMSF and proteinase inhibitors (50 mg mL^{-1} TPCK and 50 mg mL^{-1} TLCK). The reaction medium consisted of 1 mL of extraction buffer supplemented with 10 mM KNO_3 and 1 mM NADH. Nitrite production was determined by adding equal volumes of the reaction solution and 1% sulphanilamide, 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride in 1.5 N HCl, and measurement of absorbance at 540 nm on a spectrophotometer. The values obtained were compared to those of a standard curve constructed using KNO_2 and normalized against protein content.

2.8. *Protein content*

The protein content was determined by the Coomassie-blue method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The readings were performed using a microplate format (Fluostar Optima Microplate Reader, BMG Labtech, Ortenberg, Germany).

2.9. *Data analysis*

The experimental design was completely randomized and two causes of variation (factors) were analyzed: water availability and genotypes. Data were subjected to the analysis of variance (ANOVA) and mean values were compared by the Tukey test when significance was detected ($p<0.05$). The results presented are the mean ± SD and the number of replicates is stated in each figure legend.

3. Results

3.1. *Leaf relative water content (RWC)*

The water deficit induced a reduction in RWC of both genotypes, with the drought-tolerant genotype IACSP95-5000 being less affected as compared to IACSP97-7065 (Fig. 1).

3.2. *Extracellular and intracellular NO release*

We first investigated the production of NO in leaves and roots of two commercially available sugarcane genotypes that have been shown to display different drought tolerance (Marchiori, 2014). Differently from IACSP95-5000, leaves of IACSP97-7065 showed a significant increase (+30.8%) in extracellular NO under water deficit (Fig. 2A). In roots tissues, the extracellular NO production increased in both genotypes under water deficit compared to well hydrated plants. Remarkably, IACSP95-5000 exhibited the highest

extracellular NO emission from roots under water deficit, being 46% higher than in IACSP97-7065 (Fig. 2B).

Intracellular NO content was monitored using the NO-sensitive probe DAF2-DA in a fluorimetric assay. Leaves of IACSP95-5000 plants showed increase in fluorescence under water deficit when compared to well-hydrated condition (Fig. 3A,B). Non-significant changes in intracellular NO production were found in IACSP97-7065, regardless water availability. However, the drought-sensitive genotype presented lower values than IACSP95-5000 under low water availability (Fig. 3B). Both genotypes exhibited increases in intracellular NO content in roots under water deficit and no differences were observed among the genotypes studied (Fig. 3C,D).

3.3. *NO synthesis and degradation*

Leaf NR activity was not affected by water deficit, regardless of which sugarcane genotype was studied (Fig. 4A). However, water deficit reduced root NR activity in both genotypes, with IACSP95-5000 presenting higher root NR activity than IACSP97-7065 under low water availability (Fig. 4B). Leaf GSNOR activity did not change by water deficit and IACSP95-5000 presented higher GSNOR activity than IACSP97-7065 in both water conditions (Fig. 4C). Root GSNOR activity was reduced by water deficit only in IACSP95-5000 (Fig. 4D).

4. Discussion

The drought-tolerant genotype IACSP95-5000 produced more NO extracellular in roots when compared to the sensitive genotype IACSP97-7065 (Fig. 2B). Such response may have a role in root formation, which would be expected under water deficit. In fact, it has been shown that NO is associated with the signaling cascades leading to root hair formation in *A. thaliana* (Lombardo et al., 2006, 2012) and with increases in root dry mass in sugarcane (Silveira et al., 2016). The main function of root hairs is to increase root surface and then improve the uptake of water and nutrients. In such context, increases in extracellular NO content could trigger root formation and improve water uptake in IACSP95-5000.

Images by confocal microscopy showed that leaves of IACSP95-5000 had also increased intracellular NO production under water deficit (Fig. 3A,B), giving additional evidence for an association between NO production and drought tolerance. It has been suggested that NO can diffuse rapidly through the cytoplasm and biomembranes, thus affecting many biochemical functions simultaneously (Lamattina et al., 2003), although this has been questioned by other (Lancaster et al., 1997).

NO synthesis in plant cells is not yet fully understood, constituting one of the major challenges to studies investigating this signaling molecule. Nitrate reductase activity, a cytosolic enzyme essential for the assimilation of nitrogen, has been suggested to play a key role in NO production in plants (Horchani et al., 2011). In this study, the tolerant genotype showed higher root NR activity than the sensitive one under water deficit (Fig. 4B). In addition, NO can also be produced by several other enzymatic and non-enzymatic pathways (Hancock, 2012). The nitrite has been considered the main substrate for NO

production and it can be reduced to NO by electrons provided by the photosynthetic system (Jasid et al., 2006) or by the mitochondrial chain (Planchet et al., 2005). Furthermore, polyamines (PAs) may induce NO biosynthesis in *Arabidopsis* seedlings, giving a new insight into PA-mediated signaling and NO as a potential mediator of PA actions (Tun et al., 2006).

NO degradation is as important as its synthesis in determining the final concentration of NO as a signaling molecule in plant cells. Herein, the drought-tolerant genotype exhibited decreases in root GSNOR activity under water deficit (Fig. 4D). As a consequence, it could be argued that GSNO is less degraded, which would improve the performance of IACSP95-5000 under water deficit. In fact, GSNO regulates NO availability acting as a natural reservoir of intracellular NO and acts particularly in *S*-nitrosation of thiol groups of proteins (Silveira et al., 2016). GSNOR can also modulate SNO levels in response to abiotic stresses, an important response for improving plant acclimation (Salgado et al., 2013). Accordingly, the drought-tolerant genotype exhibited higher leaf GSNOR activity than the sensitive one in both water regimes (Fig. 4A).

In this study, we demonstrated that NO metabolism is more active in IACSP95-5000 than in IACSP97-7065, with the drought-tolerant IACSP95-5000 presenting higher root extracellular NO content, higher root NR activity and lower root GSNOR activity as compared to IACSP97-7065. IACSP95-5000 had also higher leaf intracellular NO content than IACSP97-7065. NO influence on metabolic and physiological processes is due to its ability in interacting and modifying multiple targets within the plant cell (Lamattina et al., 2003), which turns the understanding of its effects on plants a hard task. The understanding of metabolic pathways controlling NO homeostasis in plants should be one of the major aims of NO research in the near future.

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Authors' contributions

NMS, LF, FCCM, JTH, IS, ECM and RVR designed the experiments. NMS and ES performed the biochemical measurements. FCCM obtained the mini stalks taken from adult plants. NMS and RVR wrote the manuscript and all authors contributed to data discussion and edited the final version of the manuscript.

Figure 1

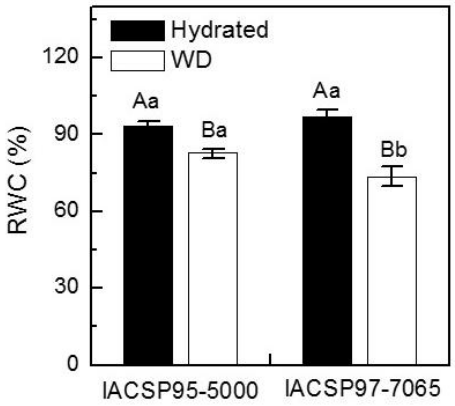

Fig. 1. Leaf relative water content (RWC) in sugarcane genotypes IACSP95-5000 and IACSP97-7065 under well-hydrated conditions (Hydrated) or water deficit (WD). The data represents the mean value of four replications ± standard deviation. Different uppercase letters indicate statistical difference ($p<0.05$) between water treatments, while different lowercase letters indicate statistical difference ($p<0.05$) between genotypes.

Figure 2

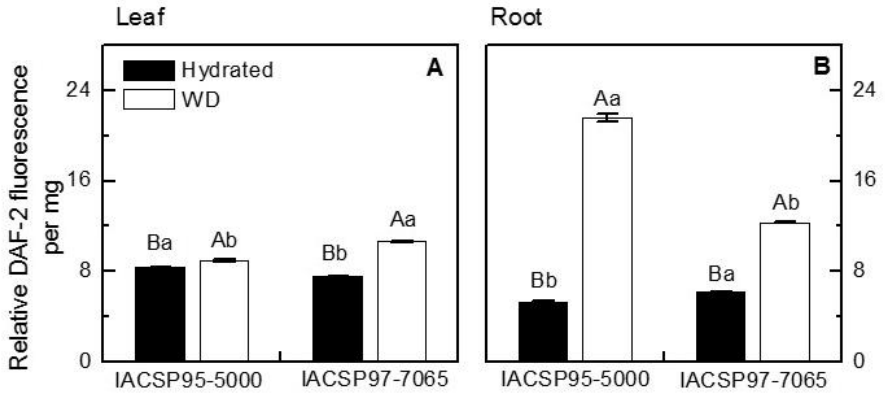

Fig. 2. Relative DAF-2 fluorescence demonstrating DAF-2-reactive compound-release (NO) in sugarcane genotypes IACSP95-5000 and IACSP97-7065 under well-hydrated conditions (Hydrated) or water deficit (WD) in leaves (A) and roots (B). The data represents the mean value of four replications ± standard deviation. Measurements of relative fluorescence were taken after 30 min. Different uppercase letters indicate statistical difference ($p<0.05$) between water treatments, while different lowercase letters indicate statistical difference ($p<0.05$) between genotypes. Data were normalized by subtracting the values of the negative controls.

Figure 3

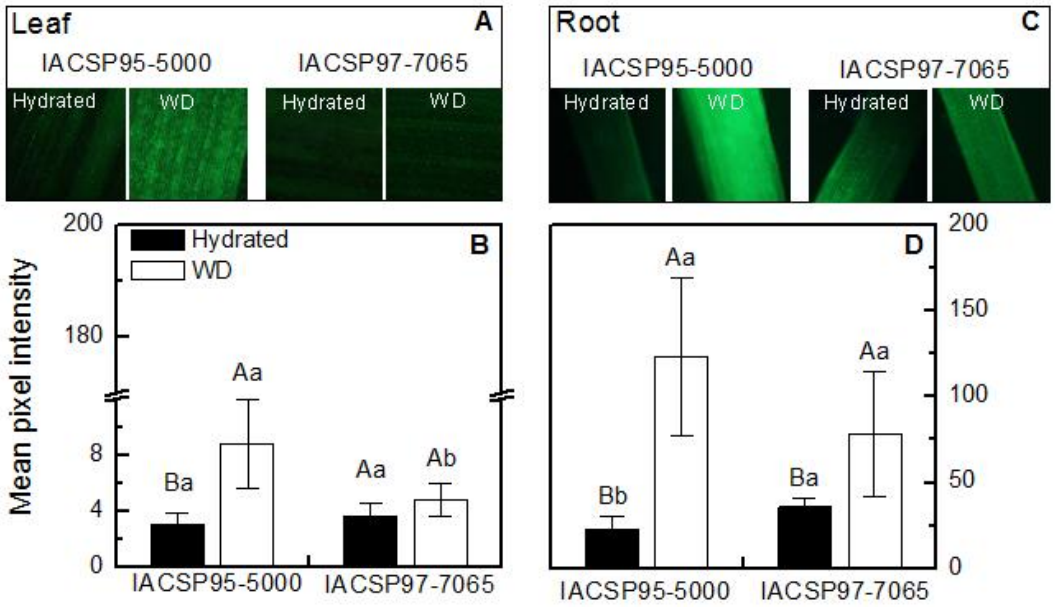

Fig. 3. Confocal microscopy images demonstrating intracellular NO synthesis in leaves (A) and roots (C) and mean pixel intensity by ImageJ in sugarcane genotypes IACSP95-5000 and IACSP97-7065 under well-hydrated conditions (Hydrated) or water deficit (WD) in leaves (B) and roots (D). The data represents the mean value of five replications ± standard deviation. Different uppercase letters indicate statistical difference ($p<0.05$) between water conditions, while different lowercase letters indicate statistical difference ($p<0.05$) between genotypes. Data were normalized by subtracting the values of the negative control.

Figure 4

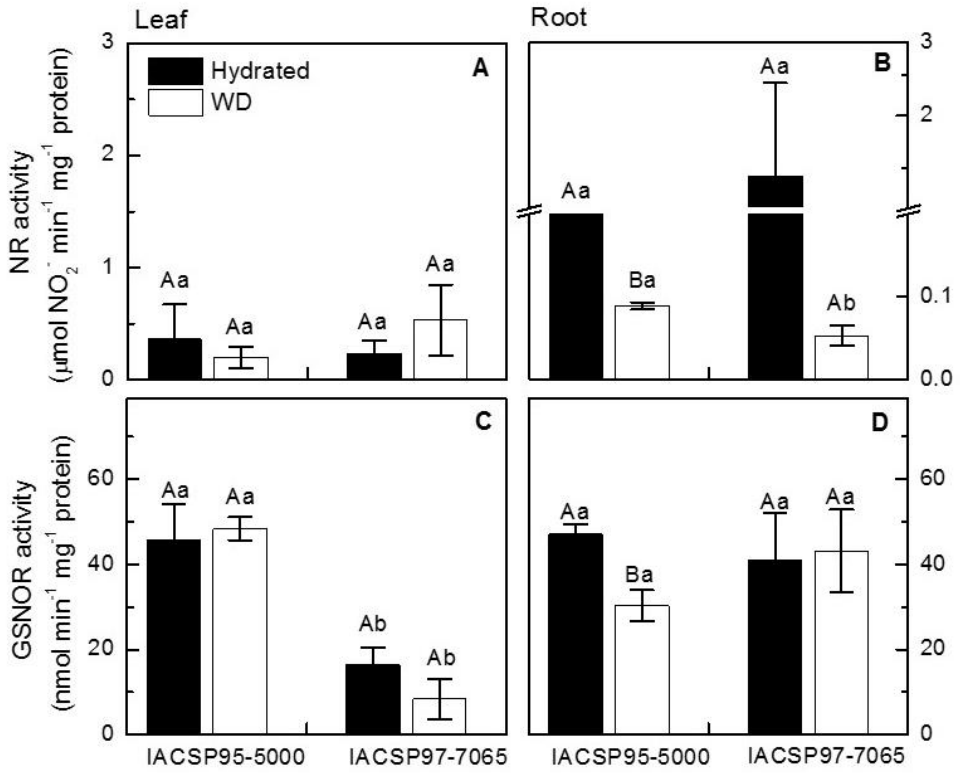

Fig. 4. Nitrate reductase activity (NR, in A,B) and *S*-nitrosoglutathione reductase activity (GSNOR, in C,D) in leaves (in A,C) and roots (in B,D) in sugarcane genotypes IACSP95-5000 and IACSP97-7065 under well-hydrated conditions (Hydrated) or water deficit (WD). The data represents the mean value of three replications ± standard deviation. Different uppercase letters indicate statistical difference ($p<0.05$) between water conditions, while different lowercase letters indicate statistical difference ($p<0.05$) between genotypes.

Supplementary material

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