Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit

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Abbreviations: A, leaf CO_2 assimilation; C_i, intercellular CO_2 concentration; ETR, apparent electron transport rate; F_V/F_M, maximum quantum efficiency of PSII; g_S, stomatal conductance; GSH, glutathione; GSNO, *S*-nitrosoglutathione; k, instantaneous carboxylation efficiency; LDM, leaf dry mass; NO, nitric oxide; NPQ, non-photochemical quenching; PEG, polyethylene glycol; PPFD, photosynthetic photon flux density; PSII, photosystem II; RDM, root dry mass; RWC, relative water content; RSNO, *S*-nitrosothiol; WD, water deficit; ϕ_{PSII}, effective quantum efficiency of PSII.

Abstract

Main conclusion **NO-mediated redox signaling plays a role in alleviating the negative impact of water stress in sugarcane plants by improving root growth and photosynthesis.**

Drought is an environmental limitation affecting sugarcane growth and yield. The redox active molecule nitric oxide (NO) is known to modulate plant responses to stressful conditions. NO may react with glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), which is considered the main reservoir of NO in cells. Here, we investigate the role of NO in alleviating the effects of water deficit on growth and photosynthesis of sugarcane plants. Well-hydrated plants were compared to plants under drought and sprayed with mock (water) or GSNO at concentrations ranging from 10 to 1000 μM. Leaf GSNO sprayed plants showed significant improvement of relative water content, and leaf and root dry matter under drought compared to mock-sprayed plants. Additionally, plants sprayed with GSNO (≥100 μM) showed higher leaf gas exchange and photochemical activity as compared to mock-sprayed plants under water deficit and after rehydration. Surprisingly, a raise in the total *S*-nitrosothiols content was observed in leaves sprayed with GSH or GSNO, suggesting a long-term role of NO-mediated responses to water deficit. Experiments with leaf discs fumigated with NO gas also suggested a role of NO in drought tolerance of sugarcane plants. Overall, our data indicate that the NO-mediated redox signaling play a role in alleviating the negative effects of water stress in sugarcane plants by protecting the photosynthetic apparatus and improving shoot and root growth.

Keywords: Drought; Photochemistry; *Saccharum* spp; *S*-nitrosoglutathione; Water stress.

Introduction

Drought is considered the main abiotic stress for plants (Parry et al. 2004; Cruz de Carvalho 2008), being the most important environmental constrain to sugarcane (Ramesh 2000). Under drought conditions, stomatal closure is a primary response to avoid water loss through leaf transpiration. However, such response also reduces the CO_2 availability for photosynthesis and then biomass production is inhibited (Machado et al. 2009; Ribeiro et al. 2013). Additionally, decreases in leaf chlorophyll content, inhibition of photochemical activity and photosynthetic enzymes of the C_4 metabolism have been reported in drought-stressed sugarcane (Machado et al. 2009; Barbosa et al. 2015). As consequence of low carboxylation capacity, there is an ineffective recycling of coenzymes ATP and NADPH produced during the light reactions and plants face excessive light energy and photoinhibition of photosynthesis, with reduction on quantum efficiency of photosystem II (Sales et al. 2013, 2015).

Nitric oxide (NO) is a redox active molecule with well-established central roles in plant development and responses to biotic and abiotic stresses (Santos-Filho et al. 2012; Salgado et al. 2013; Frungillo et al. 2014; Kneeshaw et al. 2014; Simontacchi et al. 2015). Intracellularly, NO may react with the antioxidant glutathione (GSH) to yield GSNO (Liu et al. 2001). GSNO has been considered a natural reservoir of NO in cells (Stamler et al. 1992; Lindermayr et al. 2005) and several lines of evidence suggest that the NO and GSNO signaling functions overlap. In fact, both NO and GSNO are able to post-transcriptionally control protein activity and localization through *S*-nitrosylation (Salgado et al. 2013; Yu et al. 2014). NO may also react with superoxide under oxidative stress and produce the potent oxidant peroxynitrite that causes permanent nitration of tyrosine residues in proteins (Radi 2004). This NO-mediated mechanism of protein modification

may also be induced during plant responses to biotic and abiotic stresses (Chaki et al. 2011). As transcription factors can also be targets of *S*-nitrosylation, NO/GSNO can change gene expression (Besson-Bard et al. 2009; Begara-Morales et al. 2014).

The phytohormone abscisic acid (ABA) is a key constituent of abiotic stress responses in plants. During water stress, biosynthesis and activation of ABA mediates stomatal closure to prevent water loss by transpiration, a processes modulated by the activity of open stomata 1 (OST1)/sucrose nonfermenting 1 (SNF1)-related protein kinase 2.6 (SnRK2.6) (Lee et al. 2006). Recently, *S*-nitrosylation of SnRK2.6 at Cys 137 was proposed to counteract ABA-induced stomatal closure in guard cells of *Arabidopsis thaliana* (Wang et al. 2015). Additionally, pharmacological and genetic evidence indicate that NO-mediated signaling increases tolerance to water stress in plants (Tian and Lei 2006; Cai et al. 2015; Foresi et al. 2015).

On the other hand, the studies regarding NO influence on the photosynthetic apparatus are not easily conciliated. Metal-induced impairment of the electron transport chain in photosynthesis was attenuated by NO in plants (Aftab et al. 2012; Yang et al. 2012). Additionally, NO was shown to induce a slow and continuous increase of the non-photochemical quenching of fluorescence, a well-known photoprotective mechanism (Ördög et al. 2013). Intriguingly, evidences suggest that NO reversibly inhibits the photosynthetic electron transport in guard cells, reducing ATP and NADPH production, starch formation and also the synthesis of malate and sucrose (Takahashi et al. 2002; Wodala et al. 2008; Ördög et al. 2013; Misra et al. 2014). It has been proposed that the protective functions of NO are likely dependent on a fine control of its cellular homeostasis under different physiological conditions and stressful conditions (Salgado et al. 2013).

Here, we have hypothesized that NO can attenuate the inhibition of growth and photosynthesis in sugarcane plants under water deficit. In addition, the underlying mechanisms leading to improved photosynthesis in NO-supplied plants under drought are also addressed in this study.

Materials and methods

Plant material and growth conditions

Sugarcane plants (*Saccharum* spp.) cv. IACSP94-2094 were propagated by placing mini-stalks from adult plants in trays containing commercial substrate (Carolina Soil of Brazil, Vera Cruz RS, Brazil). Four-week-old plants with three to four leaves were transferred to plastic pots (5 L) containing soil and irrigated daily under greenhouse conditions, where the air temperature varied between 18 °C and 37 °C and the maximum photosynthetic photon flux density (PPFD) was about 1100 μmol m^{-2} s^{-1}. Another group of similar plants was transferred to modified Sarruge (1975) nutrient solution [0.31 g L^{-1} KNO_3, 1.20 g L^{-1} $Ca(NO_3)_2$, 0.50 g L^{-1} $MgSO_4$, 0.08 g L^{-1} NH_4NO_3, 0.14 g L^{-1} KH_2PO_4, 0.06 g L^{-1} $KClO_3$, 0.07 g L^{-1} Na_2EDTA, 0.07 g L^{-1} $FeSO_4$, 1.69 mg L^{-1} H_3BO_3, 1.10 mg L^{-1} $ZnSO_4$, 0.16 mg L^{-1} Cu_2SO_4, 0.92 mg L^{-1} $MnSO_4$, 2.32 mg L^{-1} $(NH_4)_2MoO_4$] and maintained hydroponically in a growth chamber (PGR15, Conviron, Winnipeg MB, Canada), at 30/20 ºC (day/night), 80% relative humidity, 12 h photoperiod (7:00 to 19:00 h) and PPFD of 800 μmol m^{-2} s^{-1}. The pH of the nutrient solution was monitored with a pHmeter Tec-3MPp (Tecnopon, Piracicaba SP, Brazil) and kept between 5.5 and 6.0 by adding sulfuric acid or sodium hydroxide. The electrical conductivity of the nutrient solution was also monitored (Tec-4MPp, Tecnopon, Piracicaba SP, Brazil) and the values

were kept between 1.53 and 1.70 mS cm^{-1} by replacing the solution. Plants were grown under the above conditions for 25 days prior to treatments.

Synthesis of *S*-nitrosoglutathione (GSNO)

GSNO was synthesized and characterized as previously described (Shishido et al. 2003; De Oliveira et al. 2002; Seabra and De Oliveira 2004; De Souza et al. 2006). Reduced glutathione (GSH) was reacted with equimolar amount of sodium nitrite in acidified aqueous solution, in an ice bath for 40 minutes, under magnetic stirring. The obtained GSNO was precipitated by the addition of acetone, filtrated, and washed with cold water. The obtained solid was freeze-dried for 24 h.

Experiment I: Water deficit induced by PEG and GSNO spraying

Sugarcane plants growing in nutrient solution were submitted to water deficit (WD) by adding polyethylene glycol (Carbowax™ PEG-8000, Dow Chemical Comp, Midland MI, USA) to the solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential as follows: -0.25 MPa with 20 mM PEG-8000 for one day; -0.50 MPa with 74 mM PEG-8000 for four days; and finally -0.75 MPa with 111 mM PEG-8000. As we did not notice any significant change in leaf gas exchange of plants grown in nutrient solution with -0.50 MPa of osmotic potential, we considered the day 1 of water deficit when the osmotic potential of nutrient solution reached -0.75 MPa. The osmotic potential of the nutrient solution was determined by the hygrometric method, using a microvoltmeter (HR-33T) and C-52 measuring chambers (Wescor Inc., Logan UT, USA). After five days under PEG-induced

water deficit (-0.75 MPa), we transferred plants to the original nutrient solution (-0.15 MPa) for rehydration during two days.

Sugarcane leaves were sprayed twice a day (at 12:00 and 18:00 h) with freshly prepared GSNO solutions at 10, 100, 500 or 1000 μM. Leaves were sprayed as follows: when the osmotic potential of nutrient solution reached -0.25 MPa; and at two consecutive days under -0.50 MPa. In this way, the last GSNO spraying was done three days before the nutrient solution reaches -0.75 MPa. GSNO spraying was done outside the growth chamber to avoid undesirable interference in other treatments. As references, we had control plants grown in original nutrient solution (-0.15 MPa) and plants subjected to water deficit (nutrient solution with osmotic potential of -0.75 MPa) and sprayed with water (WD + mock). Four plants composed each treatment, with each plant representing one biological replicate. In all treatments plants were sprayed with similar volumes of about 25 mL of GSNO solutions or water.

Experiment II: Water deficit induced by leaf disc dehydration

Leaf discs (2 cm of diameter) were detached from plants grown in pots and placed on moistened (Milli-Q water) filter paper in Petri dishes. They were maintained under 22°C and PPFD of 80 μmol m^{-2} s^{-1} for three days for dehydration. Before detaching leaf discs, plants were sprayed twice a day for three days with a freshly made GSNO or GSH solutions at 100 μM. As reference, plants were sprayed with water (mock). Approximately, 50 mL of GSNO and GSH solutions or water were sprayed on plants.

In another essay, leaf discs were taken as previously and submitted to an NO atmosphere as done by Vitor et al. (2013). Briefly, leaf discs were placed on moistened (Milli-Q water) filter paper in Petri dishes inside an acrylic fumigation chamber, which

was properly sealed with a transparent cover containing tubes for the gases to enter and exit. A continuous flow of NO gas (60 mL min^{-1}) mixed with commercial air (240 mL min^{-1}), equivalent to 60 μmol mol^{-1} of NO, was applied for 6 h. As reference, leaf discs were exposed to a flow of commercial air (300 mL min^{-1}). The commercial air was composed by oxygen (21%) and nitrogen (79%). After fumigation, the leaf discs were transferred to moistened filter paper in Petri dishes and kept at 22 °C and PPFD of 80 μmol m^{-2} s^{-1} for natural dehydration.

Leaf gas exchange and photochemistry

In plants growing in nutrient solution, gas exchange of the first fully expanded leaf with visible ligule was measured daily using an infrared gas analyzer (Li-6400, Licor, Lincoln NE, USA) attached to a modulated fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO_2 assimilation (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured under PPFD of 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 400 μmol mol^{-1}. The measurements were performed between 10:00 and 13:00 h, following the procedures recommended by Long and Bernacchi (2003). The vapor pressure difference between leaf and air (VPDL) was 2.2±0.3 kPa and leaf temperature was 29±1 ºC during the evaluations. The instantaneous carboxylation efficiency ($k=A/C_i$) was calculated according to Machado et al. (2009).

Chlorophyll fluorescence was evaluated simultaneously to the leaf gas exchange and the apparent electron transport rate (ETR) estimated as $ETR= \phi_{PSII} \times PPFD \times 0.85 \times 0.4$, in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light absorption and 0.4 is the fraction of light energy partitioned to PSII (Edwards and

199 Baker 1993; Baker 2008). Additionally, the non-photochemical quenching of
200 fluorescence (NPQ) was evaluated with the 6400-40 LCF.

201 The potential quantum efficiency of photosystem II (F_v/F_m) was estimated in leaf
202 discs by using the fluorometer PAM-2000 (Heinz Walz GmbH, Effeltrich, Germany) and
203 the chlorophyll content by using a portable chlorophyll meter SPAD-502 (Konica
204 Minolta, Tokyo, Japan), following the manufactory instructions.

205

206 **Relative water content**

207

208 The fresh (FW), turgid (TW) and dry (DW) weights of leaf discs were determined
209 and the relative water content (RWC) calculated according to Jamaux et al. (1997): RWC
210 = 100*[(FW-DW)/(TW-DW)].

211

212 **Biometry**

213

214 At the end of the experiment I (nutrient solution), roots and all leaves were
215 harvested and the dry matter determined after drying samples in an oven (60 ºC) with
216 forced-air circulation until constant weight.

217

218 **Estimation of leaf *S*-nitrosothiols content**

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220 Total leaf protein was extracted in mili-Q water and the resulting homogenate used
221 for the amperometric estimation of *S*-nitrosothiol content as previous described (Santos
222 et al. 2016; Zhang et al. 2000). Measurements were carried out with the WPI
223 TBR4100/1025 amperometer (World Precision Instruments Inc., Sarasota FL, USA) and

a nitric oxide specific ISO-NOP sensor (2 mm). Aliquots of 0.2 mL of aqueous suspension were added to the sampling compartment, which contained 10 mL of aqueous solution of copper chloride (0.1 mol L^{-1}). This condition allowed for the detection of free NO released from the *S*-nitrosothiol present in the leaf protein homogenate. The experiments were performed in triplicate and the calibration curves were obtained with aqueous solutions of freshly prepared GSNO (data not shown). Data was compared to a standard curve obtained with GSNO and normalized against leaf FW.

Data analysis

Data was subjected to the ANOVA procedure and the Student's t-test ($P<0.05$) was used to compare treatments. The results presented are the mean ± SD and the number of replicates is stated in each figure legend.

Results

GSNO alleviates negative effects of water deficit in sugarcane phenotype

The water deficit caused significant reduction in leaf (-62%) and root (-47%) dry matter of sugarcane plants (Fig. 1a,b). Accordingly, the leaf relative water content was also reduced (-13%) in water-stressed plants as compared to well-hydrated ones (Fig. 1c). Interestingly, we found a protective effect on plants that were sprayed with GSNO when considering biomass accumulation and leaf water status (Fig. 1). Such effect was found even after 11 days of the last GSNO application. Plants subjected to water deficit and sprayed with 100 µM GSNO solution presented similar ($P>0.05$) root and leaf dry matter

and leaf relative water content to plants under well-watered conditions (Fig. 1). GSNO concentrations lower or higher than 100 μM caused mild protective effects in root growth. These findings suggest a role of GSNO in alleviating the negative effects of dehydration in sugarcane plants.

Protective role of GSNO on leaf gas exchange

As plant growth was improved under water deficit by GSNO spraying, we hypothesized that leaf GSNO spray affects the leaf gas exchange. Whereas water deficit induced a large reduction (-79%) in leaf CO_2 assimilation in sugarcane plants as compared to the control, spraying plants with 100 μM GSNO or higher concentrations significantly restored leaf CO_2 assimilation (Fig. 2a). For instance, leaf CO_2 assimilation of GSNO sprayed plants (> 100 μM) under water deficit was similar ($P>0.05$) to one found in control plants at the 4^{th} day of water deficit and at the 1^{st} and 2^{th} day of rehydration (recovery). Stomatal conductance was nearly suppressed in sugarcane plants under water deficit (-83%) and strongly inhibited during the rehydration (-73%); however, spraying plants with 100 μM GSNO or higher concentrations kept the stomatal conductance of plants under water deficit similar ($P>0.05$) to one found in control plants (Fig. 2b). The instantaneous carboxylation efficiency, given by the rate between leaf CO_2 assimilation and intracellular CO_2 partial pressure, was significantly reduced by water deficit (Fig. 2c). Such negative effect was partially alleviated by spraying 1000 μM GSNO and no differences ($P>0.05$) between treatments were found after two days of rehydration (Fig. 2c). Overall, these data suggest that GSNO plays a role in alleviating the negative effect of water deficit on leaf photosynthesis, stimulating the stomatal aperture during both water shortage and rehydration.

GSNO improves photochemistry in sugarcane plants under water deficit

The apparent electron transport rate and the effective quantum efficiency of PSII were drastically reduced (-51% and -41%, respectively) in plants under water deficit as compared to well-hydrated ones, indicating inhibition of the primary photochemistry in sugarcane (Fig. 3a,b). However, such deleterious effects of water deficit were completely offset by GSNO spraying (Fig. 3a,b). The non-photochemical quenching was increased by water deficit (+62%) as compared to plants under well-hydrated conditions (Fig. 3c). Notably, leaf spraying with 100 μM GSNO or higher concentrations reduced the non-photochemical quenching under water deficit (Fig. 3c), suggesting that GSNO was effective in protecting sugarcane plants of excessive light energy at the PSII. Taken together, these data indicate that leaf GSNO spraying has positive effects on sugarcane by improving photochemistry under water deficit. At the 2^{th} day of rehydration (recovery), the photochemical activity was similar ($P>0.05$) in plants previously exposed to water deficit and sprayed with GSNO and well-hydrated plants (data not shown).

Effects of the redox active molecules GSH and GSNO during leaf dehydration

Non-enzymatic catabolism of GSNO may yield the antioxidant GSH and the free radical NO. To test a possible role of GSH on the protective effects found when spraying GSNO on sugarcane plants, we followed the dehydration of leaf discs taken from plants sprayed with GSH or GSNO. As a biological NO donor, GSNO is known to cause *S*-nitrosylation of proteins. We first estimated the level of *S*-nitrosylated proteins in leaf extracts of plants sprayed with water (mock), GSH or GSNO solutions. There was a sharp increase in *S*-nitrosothiol concentration of leaf discs taken from GSNO sprayed plants

(Fig. 4a). Surprisingly, increase in *S*-nitrosothiol concentration was also found in plants sprayed with GSH (Fig. 4a). Although not expected, we may argue that increasing GSH availability due to leaf spraying may shift the equilibrium towards GSNO formation, thus causing increased *S*-nitrosothiol content in GSH sprayed plants. Further analysis revealed that the chlorophyll content was higher in plants sprayed with GSNO as compared to water or GSH sprayed ones (Fig. 4b).

To assess the leaf disc functionality, the potential quantum efficiency of PSII was measured during dehydration and significant increase in this physiological index was observed in leaf discs taken from plants sprayed with GSNO as compared to those ones sprayed with water or GSH (Fig. 5a). In accordance to the possible long-term protective role of GSH, the potential quantum efficiency of PSII was higher in plants sprayed with GSH than in ones sprayed with water at the 3rd day of dehydration (Fig. 5a). Importantly, when we exposed the leaf discs to a NO atmosphere, similar results were obtained when considering the protective role of NO on photochemistry (Fig. 5b). These findings highlight the NO-mediated signaling in alleviating the negative effects of dehydration in sugarcane plants.

Discussion

Due to the sugarcane importance as a bioenergy crop, physiological strategies aiming to improve sugarcane growth and development are of great interest, mainly under limiting environmental conditions. Field-grown sugarcane plants commonly face periods of water shortage that negatively affects plant growth and reduces sucrose production (Ribeiro et al. 2013; Barbosa et al. 2015). Our findings show that leaf GSNO spray improves sugarcane tolerance to water deficit by improving plant growth and

photosynthetic rate. We also sprayed GSNO on well-hydrated plants (Suppl. Fig. S1), but the beneficial effects of GSNO on photosynthesis were found only in sugarcane plants under water deficit (Fig. 2a), indicating that the role of NO is dependent on stress occurrence.

By decreasing the water potential of the nutrient solution through the sequential addition of PEG, we imposed a water deficit to sugarcane plants hydroponically cultivated, avoiding any osmotic shock. This protocol is an advantageous strategy to study plant responses to water deficit because of its similarity to the actual desiccation that occurs in field, where the water potential is gradually reduced and plants are able to trigger metabolic acclimation (Farrant et al. 2015). At the end of the experiment, we observed a significant reduction in biomass accumulation and leaf relative water content of plants not supplied with GSNO (Fig. 1), indicating that plants were facing water shortage. Interestingly, we found a significant alleviation of water stress on biomass accumulation of plants by spraying GSNO several days prior the water deficit imposition.

Plants trigger several physiological processes in response to water deficit (revised by Fang and Xiong 2015; Santisree et al. 2015) and the stomatal closure is a well established and primordial response aiming to protect plants from water loss through transpiration (García-Mata and Lamattina 2001). Although reduction in stomatal conductance protects plants from desiccation, it negatively affects photosynthesis by reducing the CO_2 availability to carboxylation processes (Sales et al. 2015). Under water deficit, we observed an inhibition of photochemistry accompanied by decreases in stomatal conductance in plants not sprayed with GSNO. While sugarcane photosynthesis seems to be limited by photochemical reactions and stomatal closure under water deficit (Figs. 2b and 3a,b), our data revealed that spraying 100 μM GSNO was able to protect plants from those negative effects of water stress. Protein *S*-nitrosylation is an important

post-translational modification, affecting the activity of proteins. Kato et al. (2013) have found *S*-nitrosylated proteins associated with photosynthesis (small and large subunits of Rubisco and oxygen-evolving system) and cellular redox status in potato leaves treated with GSNO. In fact, GSNO was effective in recovering the photosynthetic rates of water-stressed plants, and plants sprayed with GSNO presented photosynthesis similar to one found in well-hydrated plants after four days under water shortage (Fig. 2a).

It has been proposed that GSNO acts as both NO reservoir and donor in biological systems (revised by Salgado et al. 2013; Yu et al. 2014). In fact, non-enzymatic cleavage of GSNO yields GSH and NO (Liu et al. 2001). NO is a redox active molecule that acts mainly through *S*-nitrosylation of proteins (Lindermayr et al. 2005; Yun et al. 2011; Frungillo et al. 2013; Kneeshaw et al. 2014; Wang et al. 2015). The covalent addition of a NO moiety to a cysteine residue in proteins, called *S*-nitrosylation, is known to frequently alter protein activity and localization (Spadaro et al. 2010; Frungillo et al. 2014). GSNO is able to directly transfer its NO moiety to thiol groups, a process referred as *S*-transnitrosylation (Salgado et al. 2013).

In this sense, the protective effect observed after leaf GSNO spraying could be caused by NO release or transfer, increase in GSH availability or both synergistically. We sought to test these possibilities by spraying plants with GSNO, GSH or mock solution and follow the dehydration of leaf discs. Surprisingly, our analyses done at the 3rd day of dehydration (at the end of the experiment) revealed similar increases in the total level of *S*-nitrosothiol in plants sprayed with GSNO and GSH (Fig. 4a). The potential quantum efficiency of PSII indicated a significant protective effect of GSNO during the first three days of dehydration compared to control and GSH sprayed plants (Fig. 5a). Interestingly, a significant protective effect of GSH was found at the 3rd day of dehydration as compared to mock discs. Such unexpected protective effect of GSH may be explained by changes

374 in GSH and NO reactions towards the formation of the product GSNO. Although further
375 analysis are necessary, we hypothesize that GSH spray indirectly increase NO half-life
376 and bioavailability in cells over time (Salgado et al. 2013), which would justify the
377 protective effect of GSH observed only after three days of dehydration (Fig. 5a). The
378 increase in NO bioavailability would then be reflected in the protective effect of GSH
379 spray on the potential quantum efficiency of PSII (Fig. 5a). It is worthy to mention that
380 the determination of the total *S*-nitrosothiols content was carried out 3 days after spraying
381 the plants. Although the levels of leaf *S*-nitrosothiols are comparable in plants sprayed
382 with GSH or GSNO, the kinetics of *S*-nitrosylation may differ. Unlike the GSH, the
383 GSNO is able to *S*-nitrosylate proteins indirectly by the release of NO or through *S*-
384 transnitrosylation.

385 Several reports indicate an intimate and complex interplay between NO signaling
386 and plant hormones. For instance, overlapping roles of the NO and the phytohormone
387 abscisic acid (ABA) have been reported in plants under water stress (García-Mata and
388 Lamattina 2001; Bright et al. 2006; Wang et al. 2015). Recently, it has been found
389 that open stomata 1 (OST1)/sucrose nonfermenting 1 (SNF1)-related protein kinase 2.6
390 (SnRK2.6) is targeted by an inhibitory *S*-nitrosylation in *Arabidopsis thaliana* guard cells
391 that led to the inhibition of the ABA-induced stomatal closure *in vivo* (Wang et al. 2015).
392 Remarkably, evidences suggest that a reactive thiol group is highly conserved throughout
393 the SnRK2 family in the plant kingdom (Wang et al. 2015). Thus, it is tempting to
394 speculate that the NO released or transferred by GSNO targets protein kinases that
395 ultimately affect the stomatal conductance in sugarcane plants sprayed with GSNO and
396 subjected to water deficit. Specifically, it can be fruitful to investigate the role of the
397 SnRK2.6 in sugarcane plants under water stress. Due the wide extent of possible targets
398 of NO in cells, we cannot exclude that the GSNO spray may impact in other process to

promote drought tolerance in sugarcane. Regarding plant tolerance to abiotic stresses, Foresi et al (2015) reported that transgenic plants expressing OtNOS accumulated higher NO concentrations compared with siblings transformed with the empty vector and displayed enhanced salt, drought and oxidative stress tolerance. Moreover, transgenic OtNOS lines exhibited increased stomatal development compared with plants transformed with the empty vector.

Additionally to its role in stomatal closure, ABA is known to promote root growth under dehydrating conditions by inhibition of ethylene production (Sharp and LeNoble 2002). By spraying sugarcane plants with GSNO under water deficit, we found a significant increase in root biomass and likely increment of water absorption area, which may allow plants to maintain their water status. In fact, the leaf relative water content was not changed by water deficit in plants sprayed with GSNO at 10, 100 and 1000 μM (Fig. 1c). This increase in root:shoot ratio can represent a strategy to explore more efficiently the soil and it aids plants to cope with water stress (Sharp 2002). In addition, it is known that NO has been appointed as an intermediate in the signaling cascade regulated by auxin, influencing the morphology and physiology of roots (Correa-Aragunde et al. 2007). Studies show that NO modulates the metabolism, transport and signaling of auxins, by raising the levels of 3-indoleacetic acid in alfalfa seedlings (Sanz et al. 2014) and promoting root growth (Gouvea et al. 1997) and the formation of adventitious (Pagnussat et al. 2002) and side (Correa-Aragunde et al. 2004) roots. Thus, it is likely that NO-mediated modulation of ABA and/or auxin signaling is shaping sugarcane responses to water stress in our experimental conditions.

In a scenario of climate changes and decreasing water resources, water shortage has become a severe bottleneck in crop yield worldwide. The development of novel agriculture practices and concepts about drought tolerance is of outmost importance to

improve crop yield and understand how plants cope with environmental challenges. Our data indicate that the NO-mediated redox signaling plays a role in promoting shoot and root growth and improving the photosynthesis in sugarcane plants under water deficit.

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Authors' contribution

NMS, LF, IS, ABS, ECM and RVR designed the experiments. NMS and FCCM performed the measurements of photosynthesis and plant growth. MTP and ABS prepared the GSNO and GSH solutions and measured *S*-nitrosothiol concentration in leaf samples. NMS, MTM and IS carried out the experiment with NO fumigation. NMS, LF and RVR wrote the manuscript and all authors contributed in data discussion and edited the final version of the manuscript.

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614

615 **Figure captions**

616

617 **Fig. 1.** Leaf (LDM, in a) and root (RDM, in b) dry mass and leaf relative water content
618 (RWC, in c) in sugarcane plants maintained well-hydrated (Control) and subjected to
619 water deficit (WD) and sprayed with water (mock) or GSNO doses (10, 100, 500 or 1000
620 μM). Data represents the mean value of four replications + standard deviation. Asterisks

indicate statistical differences between a specific condition and the WD+mock treatment (Student's t-test, $P<0.05$).

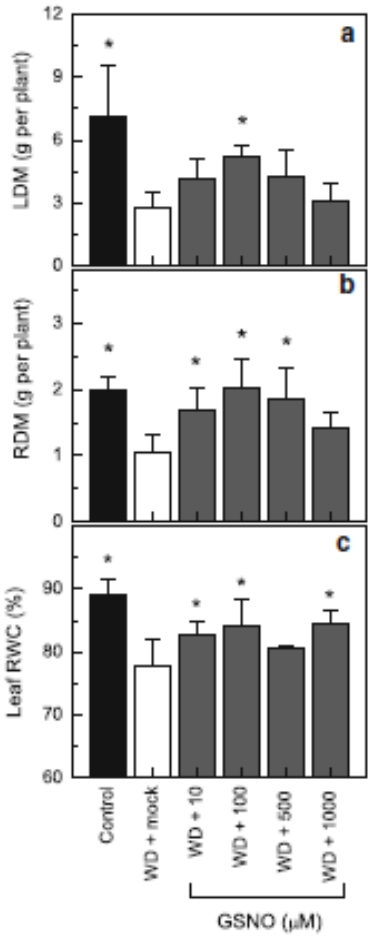
Fig. 2. Changes in leaf CO_2 assimilation (A, in a), stomatal conductance (g_S, in b) and the instantaneous carboxylation efficiency (k, in c) in sugarcane plants maintained well-hydrated (Control) and subjected to water deficit (WD) and sprayed with water (mock) or GSNO doses (10, 100, 500 or 1000 μM). Data represents the mean value of four replications ± standard deviation. In b and c, we show measurements taken after four days of water deficit and two days of rehydration (recovery). Asterisks indicate significant differences between a specific condition and the WD+mock treatment (Student's t-test, $P<0.05$).

Fig. 3. The apparent electron transport rate (ETR, in a), effective quantum efficiency of PSII (ϕ_{PSII}, in b) and non-photochemical quenching (NPQ, in c) in sugarcane plants maintained well-hydrated (Control) and subjected to water deficit (WD) and sprayed with water (mock) or GSNO doses (10, 100, 500 or 1000 μM). Data represents the mean value of four replications + standard deviation. Measurements were taken after four days of water deficit. Asterisks indicate significant differences between a specific condition and the WD+mock treatment (Student's t-test, $P<0.05$).

Fig. 4. The *S*-nitrosothiol concentration (a) and chlorophyll content (b) in leaf discs of sugarcane plants under dehydration. Plants were sprayed with water (mock), 100 μM GSNO and 100 μM GSH. The data represents the mean value + standard deviation. The number of replications varied as follows: n=6 in a; n=12 in b. Asterisks indicate

645 significant differences between a specific condition and the mock treatment (Student's t-
646 test, $P<0.05$).
647
648 **Fig. 5.** The potential quantum efficiency of PSII (F_V/F_M) in leaf discs of sugarcane plants
649 under dehydration. In a, plants were sprayed with water (mock), 100 μM GSNO and 100
650 μM GSH. In b, plants were fumigated with gaseous NO or commercial air (Reference).
651 The data represents the mean value ± standard deviation. The number of replications
652 varied as follows: n=8 in a; and n=3 in b. Asterisks indicate significant differences
653 (Student's t-test, $P<0.05$) between a specific condition and the mock (in a) or between a
654 specific condition and the reference (in b).
655

656 Figure 1



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659 Figure 2

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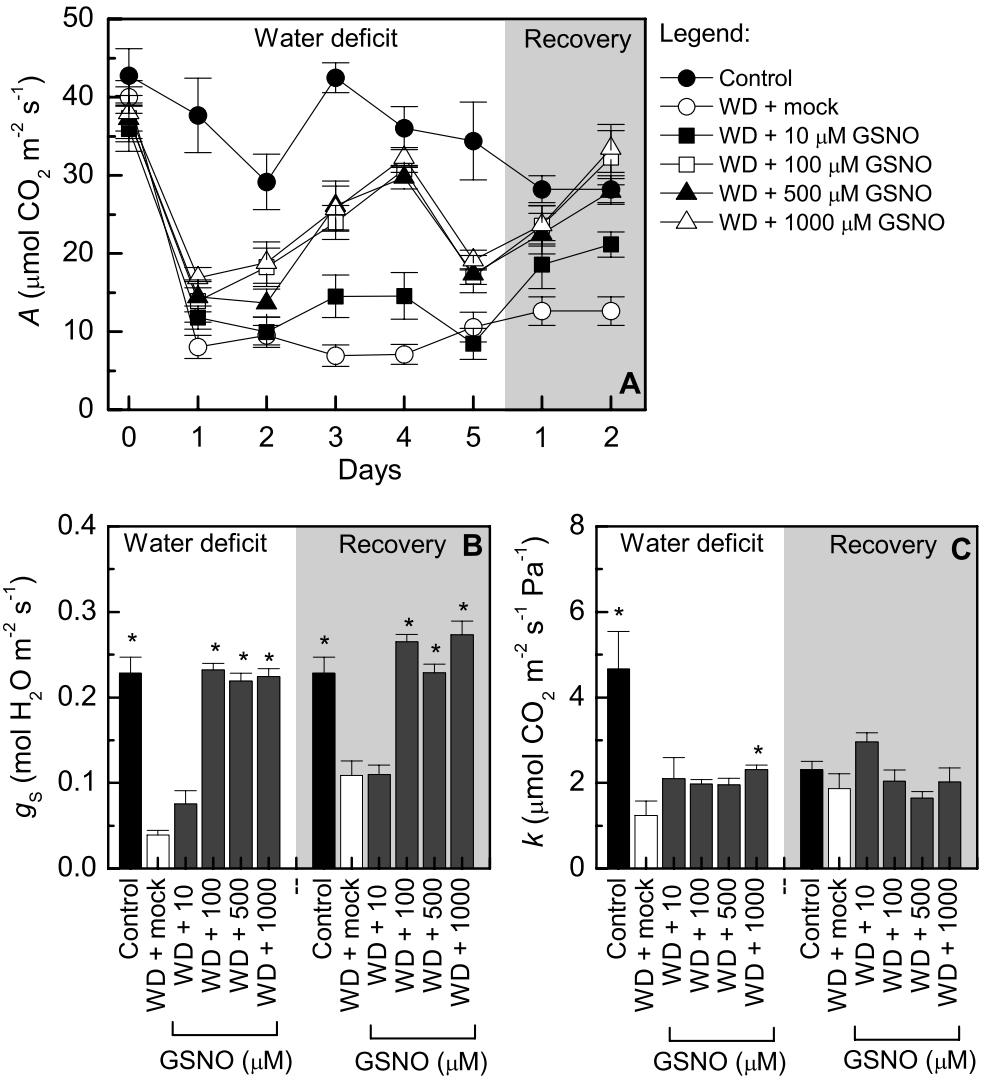

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663 Figure 3

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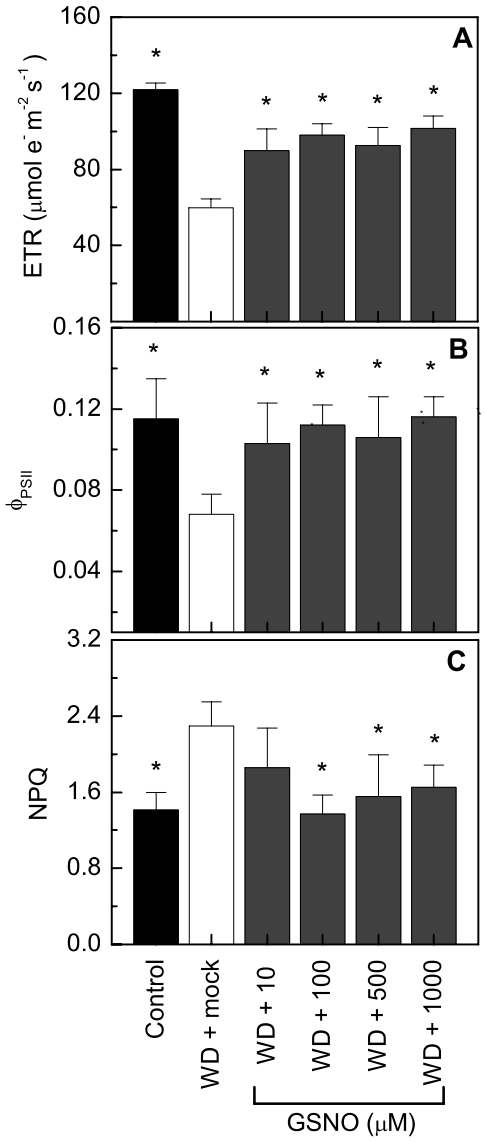

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667 Figure 4

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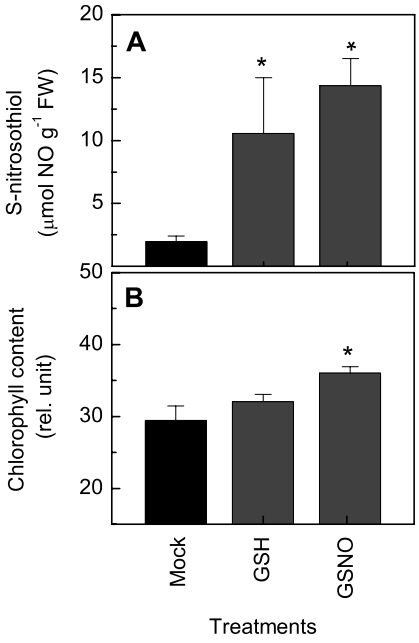

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672 Figure 5

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