



S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit

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Key Words:	Antioxidant enzymes, Drought, Photosynthesis, Nitric oxide, Saccharum

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3 **1 S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and**
4 **2 antioxidant defense in both leaves and roots of sugarcane plants under water deficit**

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38 20 **Abbreviations:**

39 21 *A*, leaf CO₂ assimilation; ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase;
40 22 *C*_i, intercellular CO₂ concentration; DAB, 3,3'-diaminobenzidine; ETR, apparent electron
41 23 transport rate; *g*_s, stomatal conductance; GSH, glutathione; GSNO, S-nitrosoglutathione;
42 24 MDA, malondialdehyde; NO, nitric oxide; O₂⁻, superoxide anion; PEG, polyethylene
43 25 glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PPF, photosynthetic
44 26 photon flux density; PSII, photosystem II; ROS, reactive oxygen species;
45 27 RuBP, ribulose-1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate
46 28 carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase; WD,
47 29 water deficit.
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3 31 **Abstract**
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8 33 Water deficit is a major environmental constraint on crop productivity and performance and
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10 34 nitric oxide (NO) is an important signaling molecule associated with many biochemical and
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12 35 physiological processes in plants under stressful conditions. This study aims to test the
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14 36 hypothesis that leaf spraying of *S*-nitrosoglutathione (GSNO), a NO donor, improves the
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16 37 antioxidant defense in both roots and leaves of sugarcane plants under water deficit, with
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18 38 positive consequences for photosynthesis. In addition, the role of key photosynthetic
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20 39 enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and
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22 40 phosphoenolpyruvate carboxylase (PEPC) in maintaining CO₂ assimilation of GSNO-
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24 41 sprayed plants under water deficit were evaluated. Sugarcane plants were sprayed with
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26 42 water or GSNO 100 μM and subjected to water deficit, by adding polyethylene glycol
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28 43 (PEG-8000) to the nutrient solution. Sugarcane plants supplied with GSNO presented
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30 44 increases in the activity of antioxidant enzymes such as SOD in leaves and CAT in roots,
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32 45 indicating higher antioxidant capacity under water deficit. Such adjustments induced by
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34 46 GSNO were sufficient to prevent oxidative damage in both organs and were associated with
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36 47 better leaf water status. As consequences, GSNO spraying alleviated the negative impact of
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38 48 water deficit on stomatal conductance and photosynthetic rates, with plants also showing
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40 49 increases in Rubisco activity under water deficit.
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51 **Keywords:** Antioxidant enzymes; Drought; Photosynthesis; Nitric oxide; *Saccharum*.
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55 Introduction

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57 Low water availability is the main abiotic stress affecting sugarcane metabolism and
58 reducing crop yield and biomass production (Ribeiro et al. 2013, Sales et al. 2013).
59 Sugarcane plants facing water deficit present reductions in CO₂ assimilation, transpiration,
60 stomatal opening, and decreases in tillering and culm length (Machado et al. 2009, Silva et
61 al. 2012). While stomatal closure reduces CO₂ diffusion into mesophyll and then limit
62 sugarcane photosynthesis under mild water deficit, metabolic limitation of photosynthesis
63 occurs under severe water deficit through decreases in the activity of key photosynthetic
64 enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and
65 phosphoenolpyruvate carboxylase (PEPC) (Ghannoum 2009, Lopes et al. 2011). These
66 responses may be associated with impairment of ATP and NADPH synthesis, reducing
67 regeneration of both ribulose-1,5-bisphosphate (RuBP) and phosphoenolpyruvate (PEP) in
68 C₄ plants (Lawlor 2002). In fact, photochemical impairment leading to low ATP and
69 NADPH synthesis is found under drought and it is a result of reduced electron transport
70 rate between PSII and PSI and damage to thylakoid membranes (Lawlor and Cornic 2002).

71 The redox status of cells is also changed under water deficit due to reduced
72 consumption ATP and NADPH in reactions of CO₂ assimilation. Thus, the water deficit
73 can disrupt cellular homeostasis and increase the production of reactive oxygen species
74 (ROS). Increase in superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂) concentrations in
75 plant cells is one of the first symptoms of exposure to drought, often accompanied by lipid
76 peroxidation in cell membranes (Yamamoto et al. 2002, Tian and Lei 2006). To prevent or
77 reduce cell damage due to ROS, plants have an antioxidant system based on enzymatic and
78 non-enzymatic reactions. While the non-enzymatic reactions are mediated by ascorbate,

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3 79 glutathione, flavonoids, carotenoids and tocopherols, the enzymatic mechanism consists of
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6 80 several enzymes located in different cellular compartments such as superoxide dismutase
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8 81 (SOD), ascorbate peroxidase (APX) and catalase (CAT). During cell detoxification, O_2^-
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10 82 produced in the mitochondria, chloroplasts and peroxisomes is dismutated to H_2O_2 by SOD,
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12 83 which is rapidly eliminated by the CAT and APX, producing H_2O and O_2 (Foyer and
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14 84 Noctor 2005, Wu et al. 2012, Huseynova 2012).

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17 85 Some studies have reported nitric oxide (NO) action in plants under drought
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19 86 (Santisree et al. 2015, Farnese et al. 2015), with this molecule cooperating in ABA-induced
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21 87 stomatal closure (Neill et al. 2003) and also increasing drought tolerance in *Vicia faba*
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23 88 (Garcia-Mata and Lamattina 2001). In fact, NO is an important plant messenger mediating
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25 89 various physiological and biochemical processes, which can directly alter protein structure
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27 90 and activity (Cai et al. 2015). NO has a multifunctional role and its effects on plants can be
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29 91 beneficial or not, depending on its concentration and type of donor when supplied.
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31 92 Recently, we found increases in root growth and maintenance of leaf water status in
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33 93 sugarcane plants under water deficit and supplied with NO. GSNO-sprayed plants exhibited
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35 94 increases in photosynthesis under water deficit, which was a consequence of high stomatal
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37 95 conductance and increased apparent carboxylation efficiency (Silveira et al. 2016).
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43 96 While high *S*-nitrosothiols content in GSNO-sprayed sugarcane plants suggests a
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45 97 long-term role of NO-mediated responses to water deficit (Silveira et al. 2016), it is unclear
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47 98 whether improved photosynthesis is related to the activity of key photosynthetic enzymes
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49 99 Rubisco and PEPC. Few studies have addressed the effects of NO on Rubisco and PEPC
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51 100 activities, which are likely dependent on plant species and NO donor. For instance, Kovacs
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53 101 (2013) showed that NO can *S*-nitrosated Rubisco in a dose-dependent manner, reducing its
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55 102 activity.
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3 103 Alternatively, improved performance of NO-supplied plants under drought may be
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5 104 due to reduced oxidative damage. NO can prevent oxidative damage by activation of
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8 105 antioxidant mechanisms and maintenance of ROS homeostasis in two turfgrass species
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10 106 (Hatamzadeh et al. 2014), increasing ascorbate levels and activity of antioxidant enzymes
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12 107 (Zhang et al. 2008). Furthermore, $O_2^{\cdot-}$ can react with NO to form peroxynitrite (ONOO-)
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15 108 and therefore control ROS accumulation (Radi et al. 2002). The protective effect of
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17 109 exogenous application of NO donor molecules has been attributed to the elimination of $O_2^{\cdot-}$
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20 110 and to increases in the activities of SOD, CAT and APX (Zhao et al. 2008). However, the
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22 111 consequences of such metabolic changes on photosynthetic metabolism remain unexplored.
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24 112 The activity of NADPH oxidase is also an important source of ROS under abiotic stress
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27 113 (Marino et al. 2012), with its activity reduced through S-nitrosylation mediated by NO
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29 114 (Yun et al. 2011).

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31 115 While there is ample evidence of the involvement of NO in plant responses to
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33 116 drought, our knowledge about the underlying physiological processes leading to improved
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35 117 performance is still limited. In such context and using GSNO as a donor of NO, we
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38 118 hypothesized that leaf GSNO spraying improves the antioxidant defense of sugarcane
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40 119 plants under water deficit, with positive consequences for photosynthesis during and after
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43 120 such stressful condition. In addition, the role of key photosynthetic enzymes Rubisco and
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45 121 PEPC in maintaining CO_2 assimilation in GSNO-sprayed plants under water deficit was
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125 **Material and methods**

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127 **Plant material and experimental conditions**

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129 Sugarcane plants (*Saccharum* spp.) cv. IACSP94-2094 developed by ProCana
130 Program (Agronomic Institute, IAC, Brazil) were propagated by placing mini-stalks from
131 adult plants in trays containing commercial substrate composed of *Sphagnum*, rice straw
132 and perlite in 7:2:1 ratio (Carolina Soil of Brazil, Vera Cruz RS, Brazil). Three-week-old
133 plants with two to three leaves were transferred to modified Sarruge (1975) nutrient
134 solution (15 mmol L⁻¹ N (7% as NH₄⁺); 4.8 mmol L⁻¹ K; 5.0 mmol L⁻¹ Ca; 2.0 mmol L⁻¹
135 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;
136 2.1 μmol L⁻¹ Zn; 1.1 μmol L⁻¹ Cu and 0.01 μmol L⁻¹ Mo) and maintained hydroponically in
137 a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) at 30/20 °C (day/night),
138 80% relative humidity, 12 h photoperiod (7:00 to 19:00 h) and PPFD of 800 μmol m⁻² s⁻¹.
139 This PPFD was the maximum intensity at plant level, inside growth chamber. Plants were
140 grown under the above conditions for 30 days prior to water deficit and GSNO spraying.

141 Sugarcane plants were subjected to water deficit (WD) by adding polyethylene glycol
142 (CarbowaxTM PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient
143 solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a
144 gradual decrease in its osmotic potential as follows: -0.25 MPa on the first day; -0.5 MPa
145 on the second day; -0.6 MPa on the third day; -0.75 MPa on the 6th day of treatment. From
146 this point, the osmotic potential of the nutrient solution was monitored daily and corrected
147 when necessary. After six days under water deficit (-0.75 MPa), plants were transferred to
148 the original nutrient solution (-0.15 MPa) for rehydration for three days.

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3 149 Sugarcane leaves were sprayed twice a day with freshly prepared GSNO solutions at
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5 150 100 μM . This concentration was based on a previous study in which significant increase of
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8 151 photosynthesis was found in sugarcane plants under water deficit (Silveira et al. 2016).
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10 152 Leaves were sprayed as follows: when the osmotic potential of nutrient solution reached -
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12 153 0.25, -0.5 and -0.6 MPa. The last GSNO spraying was done three days after reaching -0.75
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14 154 MPa (9th day). GSNO spraying was done outside the growth chamber to avoid undesirable
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16 155 interference with other treatments. Plants were then subjected to the following treatments:
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18 156 control, under nutrient solution with osmotic potential of -0.15 MPa + water spray; water
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20 157 deficit (WD), under nutrient solution with osmotic potential of -0.75 MPa + water spray;
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22 158 and WD + GSNO spray (WDG).
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27 159 Sampling was done at the maximum water deficit (12th day after adding PEG-8000 to
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29 160 the nutrient solution) and also at the recovery period (3rd day after returning plants to the
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31 161 control nutrient solution). Samples of leaves and roots were collected, immediately
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33 162 immersed in liquid nitrogen and then stored at -80°C for further enzymatic analyses.
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39 164 **Synthesis of *S*-nitrosoglutathione (GSNO)**

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43 166 *S*-nitrosoglutathione (GSNO) is an important donor of NO, which is a *S*-nitrosated
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45 167 derivative of the most abundant cellular thiol, glutathione (GSH). GSNO has been
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47 168 considered an internal source of NO and as an essential component of NO-dependent signal
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49 169 transduction pathway (Broniowska et al. 2013). GSNO was synthesized and characterized
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51 170 as previously described (Silveira et al. 2016). Briefly, the reduced glutathione (GSH) was
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53 171 reacted with equimolar sodium nitrite in acidified aqueous solution, acetone was added, and
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3 172 the solution was washed with cold water, filtered and the obtained GSNO was freeze-dried
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10 175 **Leaf gas exchange and photochemical activity**

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15 177 Gas exchange of the first fully expanded leaf with visible ligule was measured daily
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17 178 using an infrared gas analyzer (Li-6400, Licor, Lincoln NE, USA) attached to a modulated
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19 179 fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO₂ assimilation (*A*), stomatal
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21 180 conductance (*g_s*) and intercellular CO₂ concentration (*C_i*) were measured under PPFD of
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23 181 2000 μmol m⁻² s⁻¹ and air CO₂ concentration of 400 μmol mol⁻¹. The measurements were
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25 182 performed between 11:00 and 13:00 h, as done by Silveira et al. (2016). The vapor pressure
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27 183 difference between leaf and air (VPDL) was 2.2±0.3 kPa and leaf temperature was 29±1 °C
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29 184 during the evaluations.
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34 185 Chlorophyll fluorescence was evaluated simultaneously to the leaf gas exchange and
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36 186 the apparent electron transport rate (ETR) was estimated as $ETR = \phi_{PSII} \times PPFD \times 0.85 \times 0.4$,
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38 187 in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light
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40 188 absorption and 0.4 is the fraction of light energy partitioned to PSII (Edwards and Baker
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42 189 1993, Baker 2008). The non-photochemical quenching was calculated as $NPQ = (F_M -$
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44 190 $F_M')/F_M'$. The relative energy excess at PSII level was calculated as $EXC = [(F_V/F_M) -$
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46 191 $\phi_{PSII}]/(F_V/F_M)$, in which F_V/F_M is the potential quantum efficiency of photosystem II
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48 192 (Bilger et al. 1995). A chlorophyllmeter (CFL 1030, Falker, Porto Alegre RS, Brazil) was
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50 193 used to evaluate chlorophyll *a* and *b* and the relative content of chlorophyll (Chl) was
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52 194 calculated as chlorophyll *a* + *b*.
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3 195 Measurements were taken at the beginning of the experiment (before reducing the
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5 196 osmotic potential of nutrient solution), when the osmotic potential of nutrient solution
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8 197 reached -0.75 MPa, at the maximum water deficit, and three days after returning plants to
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10 198 the control condition (recovery period).
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14 15 200 **Relative water content**

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20 202 The relative water content (RWC) was calculated in leaf discs according to Jamaux et
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22 203 al. (1997): $RWC = 100 * [(FW - DW) / (TW - DW)]$, in which FW, TW and DW are the fresh,
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24 204 turgid and dry weights, respectively.
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28 29 206 **Activity of photosynthetic enzymes**

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34 208 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39)

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39 210 Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of
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41 211 Bicine-NaOH buffer 100 mM (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM
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43 212 $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10
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46 213 μM leupeptin. The resulting solution was centrifuged at 14000 g for 5 min at 4 °C. The
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48 214 initial activity of Rubisco was measured using as medium 100 mM bicine-NaOH (pH 8.0)
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50 215 containing 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25
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53 216 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-
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55 217 phosphoglyceric phosphokinase and 80 nkat creatine phosphokinase, incubated at 25 °C.
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58 218 The oxidation of NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate
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3 219 (RuBP). A similar aliquot was incubated with the reaction medium for 10 min at 25 °C and
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5 220 total Rubisco activity was measured after adding RuBP. The reduction of absorbance at 340
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8 221 nm was monitored for 3 min. The activation state of Rubisco was calculated as the ratio
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10 222 between the initial and total activities (Sage et al. 1988, Reid et al. 1997).

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14 224 Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31)

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18 226 Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of
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20 227 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA and 1 mM PMSF and
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22 228 centrifuged at 14000 g for 25 min at 4 °C and the supernatant collected. The reaction
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24 229 medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM MgCl₂, 5
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26 230 mM glucose 6-phosphate, 10 mM NaHCO₃, 33 nkat malic dehydrogenase and 0.3 mM
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28 231 NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate and incubated at
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30 232 30 °C. The oxidation of NADH was monitored for 1 min (Degl'innocenti et al. 2002).

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33 234 **Reactive oxygen species**

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37 236 The quantification of hydrogen peroxide (H₂O₂) in plant material was performed
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39 237 following the method of Alexieva et al. (2001). Homogenates were obtained from 0.1 g of
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41 238 fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP)
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43 239 and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at
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45 240 10000 g and 4 °C for 15 min. The reaction medium consisted of 1.2 mL of KI 1 mM,
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47 241 potassium phosphate buffer (pH 7.5 and 0.1 M) and crude extract. The microtubes were
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49 242 incubated on ice under dark for 1 h. After this period, the absorbance was read at 390 nm.

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3 243 A standard curve was obtained with H₂O₂ and the results were expressed as μmol H₂O₂ g⁻¹
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5 244 FW.

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8 245 To determine the concentration of the superoxide anion (O₂^{•-}), 50 mg of samples were
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10 246 incubated in an extraction medium consisting of 100 μM EDTA, 20 μM NADH, and 20
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12 247 mM sodium phosphate buffer, pH 7.8 (Kuo and Kao 2003). The reaction was initiated by
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15 248 adding 100 μL of 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28 °C
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17 249 under stirring for 5 min and the absorbance was read at 480 nm for 5 min. Superoxide anion
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20 250 production was assessed by the accumulation of adrenochrome using a molar absorption
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22 251 coefficient of 4.0×10³ M⁻¹ (Gay and Gebicki 2000).
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26 27 253 **Lipid peroxidation**

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31 255 The concentration of malondialdehyde (MDA) was measured and used as a proxy of
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33 256 lipid peroxidation. One hundred fifty mg of plant tissue were macerated in 2 mL of 0.1%
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35 257 TCA (w/v) and centrifuged at 10000 g for 15 min. The supernatant was added to 1.0 mL of
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37 258 0.5 % thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture was incubated at 95 °C.
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39 259 Two hours after the reaction, the absorbance was measured at 532 and 600 nm. The
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41 260 absorbance at 600 nm was subtracted from the absorbance at 532 nm and the MDA
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43 261 concentration was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹ (Heath and
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45 262 Packer 1968).
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50 51 264 **Protein extraction and antioxidant activity**

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3 266 Plant extracts were obtained from the macerate of 0.1 g of fresh tissue (roots or
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5 267 leaves) with liquid nitrogen, 1% PVPP and 2 mL of extraction medium containing 0.1 M
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8 268 potassium phosphate buffer (pH 6.8), 0.1 mM EDTA and 1 mM PMSF. After
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10 269 centrifugation of the homogenates at 15000 g for 15 min and 4 °C, supernatants (crude
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12 270 extracts) were collected and preserved on ice. The protein levels of the enzymatic extracts
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15 271 were determined by the Bradford method (Bradford 1976) using bovine serum albumin
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17 272 (BSA) as the standard.

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20 273 Catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure
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22 274 described in Havir and McHale (1987). The reaction medium consisted of 3 mL of 100 mM
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24 275 potassium phosphate buffer (pH 6.8), deionized water, 125 mM H₂O₂ and crude extract.
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27 276 The reaction was carried out in a water bath at 25 °C for 2 min and activity assessed by the
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29 277 decrease in absorbance at 240 nm. The CAT activity was calculated using the molar
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31 278 extinction coefficient of 36 M⁻¹ cm⁻¹ and expressed as nmol min⁻¹ mg⁻¹ of protein.

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34 279 Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by
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36 280 Nakano and Asada (1981). The reaction medium was composed by 3 mL of 100 mM
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38 281 potassium phosphate buffer (pH 6.0), deionized water, 10 mM ascorbic acid, 10 mM H₂O₂
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40 282 and crude extract. The reaction was carried out in a water bath at 25 °C for 2 min and
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42 283 activity monitored by the decrease in absorbance at 290 nm, using the molar extinction
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44 284 coefficient of 2.8 M⁻¹ cm⁻¹ to measure the APX activity, which was expressed as μmol
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46 285 min⁻¹ mg⁻¹ of protein.

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50 286 Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to
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52 287 Giannopolitis and Ries (1977). The reaction medium consisted of 3 mL of 100 mM sodium
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54 288 phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, crude
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56 289 extract, 100 μM riboflavin and 1 mM nitro blue tetrazolium chloride (NBT). A group of
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3 290 tubes was exposed to light (fluorescent lamp of 30 W) for 8 min, and another group
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5 291 remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the
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8 292 amount of enzyme required to inhibit the NBT photoreduction in 50%, being expressed as
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10 293 $\text{U min}^{-1} \text{mg}^{-1}$ of protein.
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15 295 **Superoxide dismutase zymogram**

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20 297 In order to check the cellular compartment in which the first line of enzymatic
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22 298 defense against oxidative damage was active, the identification of SOD isoforms was
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24 299 performed by native-PAGE electrophoresis in polyacrylamide gel (12.5%) at 4 °C under
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27 300 constant current of 40 mA in Tris-HCl (pH 8.3) and glycine buffer. The protein extract was
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29 301 obtained after maceration of a composite leaf sample (0.4 g, composed by ~0.1 g per
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31 302 replicate) (Ferreira et al. 2002). SOD isoforms were identified through specific inhibition
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34 303 by H_2O_2 and KCN, with isoforms being classified as Mn-SOD (resistant to both inhibitors),
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36 304 Fe-SOD (resistant only to KCN) and Cu/Zn-SOD (inhibited by both inhibitors) (Azevedo et
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38 305 al. 1998).
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43 307 **Histochemical analysis**

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48 309 Samples of three leaves per plant (1 cm^2) in three plants per treatment were cut and
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50 310 immediately immersed in a solution of 1 mg mL^{-1} of DAB-HCl, adjusted to pH 5.6 with
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53 311 NaOH and incubated in dark for 8 h. Samples were taken at the maximum water deficit and
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55 312 they were cleared in 96% ethanol (Faoro and Iriti 2005) before transversal hand cuts.
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58 313 Samples were examined with a light microscope Olympus BX41 (Tokyo, Japan) fitted with
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3 314 a digital camera (Media Cybernetics PL-A624, Bethesda MD, USA). H₂O₂ was visualized
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5 315 as a reddish-brown coloration. As a negative control, DAB solution was supplemented with
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8 316 10 mM ascorbic acid (Faoro et al. 2001).
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11 12 318 **Data analysis**

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17 320 The experimental design was completely randomized and data were subjected to the
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20 321 ANOVA procedure. The Scott-Knott test was used to compare treatments when
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22 322 significance was found (P<0.05). The results presented are the mean ± SD and the number
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24 323 of replicates is stated in each figure caption.
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28 29 325 **Results**

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33 34 327 **GSNO improves leaf water status and chlorophyll content under water deficit**

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39 329 As compared to well-hydrated plants, water deficit caused significant reduction in
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41 330 relative water content (RWC) of sugarcane plants at the maximum water deficit. Such
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43 331 reduction in RWC was alleviated when plants were sprayed with GSNO (Fig. 1A). Low
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46 332 water availability also reduced the chlorophyll content of sugarcane plants at the maximum
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48 333 water deficit and recovery period. Again, GSNO spraying alleviated the decreases in
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50 334 chlorophyll content caused by water deficit (Fig. 1B).
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54 55 336 **GSNO improves leaf gas exchange, photochemistry and increases Rubisco activity** 56 57 337 **under water deficit** 58 59 60

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339 The water deficit induced a large reduction in leaf CO₂ assimilation of sugarcane
340 plants from the 4th day of treatment, with GSNO treatment improving leaf CO₂ assimilation
341 during the water deficit (Fig. 2A). The stomatal conductance was affected by water deficit
342 and GSNO supplying in a similar way as compared to leaf CO₂ assimilation (Fig. 2B).
343 There was a significant increase in intercellular CO₂ concentration (C_i) at the maximum
344 water deficit when plants were not supplied with GSNO and subjected to water deficit (Fig.
345 2C). The apparent electron transport rate (ETR) was reduced due to water deficit. However,
346 such negative effect of water deficit was nulled by GSNO only at the beginning of water
347 deficit treatment (Fig. 2D). The consumption of electrons per CO₂ assimilated (ETR/ A) was
348 increased in plants under water deficit conditions. However, plants sprayed with GSNO
349 presented lower ETR/ A than plants under low water availability and without GSNO
350 supplying, mainly at the maximum water deficit (Fig. 2E). Leaf GSNO spraying caused the
351 lowest non-photochemical quenching at the maximum water deficit (Fig. 2F). While water
352 deficit caused increases in excessive light energy at PSII (EXC), GSNO spraying led to
353 reductions in EXC and then control plants and those treated with GSNO presented similar
354 values during the first days of water deficit (data not shown).

355 Rubisco activity was decreased by 65% in plants under water deficit and increased
356 above 82% from controls by GSNO supplying (Fig. 3A). On average, GSNO-supplied
357 plants presented Rubisco activity almost two-fold higher than control ones at the maximum
358 water deficit (Fig. 3A). On the other hand, the Rubisco activation state (Fig. 3A, insert) and
359 PEPC activity were not affected by water deficit or GSNO supplying (Fig. 3B).

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361 **GSNO and oxidative damage**

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Even without a statistical difference in leaf H_2O_2 levels among treatments (data not shown), the histochemical analysis revealed H_2O_2 close to the stomata of plants under water deficit and without GSNO supplying (Fig. 4). At the maximum water deficit and recovery period, the highest O_2^- concentrations in both leaves and roots were found in plants subjected to water deficit. Interestingly, plants supplied with GSNO and control ones presented similar leaf and root O_2^- concentrations (Fig. 5A,B). Leaf lipid peroxidation was not changed by treatments. However, there was an increase in root MDA content of plants under water deficit without GSNO supplying at the recovery period (Fig. 5C,D).

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GSNO stimulates the antioxidant system under water deficit

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In plants sprayed with GSNO, root CAT activity increased by 44% at the maximum water deficit and by 49% at the recovery period, as compared to control ones (Fig. 6B). Leaf CAT activity decreased due to water deficit, regardless whether GSNO was supplied or with evaluation time (Fig. 6A). At the maximum water deficit, leaf APX activity was increased only in plants subjected to water deficit and without GSNO treatment (Fig. 6C). In roots, APX activity decreased due to water deficit and GSNO spraying did not modify this response at the maximum water deficit and recovery period (Fig. 6D). Compared to the control condition, leaf SOD activity was significantly increased by GSNO spraying at the maximum water deficit (Fig. 6E). Root SOD activity was increased by water deficit, regardless GSNO spraying and evaluation time (Fig. 6F).

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The SOD zymogram revealed details about the activities of SOD isoforms, which were numbered arbitrarily based on the order of appearance on the gels. When considering

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3 386 leaf SOD isoforms, five isoforms were identified in control treatment and seven in water
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5 387 deficit, regardless of GSNO spraying (Fig. 7). There were four and seven root SOD
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8 388 isoforms in control and water deficit conditions, respectively. In leaves, Cu/Zn-SOD
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10 389 isoforms *iii* and *iv* were detected only under water deficit, regardless of GSNO spraying.
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12 390 However, GSNO increased the activity of all SOD isoforms when compared to the other
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15 391 treatments. In roots, Mn-SOD isoform *iii* and Cu/Zn-SOD isoforms *v* and *vi* were detected
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17 392 only in plants under water deficit, regardless of GSNO supplying (Fig. 7).
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21 394 **Discussion**

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23 396 **How does GSNO improve photosynthesis under water deficit?**

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25 398 Our findings clearly show that leaf GSNO spraying improves sugarcane tolerance to
26 399 water deficit by improving photosynthetic rates. Such improvement in leaf CO₂
27 400 assimilation was associated, in part, with higher RWC and higher stomatal conductance
28 401 under water deficit (Figs. 1A, 2B). For instance, GSNO treatment increased by seven times
29 402 the stomatal conductance at the maximum water deficit as compared to plants sprayed with
30 403 water (Fig. 2B). It could be argued that improvements in leaf water status were concomitant
31 404 with the stomatal conductance in GSNO-sprayed plants under water deficit, which was
32 405 lower than in control plants (Fig. 2B). As consequence, GSNO-sprayed plants would
33 406 minimize water vapor loss through transpiration (data not shown) and maximize CO₂
34 407 availability in intercellular spaces. Accordingly, control plants and those sprayed with
35 408 GSNO presented similar C_i values throughout the experimental period (Fig. 2C).
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3 409 Besides the diffusive limitation of photosynthesis imposed by stomatal closure under
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5 410 water deficit, plants may face biochemical limitation under severe drought. Then, GSNO
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7 411 would also improve CO₂ uptake under water deficit through changes in the activity of key
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9 412 photosynthetic enzymes. In fact, plants supplied with GSNO exhibited an increase in
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11 413 Rubisco activity under water deficit (Fig. 3A). Beligni and Lamattina (2002) found that the
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13 414 NO delayed Rubisco mRNA loss caused by treatment with the herbicide Diquat in potato
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15 415 leaves. On the other hand, NO can also *S*-nitrosylate Rubisco causing reduced Rubisco
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17 416 activity in a dose-dependent manner (Kovacs et al. 2013, Santisree et al. 2015). As possible
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19 417 explanation for contradictory effects of NO on Rubisco, we would consider the NO donor
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21 418 and its concentration as well as plant sensitivity, which likely vary among plant species. By
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23 419 spraying GSNO 100 μM, our data clearly show improvements in sugarcane photosynthesis
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25 420 through different techniques and approaches, i.e., measuring leaf gas exchange and using
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27 421 biochemical assays.

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29 422 GSNO also alleviated chlorophyll degradation (Fig. 1B), which is in agreement with
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31 423 previous studies showing that exogenous application of NO may attenuate chlorophyll
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33 424 degradation under stressful conditions (Eum et al. 2009, Wang et al. 2015). Accordingly,
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35 425 plants sprayed with GSNO presented higher photochemical activity under water deficit as
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37 426 compared to those sprayed with only water and also subjected to water deficit. Besides
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39 427 showing ETR values similar to control plants during the beginning of water deficit (Fig.
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41 428 2D), plants sprayed with GSNO presented less activity of alternative electrons sinks under
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43 429 water deficit (Fig. 2E). Low non-photochemical quenching of chlorophyll fluorescence also
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45 430 suggests less excessive light energy at the PSII level (Fig. 2F).

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3 431 Taken together, we may argue that GSNO spraying benefited plants through
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5 432 improvements in leaf water status, which may be a consequence of better water balance
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8 433 caused by increases in water supply by roots and/or reduced water loss by stomatal
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10 434 conductance while maintaining CO₂ availability for photosynthesis. Such higher
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12 435 photosynthetic rates may also be due to increases in Rubisco activity in GSNO-sprayed
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14 436 plants with an important regulatory role at the PSII level as the main sink of ATP and
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16 437 NADPH. While less energetic pressure at PSII level can be maintained by using of
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18 438 reducing power and ATP in CO₂ assimilatory reactions under low water availability, an
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20 439 active antioxidant system would also prevent oxidative damage induced by drought.
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22 440 Therefore, GSNO effects on antioxidant metabolism were further investigated in both roots
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24 441 and leaves of sugarcane under water deficit.
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31 443 **GSNO improves antioxidant defense in both roots and leaves under water deficit**

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36 445 Water deficit induced leaf O₂⁻ accumulation (Fig. 5A) without a concomitant increase
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38 446 in SOD activity (Fig. 6E) in plants without GSNO spraying. Presence of H₂O₂ in leaves of
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40 447 plants under water deficit (Fig. 4B) was also found even with increases in APX activity
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42 448 (Fig. 6C). As CAT activity was reduced and leaves presented H₂O₂ under water deficit, one
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44 449 would argue that the main H₂O₂ detoxification pathway in sugarcane is through CAT
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46 450 activity. In roots, accumulation of O₂⁻ was also found under water deficit, with increases in
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48 451 SOD activity and decreases in APX activity (Figs. 5A, 6D,F). Interestingly, GSNO-sprayed
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50 452 plants did not present any increase in O₂⁻ and presence of H₂O₂ in leaves (Figs. 4, 5A,B)
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52 453 and GSNO stimulated leaf SOD activity and root CAT activity under water deficit (Figs.
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54 454 4C, 6B,E).
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3 455 The increase in total SOD activity (Fig. 6E) in leaves sprayed with GSNO was
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5 456 associated with increases in the activity of mitochondrial (*i*-Mn-SOD), chloroplastial (*ii*-
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7 457 Fe-SOD) and cytosolic (*iii*-Cu/Zn-SOD) isoforms (Fig. 7). As a product of SOD activity,
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9 458 accumulation of H₂O₂ in leaves would be expected. However, evidence of H₂O₂ production
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11 459 was not found in GSNO-sprayed plants under water deficit. It is known that the
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13 460 detoxification of H₂O₂ can also be achieved by non-enzymatic antioxidants such as non-
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15 461 protein thiol, ascorbate, GSH or proline. Therefore, the protective effect observed by leaf
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17 462 GSNO spraying could be also associated with increases in GSH availability. GSNO is
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19 463 decomposed by GSNO reductase to oxidized glutathione (GSSG), which is the substrate of
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21 464 the glutathione reductase (GR) that regenerates GSH (Corpas et al. 2013). In fact, GSH
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23 465 spraying improved photochemical activity in sugarcane leaf discs subjected to natural
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25 466 drying (Silveira et al. 2016).

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27 467 Although we have not found evidence of membrane damage under water deficit in
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29 468 both leaves and roots (Figs. 5C,D), effects of oxidative stress on protein and DNA integrity
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31 469 and function cannot be ruled out (Oliveira et al. 2010). Leaf and root O₂⁻ concentrations
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33 470 remained high even after the recovery period in plants previously exposed to water deficit,
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35 471 with increased lipid peroxidation in roots (Figs. 5A,B,D). The production of ROS may
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37 472 occur by the action of NADPH oxidase and peroxidases, causing an oxidative burst during
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39 473 rehydration (Colville and Kranner 2010), which was also avoided in both leaves and roots
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41 474 by spraying GSNO on leaves.

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43 475 The changes observed in antioxidant metabolism indicate that leaf GSNO spraying
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45 476 increased the antioxidant responses of both leaves and roots, avoiding oxidative damage
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47 477 induced by O₂⁻ and H₂O₂ under water deficit. Such responses may be related to plant water
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49 478 status as H₂O₂ may inhibit aquaporin activity and reduce water transport (Liu et al. 2015).
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3 479 In fact, higher RWC and stomatal conductance in GSNO-sprayed plants were noticed (Figs.
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5 480 1A, 2B). Silva et al. (2015) have already reported that sugarcane genotypes showing an
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7 481 active antioxidant metabolism are able to maintain root hydraulic conductance and leaf gas
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9 482 exchange under water deficit. Additionally, NO by itself has been reported to play an
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11 483 important role in aquaporin regulation by stimulating the transcription of *OsPIP1;1*,
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13 484 *OsPIP1;2*, *OsPIP1;3* and *OsPIP2;8* isoforms during rice seed germination (Liu et al. 2007).
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15 485 Further studies about the potential effect of NO on plant hydraulic conductance and
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17 486 aquaporin expression and activity should reveal another interesting interaction among this
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19 487 radical, antioxidant metabolism and plant water balance.
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27 489 **Conclusion**

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31 491 Sugarcane plants supplied with GSNO presented increases in the activity of SOD in
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33 492 leaves and CAT in roots, indicating higher antioxidant capacity under water deficit. Such
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35 493 adjustments induced by GSNO were sufficient to prevent oxidative damage in both organs
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37 494 and were associated with better leaf water status. As consequence, GSNO spraying
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39 495 alleviated the negative impact of water deficit on stomatal conductance and photosynthetic
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41 496 rates, with plants also showing increases in Rubisco activity under water deficit. Herein, we
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43 497 not only gave one step more toward the understanding of how NO may modulate plant
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45 498 responses to water deficit but also we provided insights about the underlying physiological
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47 499 processes supporting improved plant performance under stressful conditions.
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55 501 **Acknowledgment**

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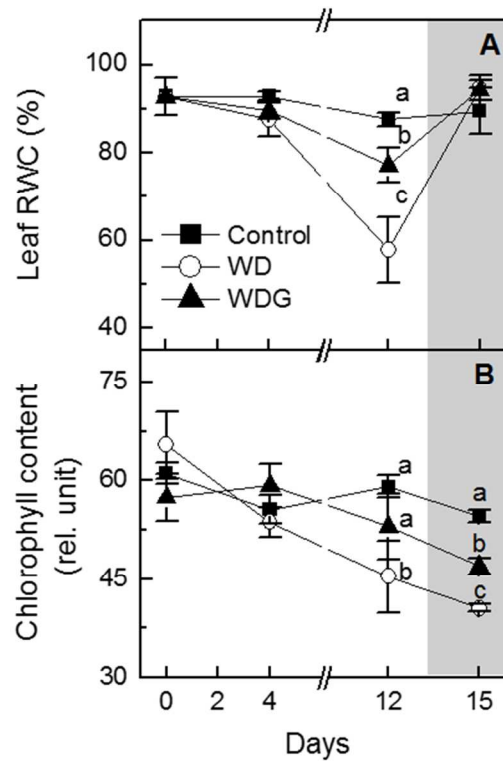
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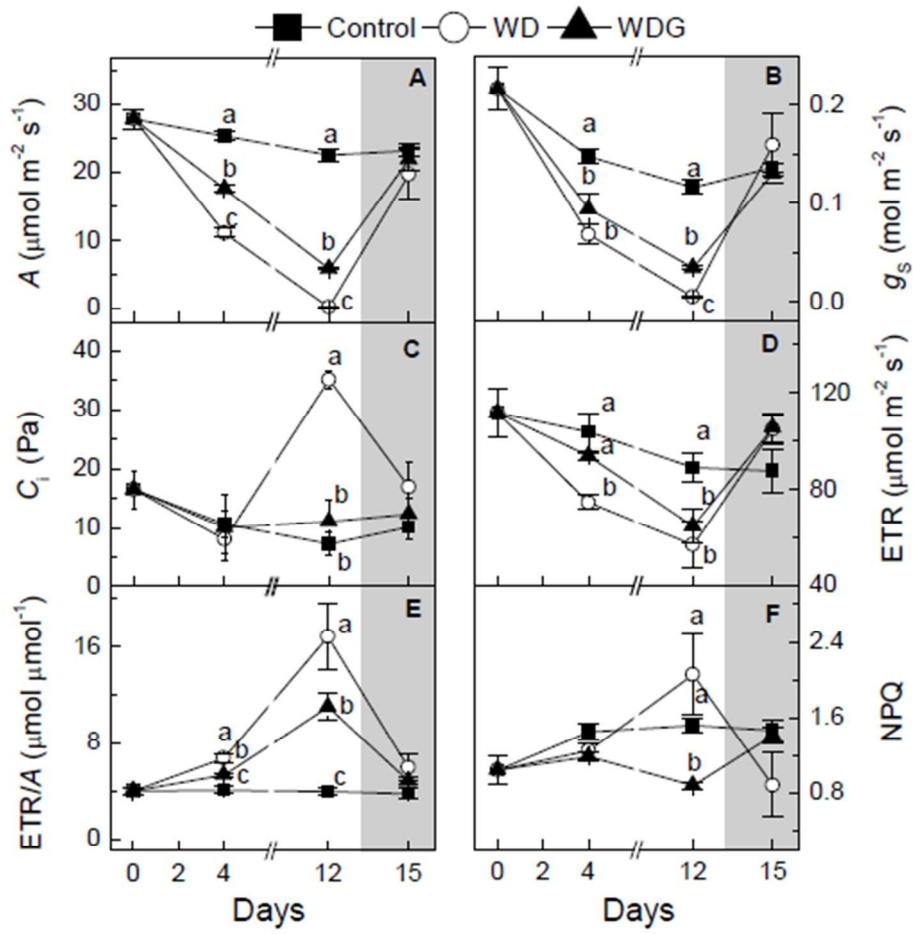
For Peer Review

Figure 1



Review

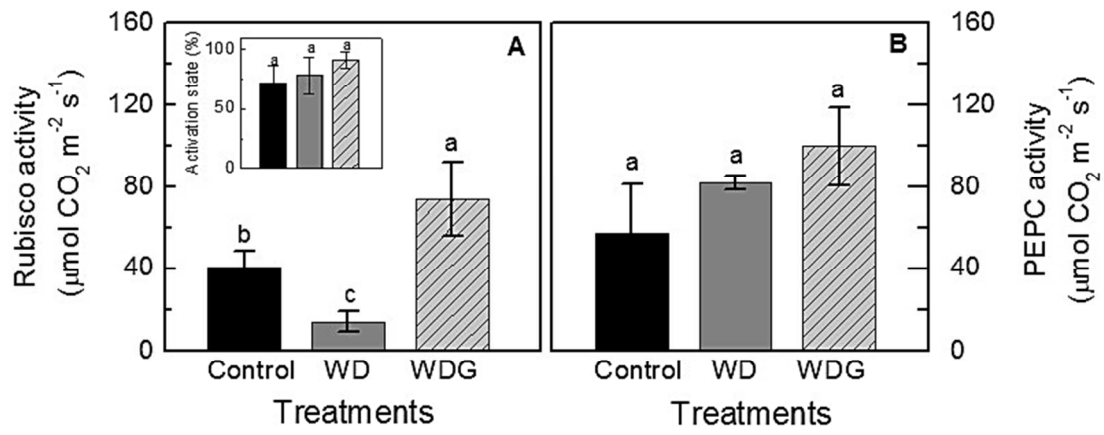
Figure 2



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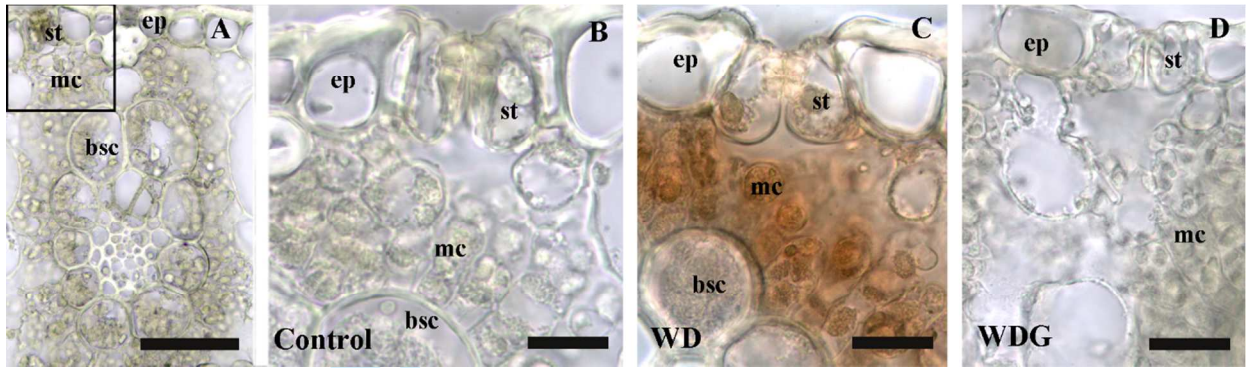
Figure 3



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Figure 4



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Figure 5

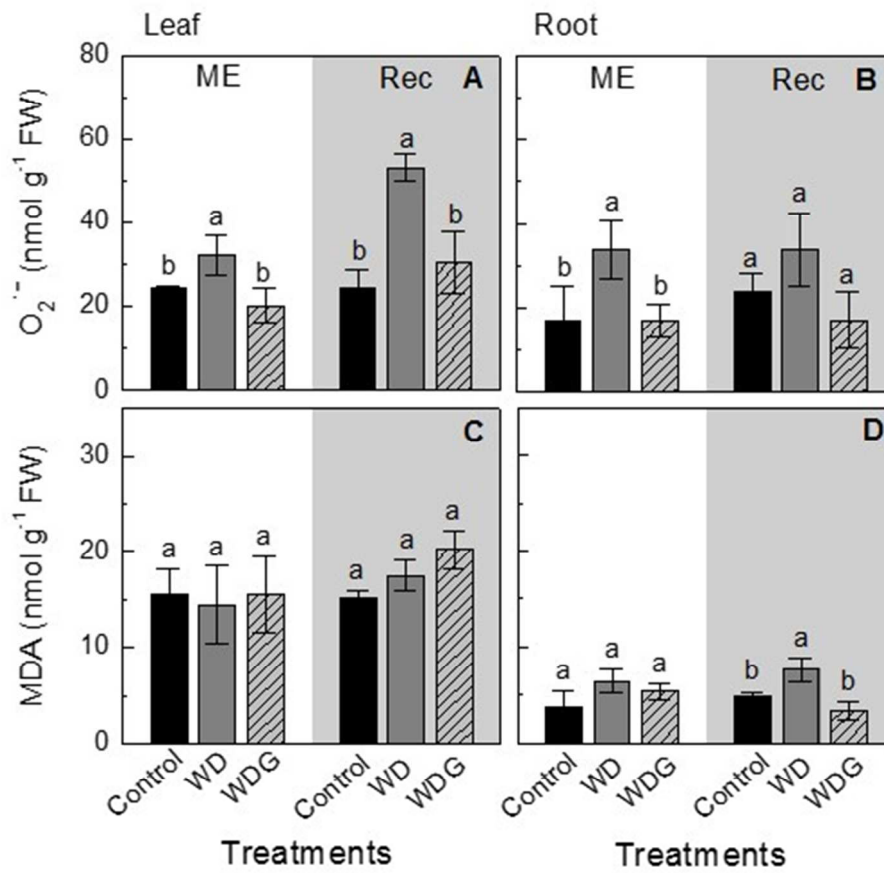


Figure 6

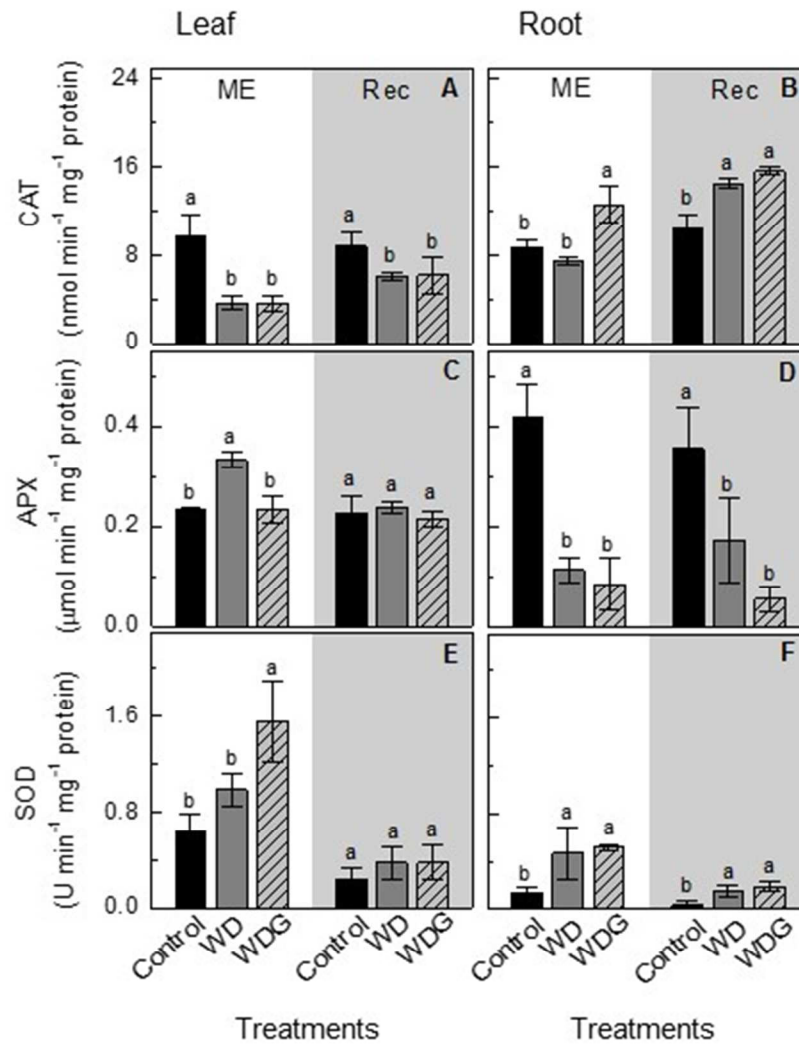
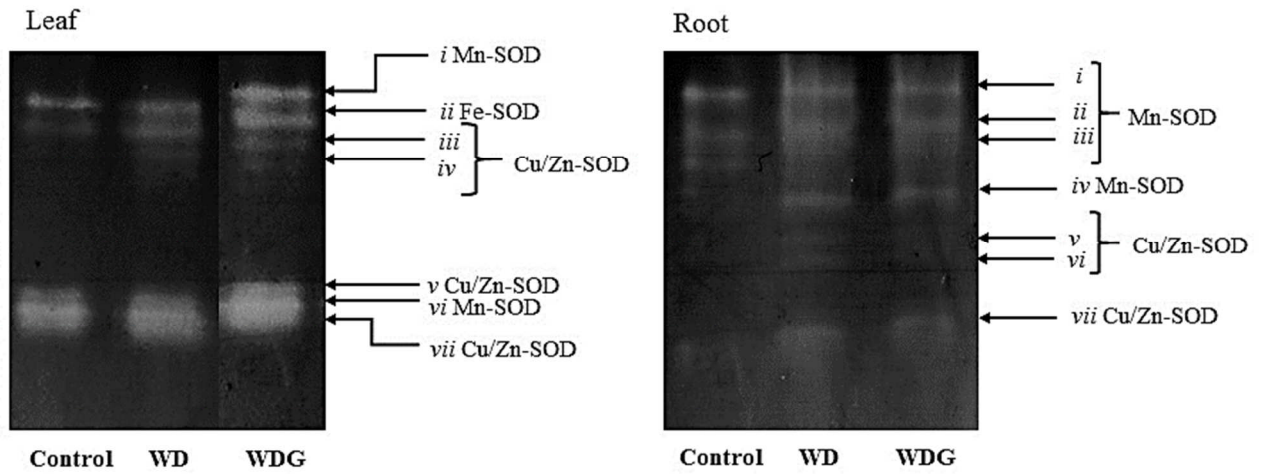


Figure 7



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3 Fig. 1. Leaf relative water content (RWC, in A) and chlorophyll content (B) in sugarcane plants
4 maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or
5 subjected to WD and sprayed with GSNO 100 μ M (WDG). The shaded area indicates recovery period,
6 when plants were moved to nutrient solution used in control treatment. Each symbol represents the
7 mean value of four replications \pm standard deviation. Different lowercase letters indicate statistical
8 difference between treatments (Scott-Knott test, $P < 0.05$).
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10 Fig. 2. Leaf CO₂ assimilation (A, in A), stomatal conductance (gS, in B), intercellular CO₂
11 concentration (C_i, in C), apparent electron transport rate (ETR, in D), ratio of electron transport rate
12 and CO₂ assimilated (ETR/A, in E), and non-photochemical quenching (NPQ, in F) in sugarcane plants
13 maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or
14 subjected to WD and sprayed with GSNO 100 μ M (WDG). The shaded area indicates recovery period,
15 when plants were moved to nutrient solution used in control treatment. Each symbol represents the
16 mean value of four replications \pm standard deviation. Different lowercase letters indicate statistical
17 difference between treatments (Scott-Knott test, $P < 0.05$).
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20 Fig. 3. In vitro activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, in A) and
21 phosphoenolpyruvate carboxylase (PEPC, in B) in sugarcane plants maintained well-hydrated
22 (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed
23 with GSNO 100 μ M (WDG). Evaluations were done at the maximum water deficit. In A, Rubisco
24 activation state is shown as insert. The data represents the mean value of three replications \pm
25 standard deviation. Different lowercase letters indicate statistical difference between treatments
26 (Scott-Knott test, $P < 0.05$).
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29 Fig. 4. Cross section of sugarcane leaves with H₂O₂ localization stained brown by DAB. A, overview of
30 samples with no H₂O₂ accumulation, the square on the top left identify the leaf region shown in
31 pictures B to D with mesophyll cells (mc) close to the stomata (st). B, well-hydrated (Control)
32 samples. C, sample subjected to water deficit (WD). D, samples subjected to WD and sprayed with
33 GSNO 100 μ M (WDG). ep: epidermis, bsc: bundle-sheath cells. Bars: A = 50 μ m, B-D = 20 μ m.
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36 Fig. 5. Concentration of superoxide anion (O₂⁻, in A and B) and malondialdehyde (MDA in C and D) in
37 leaves (A and C) and roots (B and D) in sugarcane plants maintained well-hydrated (Control),
38 subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO
39 100 μ M (WDG). ME indicates maximum water deficit and Rec and shaded area indicate recovery
40 period. The data represents the mean value of three replications \pm standard deviation. Different
41 lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P < 0.05$).
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44 Fig. 6. Activity of catalase (CAT, in A and B), ascorbate peroxidase (APX, C and D) and superoxide
45 dismutase (SOD, in E and F) in leaves (A, C and E) and roots (in B, D and F) in sugarcane plants
46 maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or
47 subjected to WD and sprayed with GSNO 100 μ M (WDG). ME indicates maximum water deficit and
48 Rec and shaded area indicate recovery period. The data represents the mean value of three
49 replications \pm standard deviation. Different lowercase letters indicate statistical difference between
50 treatments (Scott-Knott test, $P < 0.05$).
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52 Fig. 7. Superoxide dismutase isoforms by non-denaturing polyacrylamide gel electrophoresis in leaves
53 and roots in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and
54 sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 μ M (WDG). Evaluations
55 were done at the maximum water deficit. Gels are representative of three runs.
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