

Elsevier Editorial System(tm) for Plant  
Physiology and Biochemistry  
Manuscript Draft

Manuscript Number: PLAPHY-D-16-01324

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

Article Type: Research Paper

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

Corresponding Author: Dr. Rafael Vasconcelos Ribeiro,

Corresponding Author's Institution: University of Campinas, Institute of Biology

First Author: Neidiquele M Silveira, MS

Order of Authors: Neidiquele M Silveira, MS; John T Hancock, Professor; Lucas Frungillo, Dr; Eleni Siasou, Dra; Fernanda C Correia Marcos, MS; Ione Salgado, Professor; Eduardo C Machado, Dr; Rafael Vasconcelos Ribeiro

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

Dear Prof. Mario De Tullio

Editor-in-Chief | Plant Physiology and Biochemistry

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

We look forward to hearing from you.

Yours sincerely,

Rafael V. Ribeiro  
Corresponding author

## Highlights

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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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

2

3 Neidiquele M. Silveira<sup>a</sup>, John T. Hancock<sup>b</sup>, Lucas Frungillo<sup>d</sup>, Eleni Siasou<sup>b</sup>,

4 Fernanda C.C. Marcos<sup>c</sup>, Ione Salgado<sup>c</sup>, Eduardo C. Machado<sup>a</sup>, Rafael V. Ribeiro<sup>c,\*</sup>

5

6 <sup>a</sup>Laboratory of Plant Physiology “Coaracy M. Franco”, Center R&D in Ecophysiology and  
7 Biophysics, Agronomic Institute (IAC), Campinas SP, Brazil

8 <sup>b</sup>Centre for Research in Plant Science, University of the West of England (UWE), Bristol,  
9 UK

10 <sup>c</sup>Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP),  
11 Campinas SP, Brazil

12 <sup>d</sup>School of Biological Sciences, Institute of Molecular Plant Sciences, University of  
13 Edinburgh, Edinburgh, UK

14 \*Corresponding author, e-mail: rvr@unicamp.br

15

16 **Abbreviations:** GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-  
17 nitrosoglutathione reductase; GSSG, oxidized glutathione; NH<sub>4</sub><sup>+</sup>, ammonium; NO, nitric  
18 oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PEG, polyethylene glycol; PPF,   
19 photosynthetic photon flux density; RSNO, *S*-nitrosothiol; RWC, relative water content;  
20 WD, water deficit.

21

22

23

24

1  
2  
3  
4 25 **Abstract**

5  
6 26

7  
8  
9 27 Nitric oxide (NO) may be formed enzymatically and non-enzymatically and the main NO  
10  
11 28 source is subject of much debate in plants. The aim of this study was to test the hypothesis  
12  
13  
14 29 that drought-tolerance in sugarcane is associated with NO production and metabolism, in  
15  
16 30 which the more drought-tolerant genotype presenting higher NO accumulation. The  
17  
18  
19 31 sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-  
20  
21 32 sensitive) were grown in growth chamber and submitted to water deficit by adding  
22  
23  
24 33 polyethylene glycol (PEG-8000) in nutrient solution to reduce the osmotic potential to -0.4  
25  
26 34 MPa. For evaluating short-time responses to water deficit, samples were taken after 24 h  
27  
28  
29 35 under water deficit. IACSP95-5000 presented higher root extracellular NO content, which  
30  
31 36 was accompanied by higher root nitrate reductase (NR) activity as compared to IACSP97-  
32  
33  
34 37 7065 under water deficit. In addition, the drought-tolerant genotype had higher leaf  
35  
36 38 intracellular NO content than the drought-sensitive one. IACSP95-5000 exhibited decreases  
37  
38  
39 39 in root *S*-nitrosoglutathione reductase (GSNOR) activity under water deficit, suggesting  
40  
41 40 that *S*-nitrosoglutathione (GSNO) is less degraded and that the drought-tolerant has a higher  
42  
43 41 natural reservoir of NO than the drought-sensitive genotype. Those differences in  
44  
45 42 intracellular and extracellular NO contents and enzymatic activities were associated with  
46  
47  
48 43 higher leaf hydration in the drought-tolerant genotype as compared to the sensitive one  
49  
50 44 under water deficit.

51  
52  
53 45

54  
55 46 **Keywords:** Nitrate reductase; *S*-nitrosoglutathione reductase; NO metabolism; genotype  
56  
57  
58 47 dependent.

59  
60 48  
61  
62  
63  
64  
65

## 1. Introduction

51

52         Despite evidence regarding the importance of nitric oxide (NO) in plant signaling, the  
53 mechanism responsible for NO synthesis is still controversial. It is now widely accepted  
54 that NO plays a key role in signaling among plant cells, however, it has been a challenge to  
55 determine the sources of NO in plants and there is considerable discussion of how exactly  
56 NO is formed in plant cells (Hancock, 2012; Salgado et al., 2013). In biological systems,  
57 NO can be formed both enzymatically and non-enzymatically. In mammals, the enzyme  
58 responsible for NO generation is NO synthase (NOS), with L-arginine being converted to  
59 citrulline, using NADPH as electron donor and O<sub>2</sub> as co-substrate and producing NO and  
60 water (Alderton et al., 2001). However, the existence of NOS remains questionable in  
61 plants. Although NO production is dependent on L-arginine and its production is sensitive  
62 to inhibitors of NOS (Moreau et al., 2010), a homologous gene for this protein has not been  
63 found in plants. A recent extensive survey of higher plant genomes failed to uncover the  
64 presence of a NOS encoding region in any species (Jeandroz et al., 2016).

65         The nitrate reductase (NR) enzyme is essential for nitrogen assimilation and also  
66 involved in NO production both *in vitro* (Rockel et al., 2002) and *in vivo* (Kaiser et al.,  
67 2002). As a secondary activity, NR reduces nitrite to NO using NADPH, being NO  
68 synthesis dependent on the nitrite and nitrate contents of plant tissues. The efficiency of this  
69 reaction for NO production is considered low and requires high concentrations of nitrite  
70 (Yamasaki and Sakihama, 2000; Rockel et al., 2002). Modolo et al. (2005) have suggested  
71 that the primary role of NR for NO production is as a pathway to provide nitrite. Electrons

1  
2  
3  
4 72 required for the reduction of nitrite to NO can be provided by the mitochondrial respiratory  
5  
6 73 chain (Planchet et al., 2005) or by the photosynthetic system (Jasid et al., 2006).  
7  
8

9 74 The NO bioavailability may be affected by glutathione (GSH), an antioxidant present  
10  
11 75 at high intracellular concentrations. Spontaneous reaction of NO with the thiol grouping of  
12  
13  
14 76 GSH will form *S*-nitrosoglutathione (GSNO). The control of intracellular GSNO is partly  
15  
16 77 regulated by degradation catalyzed by *S*-nitrosoglutathione reductase (GSNOR) (Frunghillo  
17  
18 78 et al., 2014). The GSNOR catabolizes GSNO to oxidized glutathione (GSSG) and  
19  
20  
21 79 ammonium (NH<sub>4</sub><sup>+</sup>), resulting in depletion of intracellular levels of GSNO and reduction of  
22  
23  
24 80 *S*-nitrosothiol (RSNO) formation by transnitrosation processes. In fact, GSNO has an  
25  
26 81 important role in *S*-nitrosation and also represents a natural intracellular reservoir of NO (Ji  
27  
28 82 et al., 1999; Liu et al., 2001).  
29  
30

31 83 Recent studies have shown that NO plays an important role in plants under stressful  
32  
33 84 conditions, such as drought (Santisree et al., 2015; Farnese et al., 2016; Silveira et al.,  
34  
35  
36 85 2016). For instance, Arasimowicz-Jeloneka et al. (2009) found that roots subjected to mild  
37  
38 86 water deficit enhanced NO synthesis in root cells of *Cucumis sativus*, with an intense NO  
39  
40  
41 87 production in elongation zone. Although several reports have shown increased NO  
42  
43 88 production under drought (Filippou et al., 2011; Fan and Liu, 2012; Xiong et al., 2012; Cai  
44  
45 89 et al., 2015), there is no information about how plant species/varieties differ in NO  
46  
47  
48 90 production and how this differential NO production is related to drought tolerance. The aim  
49  
50  
51 91 of this work was to test the hypothesis that drought-tolerance in sugarcane is associated  
52  
53 92 with NO production and metabolism, with the more drought-tolerant genotype presenting  
54  
55 93 higher NO accumulation.  
56

57  
58 94  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 95 **2. Material and methods**

5  
6 96

7  
8  
9 97 *2.1. Plant material and growth conditions*

10  
11 98 Two sugarcane genotypes (*Saccharum* spp.) developed by the Sugarcane Breeding  
12  
13  
14 99 Program of the Agronomic Institute (ProCana, IAC, Brazil) with differential biomass  
15  
16 100 production and drought tolerance were studied: IACSP95-5000 is a drought-tolerant  
17  
18 101 genotype (Marchiori, 2014), whereas IACSP97-7065 is sensitive to water deficit (Oliveira,  
19  
20  
21 102 2012; Sales et al., 2013). The plants these two genotypes were obtained from mini-stalks  
22  
23  
24 103 taken from adult plants and planted in commercial substrate (Levington M2 Compost,  
25  
26 104 Heerlen UK). After 50 days, plants with five to six leaves were transferred to modified  
27  
28 105 Sarruge (1975) nutrient solution which is composed of 15 mmol L<sup>-1</sup> N (7% as NH<sub>4</sub><sup>+</sup>); 4.8  
29  
30 106 mmol L<sup>-1</sup> K; 5.0 mmol L<sup>-1</sup> Ca; 2.0 mmol L<sup>-1</sup> Mg; 1.0 mmol L<sup>-1</sup> P; 1.2 mmol L<sup>-1</sup> S; 28.0  
31  
32 107 μmol L<sup>-1</sup> B; 54.0 μmol L<sup>-1</sup> Fe; 5.5 μmol L<sup>-1</sup> Mn; 2.1 μmol L<sup>-1</sup> Zn; 1.1 μmol L<sup>-1</sup> Cu and 0.01  
33  
34 108 μmol L<sup>-1</sup> Mo; the pH of nutrient solution was kept between 5.5 and 6.0 and its electrical  
35  
36 109 conductivity between 1.53 and 1.70 mS cm<sup>-1</sup> by weekly monitoring and corrected when  
37  
38 110 necessary. Plants were grown in growth chamber, with a 12-h photoperiod, air temperature  
39  
40  
41 111 of 30/20°C (day/night), air relative humidity of 80% and the photosynthetic photon flux  
42  
43  
44 112 density (PPFD) about 700 μmol m<sup>-2</sup> s<sup>-1</sup>.

45  
46  
47  
48 113

49  
50 114 *2.2. Water deficit induced by PEG*

51  
52  
53 115

54  
55 116 Sugarcane plants growing in nutrient solution were submitted to water deficit (WD)  
56  
57  
58 117 by adding polyethylene glycol (PEG-8000, Fisher Scientific, Leicestershire, UK) to the  
59  
60 118 solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a  
61  
62  
63  
64  
65



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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

### 124 2.3. *Relative water content*

125  
126 The relative water content was calculated using the fresh (FW), turgid (TW) and dry  
127 (DW) weights of leaf discs according to Jamaux et al. (1997):  
128  $RWC=100\times[(FW-DW)/(TW-DW)]$ .

### 130 2.4. *DAF2 fluorimetric assay for extracellular NO*

131  
132 Leaf and root samples (100 mg) were incubated in 10 mM Tris, 50 mM KCl, pH 7.2  
133 buffer in 1 mL microcentrifuge tubes for 40 min, before the addition of 5 μM 4,5-  
134 diaminofluorescein diacetate (DAF2). The sample was placed into a quartz cuvette and  
135 fluorescence measured for 30 min (Suppl. Fig. S1) using a fluorescence spectrophotometer  
136 (F-2500, Hitachi - Science & Technology, Berkshire, UK) with excitation and emission at  
137 488 and 512 nm, respectively (Bright et al., 2009). For the negative control, samples were  
138 incubated in the absence of DAF2. Data are shown as average value (n=3) for each  
139 treatment and they represent the fluorescence signal after 30 min, considering the negative  
140 control (data shown = sample – negative control).

141  
142

1  
2  
3  
4 143 2.5. *DAF2-DA detection of intracellular NO*

5  
6 144

7  
8  
9 145 Intracellular NO was visualized using the cell permeable NO-specific dye 4,5  
10  
11 146 diaminofluorescein-2 diacetate (DAF2-DA). Leaf and root segments were incubated in  
12  
13  
14 147 MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM CaCl<sub>2</sub>, pH 6.15), at room  
15  
16 148 temperature for 15 min. Then, these segments were incubated in solution of 10 μM DAF2-  
17  
18  
19 149 DA, mixing gently per 40 min in dark and at room temperature (Desikan et al., 2002;  
20  
21 150 Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-  
22  
23  
24 151 DA and placed onto a glass slide and covered with a glass slip before observing  
25  
26 152 fluorescence using laser-scanning microscopy with excitation at 488 nm and emission at  
27  
28  
29 153 515 nm (Nikon PCM 2000, Nikon, Kingston-upon-Thames, UK). Photos were taken with a  
30  
31 154 10x magnification, 15 s exposure and 1x gain. Images were analyzed using ImageJ  
32  
33 155 software (NIH, Bethesda, MD, USA) and data are presented as mean pixel intensities.  
34

35  
36 156

37  
38 157 2.6. *S-nitrosogluthathione reductase (GSNOR) activity*

39  
40  
41 158

42  
43 159 Leaf and root GSNO reductase activity was estimated spectrophotometrically as the  
44  
45 160 rate of NADH oxidation in presence of GSNO as described previously (Frungillo et al.,  
46  
47  
48 161 2014). Briefly, 0.1 g of fresh tissue was grounded with liquid nitrogen, resuspended in 20  
49  
50 162 mM HEPES buffer, pH 8.0, 0.5 mM EDTA, 0.5 mM PMSF and proteinase inhibitors (50  
51  
52  
53 163 mg mL<sup>-1</sup> TPCK and 50 mg mL<sup>-1</sup> TLCK) and centrifuged for 10 min at 10,000 xg at 4 °C.  
54  
55 164 The protein extract was then incubated with 20 mM HEPES buffer, pH 8.0, 350 μM NADH  
56  
57  
58 165 in the presence or not of 350 μM GSNO. GSNO reductase activity was estimated by  
59  
60 166 subtracting the rate of NADH oxidation in the absence of GSNO from that in the presence  
61  
62  
63  
64  
65

1  
2  
3  
4 167 of GSNO by using the NADH molar extinction coefficient ( $6.22 \text{ M}^{-1} \text{ cm}^{-1}$ ) and normalized  
5  
6 168 by protein content.  
7  
8

9 169

## 10 11 170 *2.7. Nitrate reductase (NR) activity* 12 13

14 171

15  
16 172 Actual NR activity was estimated as the rate of  $\text{NO}_2^-$  production as described before  
17  
18 173 (Frunghillo et al., 2014). Protein extract was obtained from the macerate of 0.1 g of fresh  
19  
20 174 tissue with liquid nitrogen in 20 mM HEPES, pH 8.0, 0.5 mM EDTA, 10 mM FAD, 5 mM  
21  
22 175  $\text{Na}_2\text{MoO}_4$ , 6 mM  $\text{MgCl}_2$ , 0.5 mM PMSF and proteinase inhibitors (50  $\text{mg mL}^{-1}$  TPCK and  
23  
24 176 50  $\text{mg mL}^{-1}$  TLCK). The reaction medium consisted of 1 mL of extraction buffer  
25  
26 177 supplemented with 10 mM  $\text{KNO}_3$  and 1 mM NADH. Nitrite production was determined by  
27  
28 178 adding equal volumes of the reaction solution and 1% sulphanilamide, 0.02% N-(1-  
29  
30 179 naphthyl) ethylenediamine dihydrochloride in 1.5 N HCl, and measurement of absorbance  
31  
32 180 at 540 nm on a spectrophotometer. The values obtained were compared to those of a  
33  
34 181 standard curve constructed using  $\text{KNO}_2$  and normalized against protein content.  
35  
36 182

## 37 38 183 *2.8. Protein content* 39 40

41 184

42  
43 185 The protein content was determined by the Coomassie-blue method (Bradford, 1976)  
44  
45 186 using bovine serum albumin (BSA) as the standard. The readings were performed using a  
46  
47 187 microplate format (Fluostar Optima Microplate Reader, BMG Labtech, Ortenberg,  
48  
49 188 Germany).  
50  
51

52 189

53 190  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

191 2.9. *Data analysis*

192

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

198

199 **3. Results**

200

201 3.1. *Leaf relative water content (RWC)*

202

203 The water deficit induced a reduction in RWC of both genotypes, with the drought-  
204 tolerant genotype IACSP95-5000 being less affected as compared to IACSP97-7065 (Fig.  
205 1).

206

207 3.2. *Extracellular and intracellular NO release*

208

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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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

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

### 226 3.3. *NO synthesis and degradation*

227  
228 Leaf NR activity was not affected by water deficit, regardless of which sugarcane  
229 genotype was studied (Fig. 4A). However, water deficit reduced root NR activity in both  
230 genotypes, with IACSP95-5000 presenting higher root NR activity than IACSP97-7065  
231 under low water availability (Fig. 4B). Leaf GSNOR activity did not change by water  
232 deficit and IACSP95-5000 presented higher GSNOR activity than IACSP97-7065 in both  
233 water conditions (Fig. 4C). Root GSNOR activity was reduced by water deficit only in  
234 IACSP95-5000 (Fig. 4D).

235

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

236 **4. Discussion**

237

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

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

253 NO synthesis in plant cells is not yet fully understood, constituting one of the major  
254 challenges to studies investigating this signaling molecule. Nitrate reductase activity, a  
255 cytosolic enzyme essential for the assimilation of nitrogen, has been suggested to play a  
256 key role in NO production in plants (Horchani et al., 2011). In this study, the tolerant  
257 genotype showed higher root NR activity than the sensitive one under water deficit (Fig.  
258 4B). In addition, NO can also be produced by several other enzymatic and non-enzymatic  
259 pathways (Hancock, 2012). The nitrite has been considered the main substrate for NO

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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

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

275 In this study, we demonstrated that NO metabolism is more active in IACSP95-5000  
276 than in IACSP97-7065, with the drought-tolerant IACSP95-5000 presenting higher root  
277 extracellular NO content, higher root NR activity and lower root GSNOR activity as  
278 compared to IACSP97-7065. IACSP95-5000 had also higher leaf intracellular NO content  
279 than IACSP97-7065. NO influence on metabolic and physiological processes is due to its  
280 ability in interacting and modifying multiple targets within the plant cell (Lamattina et al.,  
281 2003), which turns the understanding of its effects on plants a hard task. The understanding  
282 of metabolic pathways controlling NO homeostasis in plants should be one of the major  
283 aims of NO research in the near future.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

284

285 **Acknowledgments**

286

287 NMS acknowledge the São Paulo Research Foundation (Fapesp, Brazil) for granting  
288 doctoral scholarship (Grant no. 2012/19167-0) and scholarship for stage and research  
289 abroad - BEPE (Grant no. 2015/21546-7). LF is a European Molecular Biology  
290 Organization (EMBO) Long Term Fellow (no. 420/2015). The authors also acknowledge  
291 the fellowships (ECM and RVR) and scholarship (FCCM) granted by the National Council  
292 for Scientific and Technological Development (CNPq, Brazil) and the support given by  
293 technicians Dave Molesworth, Rhiannon Davies and Jeff Davey, Centre for Research in  
294 Biosciences at University of the West of England (UWE).

295

296 **References**

297

298 Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure,  
299 function and inhibition. *Biochem J.* 357, 593-615.

300 Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Kubis, J., 2009. Involvement of nitric  
301 oxide in water stress-induced responses of cucumber roots. *Plant Sci.* 177, 682-690.

302 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram  
303 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72, 248-  
304 254.

305 Bright, J., Hiscock, S.J., James, P.E., Hancock, J.T., 2009. Pollen generates nitric oxide and  
306 nitrite: a possible link to pollen-induced allergic responses. *Plant Physiol Biochem.* 47, 49-  
307 55.



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

308 Cai, W., Liu, W., Wang, W.S., Fu, Z.W., Han, T.T., Lu, Y.T., 2015. Overexpression of rat  
309 neurons nitric oxide synthase in rice enhances drought and salt tolerance. PLoS One. 10,  
310 e0131599. doi:10.1371/journal.pone.0131599.

311 Desikan, R., Griffiths, R., Hancock, J.T., Neill, S., 2002. A new role for an old enzyme:  
312 Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced  
313 stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 99, 16319-16318.

314 Fan, Q.J., Liu, J.H., 2012. Nitric oxide is involved in dehydration/drought tolerance in  
315 *Poncirus trifoliata* seedlings through regulation of antioxidant systems and stomatal  
316 response. Plant Cell Rep. 31, 145-154.

317 Farnese, F.S., Menezes-Silva, P.E., Gusman, G.S., Oliveira, J.A., 2016. When bad guys  
318 become good ones: the key role of reactive oxygen species and nitric oxide in the plant  
319 responses to abiotic stress. Front Plant Sci. 7: 471-486, 2016.

320 Filippou, P., Antoniou, C., Fotopoulos, V., 2011. Effect of drought and rewatering on the  
321 cellular status and antioxidant response of *Medicago truncatula* plants. Plant Signal Behav.  
322 6, 270-277.

323 Frungillo, L., Skelly, M.J., Loake, G.J., Spoel, S.H., Salgado, I., 2014. S-Nitrosothiols  
324 regulate nitric oxide production and storage in plants through the nitrogen assimilation  
325 pathway. Nature Commun. 5, 5401-5410.

326 Hancock, J.T., 2012. NO synthase in plants? Period Biol. 114, 19-24.

327 Horchani, F., Prévot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., Raymond  
328 P., Boncompagni, E., Aschi-Smiti, S., Puppo, A., Brouquisse, R., 2011. Both plant and  
329 bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula*  
330 nitrogen-fixing nodules. Plant Physiol. 155, 1023-1036.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

331 Jamaux, I., Steinmetz, A., Belhassen, E., 1997. Looking for molecular and physiological  
332 markers of osmotic adjustment in sunflower. *New Phytol.* 137, 117-127.

333 Jasid, S., Simontacchi, M., Bartoli, C.G., Puntarulo, S., 2006. Chloroplasts as a nitric oxide  
334 cellular source. Effect of reactive nitrogen species and chloroplastic lipids and proteins.  
335 *Plant Physiol.* 142, 1246-1255.

336 Jeandroz, S., Wipf, D., Stuehr, D.J., Lamattina, L., Melkonian, M., Tian, Z., Zhu, Y.,  
337 Carpenter, E.J., Wong, G. K.-S., Wendehenne, D., 2016. Occurrence, structure and  
338 evolution of nitric oxide synthase-like proteins in the plant kingdom. *Sci Signal.* 9: re2.

339 Ji, Y., Akerboom, T.P., Sies, H., Thomas, J.A., 1999. *S*-nitrosylation and *S*-glutathiolation  
340 of protein sulfhydryls by *S*-nitrosoglutathione. *Arch Biochem Biophys.* 362, 67-78.

341 Kaiser, W.M., Weiner, H., Kandlbinder, A., Tsai, C.B., Rockel, P., Sonoda, M., Planchet,  
342 E., 2002. Modulation of nitrate reductase: some new insights, an unusual case and a  
343 potentially important side reaction. *J Exp Bot.* 53, 875-882.

344 Lamattina, L., Garcìa-Mata, C., Graziano, M., Pagnussat, G., 2003. Nitric oxide: the  
345 versatility of an extensive signal molecule. *Annu Rev Plant Biol.* 54, 109-36.

346 Lancaster Jr, J.R., 1997. A tutorial on the diffusibility and reactivity of free nitric oxide.  
347 *Nitric oxide-Biol Ch.* 1, 18-30.

348 Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J., Stamler, J.S., 2001. A metabolic  
349 enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature.* 410, 490-494.

350 Lombardo, M.C., Graziano, M., Pola, C.C.O., J.C., Lamattina, L., 2006. Nitric oxide  
351 functions as a positive regulator of root hair development. *Plant Signal Behav.* 1, 28-33.

352 Lombardo, M.C., Lamattina, L., 2012. Nitric oxide is essential for vesicle formation and  
353 trafficking in *Arabidopsis* root hair growth. *J Exp Bot.* 63, 4875-4885.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

354 Marchiori P.E.R., 2014. Fisiologia de cana-de-açúcar sob déficit hídrico: plasticidade  
355 fenotípica, transporte de água, metabolismo antioxidante e fotossíntese. Thesis, Agronomic  
356 Institute of Campinas (in Port.).

357 Modolo, L.V., Augusto, O., Almeida, I.M., Magalhaes, J.R., Salgado, I., 2005. Nitrite as  
358 the major source of nitric oxide production by *Arabidopsis thaliana* in response to  
359 *Pseudomonas syringae*. FEBS Lett. 579, 3814-3820.

360 Moreau, M., 2010. NO synthesis and signaling in plants – where do we stand? *Physiol*  
361 *Plant*. 138, 372-383.

362 Oliveira, J.F.N.C., 2012. Physiological characterization and gene expression profiling of  
363 sugarcane (*Saccharum* spp) genotypes contrasting for drought tolerance. Thesis, University  
364 of São Paulo (in Port.).

365 Planchet, E., Jagadis Gupta, K., Sonoda, M., Kaiser, W.M., 2005. Nitric oxide emission  
366 from tobacco leaves and cell suspensions: rate limiting factors and evidence for the  
367 involvement of mitochondrial electron transport. *Plant J*. 41, 732-743.

368 Rockel, P., Strube, F., Rockel, A., Wildt, J., Kaiser, W.M., 2002. Regulation of nitric oxide  
369 (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *J Exp Bot*. 53, 103-110.

370 Sales, C.R.G., Ribeiro, R.V., Silveira, J.A.G., Machado, E.C., Martins, O.M., Lagôa,  
371 A.M.M.A., 2013. Superoxide dismutase and ascorbate peroxidase improve the recovery of  
372 photosynthesis in sugarcane plants subjected to water deficit and low substrate temperature.  
373 *Plant Physiol Biochem*. 73, 326-336.

374 Salgado, I., Martínez, M.C., Oliveira, H.C., Frungillo, L., 2013. Nitric oxide signaling and  
375 homeostasis in plants: a focus on nitrate reductase and *S*-nitrosoglutathione reductase in  
376 stress-related responses. *Braz J Bot*. 36, 89-98.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

377 Santisree, P., Bhatnagar-Mathur, P., Sharma, K.K., 2015. NO to drought multifunctional  
378 role of nitric oxide in plant drought: do we have all the answers? *Plant Sci.* 239, 44-55.

379 Silveira, N.M., Frungillo L., Marcos F.C.C., Pelegriño, M.T., Miranda, M.T., Seabra, A.B.,  
380 Salgado, I., Machado, E.C., Ribeiro, R.V., 2016. Exogenous nitric oxide improves  
381 sugarcane growth and photosynthesis under water deficit. *Planta.* 244, 181-190.

382 Tun, N.N., Santa-Catarina, C., Begum, T., Silveira, V., Handrow, Flohe, I., Scherer, G.F.,  
383 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana*  
384 seedlings. *Plant Cell Physiol.* 47, 346-354.

385 Xiong, J., Zhang, L., Fu, G., Yang, Y., 2012. Drought-induced proline accumulation is  
386 uninvolved with increased nitric oxide, which alleviates drought stress by decreasing  
387 transpiration in rice. *J Plant Res.* 125, 155-164.

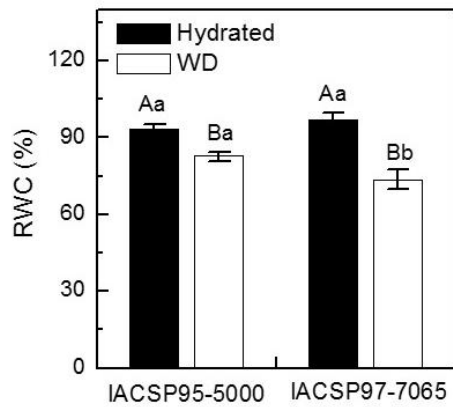
388 Yamasaki, H., Sakihama, Y., 2000. Simultaneous production of nitric oxide and  
389 peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation  
390 of active nitrogen species. *FEBS Lett.* 468, 89-92.

1 **Authors' contributions**

2

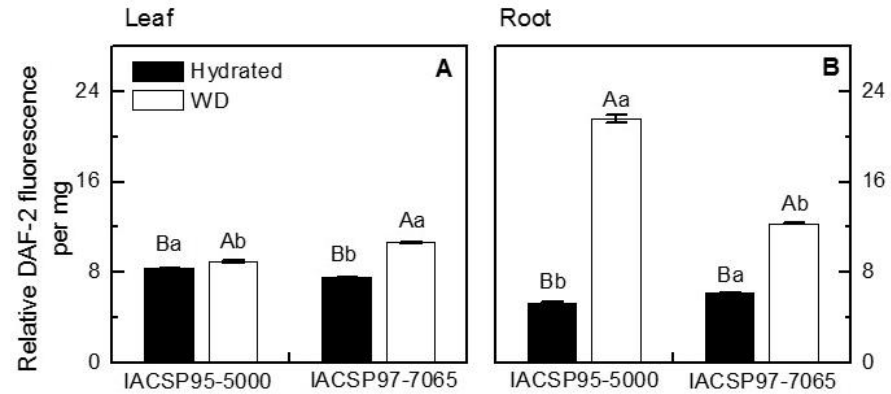
3 NMS, LF, FCCM, JTH, IS, ECM and RVR designed the experiments. NMS and ES  
4 performed the biochemical measurements. FCCM obtained the mini stalks taken from adult  
5 plants. NMS and RVR wrote the manuscript and all authors contributed to data discussion  
6 and edited the final version of the manuscript.

Figure 1



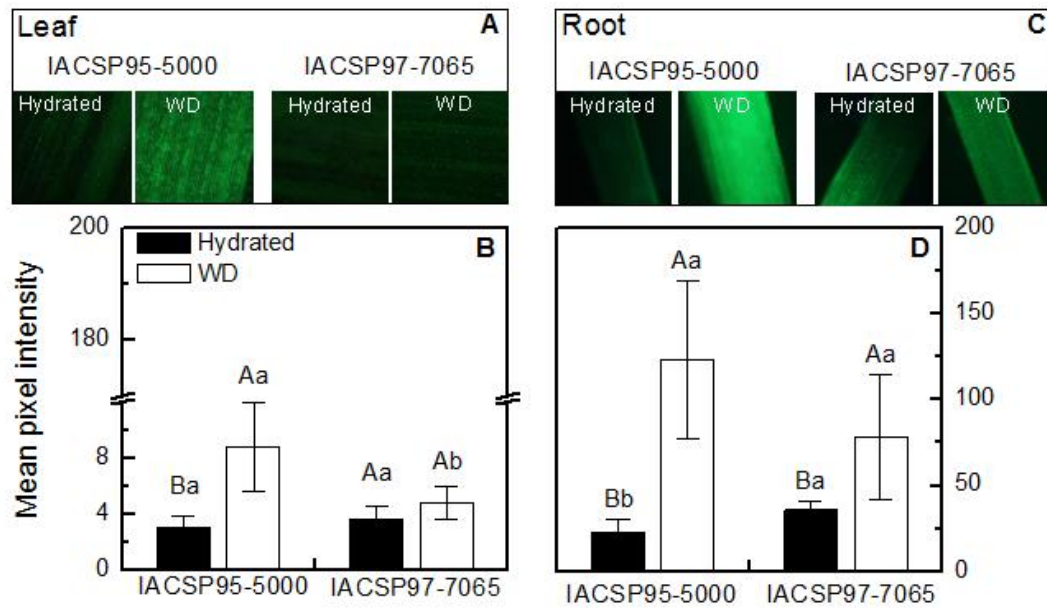
**Fig. 1.** Leaf relative water content (RWC) in sugarcane genotypes IACSP95-5000 and IACSP97-7065 under well-hydrated conditions (Hydrated) or water deficit (WD). The data represents the mean value of four replications  $\pm$  standard deviation. Different uppercase letters indicate statistical difference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

Figure 2



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

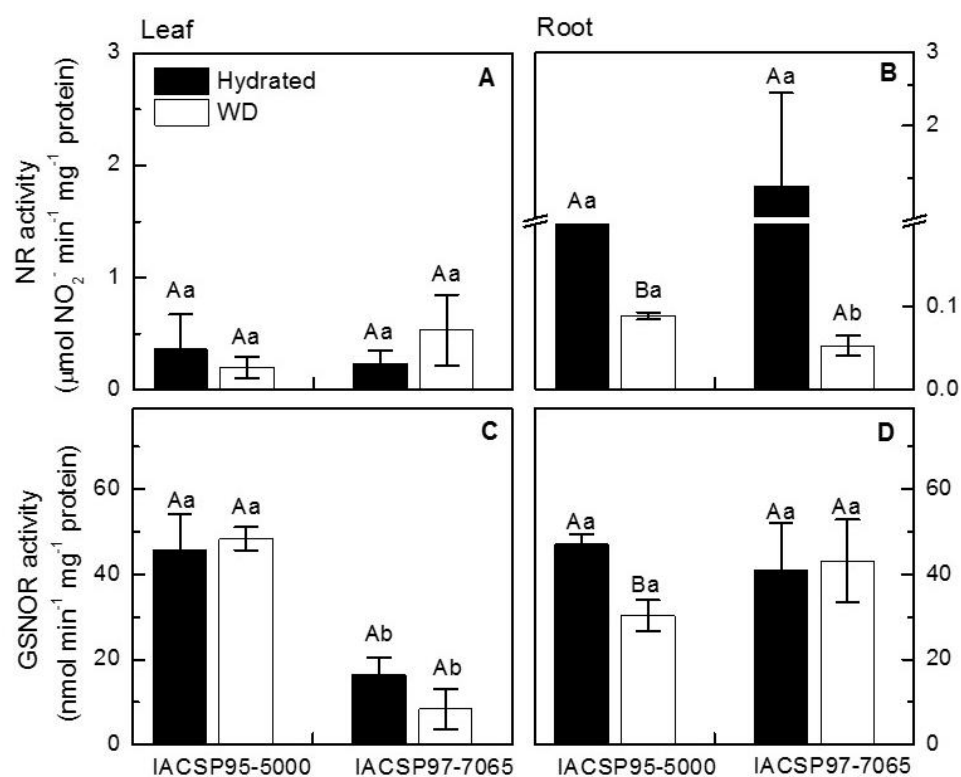
Figure 3



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



Figure 4



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

**Supplementary material**

[Click here to download Supplementary material: SupplMat\\_PPB.docx](#)