Running title: Nitrate supply increases nitric oxide synthesis in sugarcane

Enhanced nitric oxide synthesis through nitrate supply improves drought tolerance of sugarcane plants

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Highlights

- Nitrate supply improves sugarcane growth under water deficit.
- Nitrate supply stimulated nitrate reductase activity and NO synthesis in sugarcane roots facing water deficit.
- Leaf gas exchange was increased by nitrate supply as well as root growth under water limiting conditions.
- Antioxidant responses were also improved in plants supplied exclusively with nitrate.
- Nitrogen management may be an interesting strategy for improving drought tolerance in sugarcane fields.

Abstract

Nitric oxide (NO) is an important signaling molecule associated with many biochemical and physiological processes in plants under stressful conditions. Nitrate reductase (NR) not only mediates the reduction of NO_3^- to NO_2^- but also reduces NO_2^- to NO, a relevant pathway for NO production in higher plants. Herein, we hypothesized that sugarcane plants supplied with more NO_3^- as a source of N would produce more NO under water deficit. Such NO would reduce oxidative damage and favor photosynthetic metabolism and growth under water limiting conditions. Sugarcane plants were grown in nutrient solution and received the same amount of nitrogen, with varying nitrate:ammonium ratios (100:0 and 70:30). Plants were then grown under well-watered or water deficit conditions, in which the osmotic potential of nutrient solution was -0.15 and -0.75 MPa, respectively. Under water deficit, plants exhibited higher root [NO_3^-] and [NO_2^-] when supplied with 100% NO_3^-. Accordingly, the same plants also showed higher root NR activity and root NO production. We also found higher photosynthetic rates and stomatal conductance in plants supplied with more NO_3^-, which improved root growth. ROS accumulation was reduced due to increases in the activity of catalase in leaves and superoxide dismutase and ascorbate peroxidase in roots of plants supplied with 100% NO_3^- and facing water deficit. Such positive responses to water deficit were offset when a NO scavenger was supplied to the plants, thus confirming that increases in leaf gas exchange and plant growth were induced by NO. Concluding, NO_3^- supply is an interesting strategy for alleviating the negative effects of water deficit on sugarcane plants, increasing drought tolerance through enhanced NO production. Our data also provide insights on how plant nutrition could improve crop tolerance against abiotic stresses, such as drought.

Keywords: Nitrate reductase, Photosynthesis, Plant growth, Reactive oxygen species, S-nitrosylation, Water deficit.

Introduction

Nitric oxide (NO) is a diatomic radical gas and important signaling molecule in animals (Bogdan, 2015), fungi (Canovas *et al.,* 2016), bacteria (Crane *et al.,* 2010) and plants (Mur *et al.,* 2013). In plants, increasing evidence indicates NO as a key component of the signaling network, controlling numerous physiological and metabolic processes such as seed germination (Albertos *et al.,* 2015), flowering (He *et al.,* 2004), root growth (Fernandez-Marcos *et al.,* 2011), respiration, stomatal conductance (Moreau *et al*., 2010; Wang *et al*., 2015) and adaptive responses to biotic and abiotic stresses (Shan *et al*., 2015; Fatma, *et al.,* 2016).

NO synthesis is increased in plants under drought and its role in promoting adaptive responses to cope with water deficit has been suggested (Cai *et al.,* 2015; Silveira *et al.,* 2017a). NO and NO-derived molecules play a critical role in intracellular redox signaling and in the activation of antioxidant defense mechanisms (Shi *et al.,* 2014; Hatamzadeh *et al.,* 2015; Silveira *et al.,* 2015). For example, NO supply conferred drought tolerance to wheat seedlings, reducing membrane damage (Garcia-Mata and Lamattina, 2001). Spraying S-nitrosogluthatione (GSNO) – a NO donor – on sugarcane plants resulted in higher photosynthesis under drought, promoting plant growth under stressful condition (Silveira *et al.,* 2016).

The protective action of exogenous NO donors has been attributed to the elimination of superoxide ($O_2^{\bullet-}$) and enhancement of the antioxidant system in sugarcane plants under drought (Silveira *et al.,* 2017b). In addition, one of the main downstream effects of NO is the post-translational regulation involving thiols (Hancock and Neill, 2019). S-nitrosylation is a redox modification consisting in the reversible attachment of NO to the thiol group of a cysteine residue in a target protein leading to the formation S-nitrosothiols (SNOs) (Astier *et al.,* 2012; Fancy *et al.,* 2016). Then, S-nitrosylation may cause a conformational change in proteins, changing their activity or function. On the other hand, NO can react with reduced glutathione (GSH), producing S-nitrosoglutathione (GSNO) – an endogenous NO reservoir and an efficient NO donor (Jahnová *et al*., 2019).

While the mechanisms of NO synthesis in animals have been well documented, NO synthesis and its regulation in plants are complex and poorly understood. In animals, NO is bio-synthesized through NO synthase (NOS), which oxidizes *L*-arginine and produces *L*-citrulline and NO (Alderton *et al.,* 2001). Although some evidence indicates the presence of NOS-like activity in many plant species, genes encoding NOS have not yet been identified in higher plants (Hancock and Neill, 2014; Santolini *et al.,* 2017; Hancock and Neill, 2019). NO production in plant species and under diverse biological conditions point to the co-existence of multiple pathways, likely functioning in distinct tissues/organs and subcellular compartments (León and Costa-Broséta, 2019).

One of the most important pathways for NO production in land plants is through nitrate reductase (NR) (Gupta *et al.,* 2011; Fancy *et al.,* 2016; Chamizo-Ampudia *et al.,* 2017; León and Costa-Broséta, 2019), a multifunctional enzyme that catalyzes NO_3^- reduction to NO_2^-, which is then reduced to NH_4^+ during the N assimilatory pathway (Heidari *et al.*, 2011). Arasimowicz-Jelonek *et al.* (2009) reported low NO concentration in cucumber seedlings treated with a NR inhibitor, suggesting its role in NO synthesis. In rice roots, NO production through NR was increased in response to NO_3^- supply (Sun *et al*., 2015). Furthermore, low NO production by *Physcomitrella patens* occurred when plants received a NR inhibitor (Andrés *et al.,* 2015). Although there are data supporting the association between NR activity and NO production in plants (Mur *et al*., 2013), some authors have argued that NO production through NR represents only a small fraction (1-2%) of total NO_3^- reduction (Yamasaki *et al.,* 1999; Rockel *et al.,* 2002). However, the role of such a NO production pathway and its sensitivity to small changes in NO_3^- supply in plants under water deficit remain unknown.

Nitrogen is the most influential plant nutrient in sugarcane cultivation (Meyer *et al.,* 2007). Nitrate (NO_3^-), ammonium (NH_4^+), and urea ($CO(NH_2)_2$) are the main forms of fertilizers and, thus, are the main sources of N for crops (Esteban *et al.,* 2016). Some crops have a preference for NH_4^+ uptake (Malagoli *et al.,* 2000), but most studies have reported stress symptoms associated with NH_4^+ toxicity (Barreto *et al.,* 2018; Boschiero *et al.,* 2019). While Robinson *et al.* (2011) reported the sugarcane preference for NH_4^+, Pissolato *et al.* (2019) found that increasing NH_4^+ supply causes biomass reduction and

photosynthesis impairment of sugarcane plants. Changing the N source, NO_3^- supply has been shown to increase the tolerance to abiotic stresses in maize (Rios-Gonzalez *et al.,* 2002; Zhang *et al*., 2012), wheat (Speer *et al*., 1994), pea (Frechilla *et al*., 2001), *Populus simonii* (Meng *et al.,* 2016) and grass species (Wang and Macko, 2011).

The literature concerning NO_3^- supply and stress tolerance, taken together, led us to hypothesize that the increased plant performance under limiting conditions could be related to NO production through NR activity. Here, our aim was to test the hypothesis that sugarcane plants that receive NO_3^- and no NH_4^+ as sources of nitrogen will have higher NR activity and thereby produce more NO, compared to plants receiving the same amount of nitrogen but as a mixture of NO_3^- (70%) and NH_4^+ (30%). As a consequence of NO production, oxidative damage will be reduced under water deficit, favoring photosynthetic metabolism and plant growth.

Materials and Methods

Plant material and growth conditions

Pre-sprouted sugarcane seedlings (*Saccharum* spp.) cv. IACSP95-5000 developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) were used. Six-week-old plants were transferred to plastic boxes (4 L) containing nutrient solution modified from De Armas *et al.* (1992): 5 mmol L^{-1} N (nitrate 90% + ammonium 10%); 9 mmol L^{-1} Ca; 0.5 mmol L^{-1} Mg; 1.2 mmol L^{-1} P; 1.2 mmol L^{-1} S; 24 µmol L^{-1} B; 16 µmol L^{-1} Fe; 9 µmol L^{-1} Mn; 3.5 µmol L^{-1} Zn; 1 µmol L^{-1} Cu; and 0.1 µmol L^{-1} Mo. Plants received this solution for two weeks until the establishment of treatments and the nutrient solution was renewed every three days throughout the experimental period.

Electrical conductivity of nutrient solution was maintained between 1.8 and 2.0 mS cm^{-1} and pH at 5.9±0.1. The pH was adjusted daily with 0.5 M ascorbic acid or 0.5 M NaOH. Both variables were monitored on a daily basis using a portable electrical conductivity meter (mCA 150P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil) and a portable pH meter (mPA 210P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil), respectively. The nutrient

168 solution volume was also checked daily and completed with water when
169 necessary. The nutrient solution was aerated continuously by using an air
170 compressor (Master Super II, Master, São Paulo SP, Brazil).
171 The experiment was carried in a growth chamber (Instalafrio, Brazil), with
172 a 12 h photoperiod, air temperature of 30/20 °C (day/night), air relative humidity
173 of 80% and photosynthetic photon flux density (PPFD) about 800 µmol m^{-2} s^{-1}.
174
175 *Experiment I: Inducing NO production under water deficit through nitrate supply*
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177 Our previous study revealed that sugarcane plants can be supplied with
178 30% NH_4^+ in nutrient solution without compromising their photosynthesis and
179 growth (Pissolato *et al.,* 2019). Thus, the NO_3^-:NH_4^+ ratios 100:0 and 70:30
180 were chosen to represent the treatments with more and less NO_3^-, while
181 supplying the same amount of nitrogen and avoiding NH_4^+ toxicity. Plants were
182 also subjected to varying water availability, according to the osmotic potential of
183 nutrient solution: -0.15 MPa (reference, well-hydrated); and -0.75 MPa (water
184 deficit, WD). The water deficit was induced by adding polyethylene glycol
185 (Carbowax^{TM} PEG-8000, Dow Chemical Comp, Midland MI, USA) to the
186 nutrient solution, seven days after imposing NO_3^-:NH_4^+ ratios. To prevent
187 osmotic shock, PEG-8000 was gradually added to the nutrient solution,
188 reducing the osmotic potential of the solution by -0.20 MPa per day, i.e. -0.75
189 MPa was reached after three days (3th day of the experiment). Plants were
190 allowed to recover from water deficit after returning them to control conditions
191 on the 7th day of the experiment. They remained for 4 days under such
192 conditions, when the experiment ended. For the biochemical analyses, leaf and
193 root samplings were collected at the maximum water deficit (7th day) and at the
194 end of the recovery period (11th day). Samples were collected, immediately
195 immersed in liquid nitrogen and then stored at -80 °C.
196
197 *Leaf gas exchange*
198
199 Gas exchange and chlorophyll fluorescence of the first fully expanded
200 leaf with visible ligule were measured throughout the experimental period using
201 an infrared gas analyzer (Li-6400, Licor, Lincoln NE, USA) equipped with a

modulated fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO_2 assimilation (A_n), stomatal conductance (g_s) and the effective quantum efficiency of photosystem II (Φ_{PSII}) were measured under PPFD of 2000 µmol m^{-2} s^{-1} and air CO_2 concentration of 400 µmol mol^{-1}. The measurements were performed between 10:30 and 12:30 h, as carried out previously (Pissolato *et al.,* 2019). The vapor pressure difference between leaf and air (VPDL) was 2.1±0.2 kPa and leaf temperature was 30±0.4ºC during the evaluations.

Chlorophyll content and leaf relative water content (RWC)

A chlorophyll meter (CFL 1030, Falker, Porto Alegre RS, Brazil) was used to assess the relative chlorophyll content (Chl). The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Jamaux *et al*. (1997): RWC=100×[(FW−DW)/(TW−DW)]. Measurements were taken at the maximum water deficit (7^{th} day), and four days after returning plants to the control condition (recovery period, 11^{th} day).

Photosynthetic enzymes

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was quantified in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM bicine-NaOH buffer (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µM leupeptin. The resulting solution was centrifuged at 14.000 *g* for 5 min at 4 °C. An aliquot of leaf extract was incubated with the reaction medium containing 100 mM bicine-NaOH (pH 8.0) 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase and 80 nkat creatine phosphokinase, for 10 min at 25 °C. The oxidation of NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate (RuBP) and total Rubisco activity was measured. The reduction of absorbance at 340 nm was monitored for 3 min (Sage *et al.,* 1988; Reid *et al.,* 1997).

The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was also evaluated in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM PMSF and centrifuged at 14.000 *g* for 25 min at 4 °C. The supernatant was collected and the reaction medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase and 0.3 mM NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate at 30 °C. The oxidation of NADH was monitored a 340 nm for 1 min (Degl'innocenti *et al.,* 2002).

Proteins were extracted from leaf samples with extraction buffer composed of 100 mM Tris, 1 mM EDTA, 5 mM DTT, 1 mM PMSF and separated by SDS-PAGE (Laemmli, 1970). The first gel was stained with Comassie Brilliant Blue and the second was used for Western blot. SDS-PAGE electrophoresis was performed with equal amounts of protein per lane. Soluble proteins were denatured using SDS and they were electrophoretically transferred to a nitrocellulose membrane (Towbin *et al.*, 1979). PEPC and Rubisco protein abundances were measured by detection of the PEPC subunit and Rubisco large subunit (RLS) using specific polyclonal antibodies (Agrisera Co, Sweden) according to the manufacturer's instructions.

Reactive oxygen species

The concentration of the superoxide anion ($O_2^{\bullet-}$) was determined in 50 mg of fresh tissue incubated in an extraction medium consisting of 100 µM EDTA, 20 µM NADH, and 20 mM sodium phosphate buffer, pH 7.8. The reaction was initiated by adding 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28 °C under stirring for 5 min and the absorbance was read at 480 nm over a further 5 min (Mohammadi and Karr, 2001). $O_2^{\bullet-}$ production was assessed by the accumulation of adrenochrome using a molar extinction coefficient of 4.0×10^3 M^{-1} cm^{-1} (Boveris, 1984).

The quantification of hydrogen peroxide (H_2O_2) was performed following Alexieva *et al.* (2001). Homogenates were obtained from 100 mg of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was

centrifuged at 10.000 *g* and 4 °C for 15 min. The reaction medium consisted of 1 mM KI, 0.1 M potassium phosphate buffer (pH 7.5) and crude extract. The microtubes were left on ice in the dark for 1 h. After this period, the absorbance was read at 390 nm. A standard curve was obtained with H_2O_2 and the results were expressed as µmol H_2O_2 g^{-1} FW.

Lipid peroxidation

The concentration of malondialdehyde (MDA) was measured and used as a proxy of lipid peroxidation. 200 mg of fresh tissue were macerated in extraction medium containing 0.1% TCA (w/v) and centrifuged at 10.000 *g* for 15 min. The supernatant was added to 0.5% thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture incubated at 95 °C for 20 min (Cakmak and Horst, 1991). After this time, the reaction was stopped in an ice bath. Then a new centrifugation was performed at 10.000 *g* for 10 min, and after 30 min at room temperature the absorbance was read at 532 and 600 nm and the non-specific absorbance at 600 nm was discounted. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968) and results were expressed as nmol MDA g^{-1} FW

Antioxidant activity and protein extraction

The crude enzymatic extracts for the determination of superoxide dismutase activity (SOD), catalase (CAT) and ascorbate peroxidase (APX) were obtained from 100 mg of plant tissue in specific medium, followed by centrifugation at 12.000 *g* for 15 min at 4 °C. The specific medium for CAT and SOD consisted of 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM PMSF and 1% PVPP, according to Peixoto *et al*. (1999). The specific medium for APX was composed of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid and 1 mM EDTA (Nakano and Asada, 1981).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The crude extract was added to the reaction medium consisting of 100 mM sodium phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, 100 µM riboflavin and 1 mM

nitro blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp, 30 W) for 10 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD defined as the amount of enzyme required to inhibit NBT photoreduction by 50%, and activity expressed as U min^{-1} mg^{-1} of protein.

Catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure described by Havir and McHale (1987). The crude extract was added to the reaction medium consisting of 100 mM potassium phosphate buffer (pH 6.8), deionized water and 125 mM H_2O_2. The reaction was carried out in a water bath at 25 °C for 2 min and CAT activity was assessed by the decrease in absorbance at 240 nm, using the molar extinction coefficient of 36 M^{-1} cm^{-1} and expressed activity as nmol min^{-1} mg^{-1} of protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by Nakano and Asada (1981). The crude extract was added in reaction medium consisting of 100 mM potassium phosphate buffer (pH 6.8), deionized water, 10 mM ascorbic acid and 10 mM H_2O_2. The reaction was carried out at 25 °C for 2 min and APX activity quantified by the decrease in absorbance at 290 nm, using the molar extinction coefficient of 2.8 M^{-1} cm^{-1} and expressing activity as µmol min^{-1} mg^{-1} of protein.

The protein levels were determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The extract used for this analysis was the same as for SOD and CAT enzymes.

Nitrate, nitrite and ammonium

Fresh plant tissue samples (500 mg) were ground in liquid nitrogen and extraction medium containing methanol:chloroform:water (12:5:3 v/v). After centrifugation at 2.000 *g* for 5 min, the supernatants were collected and chloroform and deionized water were added to them. The mixture was shaken vigorously and then centrifuged for 3 min at 2.000 *g* for phase separation. The upper aqueous phase was collected and maintained in a water bath at 37 °C to remove traces of chloroform and then the extracts were stored at -20 ºC (Bieleski and Turner, 1966).

For nitrate determination, an aliquot of the extract was pipetted into test tubes containing reaction medium (5% salicylic acid in conc. H_2SO_4). After 20 min, 2 N NaOH was added and the solution stirred. After cooling to room temperature, the absorbance was read in a spectrophotometer at 410 nm and the nitrate content calculated from a standard curve using KNO_3 (100-1000 nmol) (Cataldo *et al.,* 1975). For nitrite, an aliquot of the extract was added to 1% sulfanilamide solution in 3 N HCl and 0.02% N-naphthyl ethylenediamine solution. The tubes were allowed to stand for 30 min in the dark at room temperature. Deionized water was added and nitrite content quantified after reading the absorbance at 540 nm (Hageman and Reed, 1980). For ammonium, the extract was added to microtubes, where solution A (1% phenol and 0.005% sodium nitroprusside) was added and followed by solution B (0.5% sodium hydroxide containing 2.52% sodium hypochlorite). The tubes were incubated for 35 min in a water bath at 37 °C and the absorbance read at 625 nm after cooling to room temperature (McCullough, 1967). A standard curve of $(NH_4)_2SO_4$ was used to estimate the ammonium content.

Nitrate reductase (NR) activity

Leaf and root nitrate reductase (NR, EC 1.7.1.1) activity was estimated as the rate of nitrite (NO_2^-) production (Cambraia *et al.,* 1989). The enzyme extract was obtained from the macerate of 200 mg of fresh tissue with liquid nitrogen and homogenized with extraction medium containing 0.1 M tris-HCl buffer (pH 8.1), 4 mM $NiSO_4$, 20 mM reduced glutathione (GSH), deionized water and 0.5 mM PMSF. Then, the crude extracts were centrifuged at 10.000 *g* for 10 min at 4 °C and the supernatant was collected and maintained on ice. The extract was added to the assay medium containing 100 mM Tris-HCl buffer (pH 7.5), 10 mM KNO_3, 0.05 mM NADH and triton 1% X-100 (v/v), mixed and incubated at 30 °C for 10 min. The reaction was quenched by adding 1% sulfanilamide solution in 1 M HCl and 0.01% N-naphthyl ethylenediamine. Nitrite production was determined by absorbance at 540 nm using a standard curve with KNO_2. The NR activity was expressed as nmol NO_2^- min^{-1} mg^{-1} protein.

S-nitrosogluthatione reductase (GSNOR) activity

Leaf and root S-nitrosogluthatione reductase (GSNOR, EC 1.2.1.1) activity was determined spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm, based on Rodríguez-Ruiz *et al.* (2017). Briefly, 200 mg of fresh tissue were grounded with liquid nitrogen, resuspended in 20 mM HEPES buffer (pH 8.0), 10 mM EDTA, 0.5 mM PMSF and centrifuged for 10 min at 10.000 *g* and 4 ºC. The enzyme extract was added in to the assay medium (20 mM HEPES buffer pH 8.0 and 1.8 mM NADH) at 25 ºC, and maintained in the dark. The reaction was started by adding 4 mM GSNO (Silveira *et al.,* 2016) and the GSNOR activity followed by NADH oxidation at 340 nm. Activity was calculated using the NADH extinction coefficient (6.22 mM^{-1} cm^{-1} at 340 nm) and expressed as nmol NADH min^{-1} mg^{-1} protein.

S-nitrosothiols content

The total leaf and root proteins were extracted in deionized water and the resulting homogenate was used to estimate the S-nitrosothiol content through an amperometer, as described by Santos *et al*. (2016) and Zhang *et al.* (2000). Measurements were performed with the WPI amperometer TBR 4100/1025 (World Precision Instruments Inc., Sarasota FL, USA) and a specific nitric oxide (NO) sensor, ISO-NOP (2 mm). Aliquots of aqueous suspension were added to the sample compartment containing aqueous copper chloride solution (0.1 mol L^{-1}). This condition allowed the detection of free NO released from the S-nitrosothiols present in the leaf and root protein homogenate. The samples were run in triplicate and the calibration curve was obtained with newly prepared GSNO solutions. The data were compared with the standard curve obtained and normalized against fresh weight. The SNO content was expressed as µmol NO g^{-1} FW.

Intracellular NO detection

NO was assayed in leaf and root segments. For the roots, it was collected approximately 1 cm from the middle part of secondary root. For the

leaves, a thin cross section was made with the aid of a scalpel. The segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM $CaCl_2$, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in solution of 10 µM DAF2-DA, mixing for 40 min in the dark at room temperature (Desikan *et al.,* 2002; Bright *et al.,* 2009). The samples were washed with buffer to remove the excess of DAF2-DA, placed onto a glass slide and covered with a glass slip before observing fluorescence using an inverted confocal microscope set for excitation at 488 nm and emission at 515 nm (Model Zeiss LSM510, Carl Zeiss AG, Germany). Photographs were taken with a 10x magnification, 15 s exposure and 1x gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data were normalized by subtracting the values of the negative control (plants well-hydrated) and presented as mean pixel intensities.

Biometry

Leaf and root dry masses were quantified after drying samples in an oven (60 °C) with forced-air circulation until constant weight. Leaf area of each plant was evaluated with a portable leaf area meter (model LI-3000, Li-Cor Inc., Lincoln NE, USA).

Experiment II: Using cPTIO to offset the benefits of NO in plants under water deficit

An additional experiment was performed to verify whether the benefits found in plants supplied with only NO_3^- and subjected to water deficit were in fact caused by NO. We used a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). cPTIO is a stable organic radical developed by Akaike and Maeda (1996), which has been widely used as a control as it oxidizes the NO molecule to form NO_2. In plants supplied with only NO_3^- as N source, the following treatments were evaluated: (a) well-watered condition, with an osmotic potential of the nutrient solution of -0.15 MPa; (b) water deficit, with an osmotic potential of nutrient solution of -0.75 MPa; and (c) same as b with 100 µM cPTIO.

First, plants were moved and roots placed in a moist chamber, where they were sprayed with cPTIO and remained in the dark for 1 hour. After this treatment, the plants were returned to the boxes with the original nutrient solution. This procedure was performed for four consecutive days from the moment the water deficit (-0.75 MPa) was installed. We also evaluated the production of intracellular NO, plant biomass, leaf CO_2 assimilation (A_n) and stomatal conductance (g_s) as described previously.

Experimental design and statistical analyses

The experimental design was completely randomized and two causes of variation were analyzed: water availability and nitrogen source. Data were then subjected to an analysis of variance (ANOVA) and when statistical significance was detected, the mean values (n=4) were compared by the Tukey test (p<0.05) using the software Assistat version 7.7 (UFCG, Campina Grande PB, Brazil).

Results

Experiment I: Sugarcane responses to water deficit as affected by NO_3^- supply

Relative water content and photosynthesis

A significant reduction in leaf relative water content was found under water deficit, as compared to well-watered conditions (Fig. 1d). The relative chlorophyll content was also reduced at the maximum water deficit, with no differences induced by NO_3^- supply (data not shown). Low water availability also caused a large reduction in leaf CO_2 assimilation (A_n); however, plants supplied with more NO_3^- exhibited higher photosynthetic rates than those under NO_3^-:NH_4^+ 70:30 (Fig. 1a). In addition, those plants showed a faster recovery of A_n when compared to ones receiving 70% NO_3^- (Fig. 1a). Similar results were found for stomatal conductance (Fig. 1b) and effective quantum efficiency of PSII (Fig. 1b,c). We did not observe any significant difference among treatments for the PEPC abundance and activity at maximum water deficit

(Suppl. Fig. S1a,c). However, both Rubisco abundance and activity were decreased under water deficit, regardless of the variation in NO_3^- supply (Suppl. Fig. S1b,d).

Nitrate and ammonium

Leaf [NO_3^-] was higher in water-stressed plants as compared to well-hydrated ones, but no difference was found due to NO_3^- supply under low water availability (Fig. 2a). Root [NO_3^-] was significantly higher in plants supplied with 100% NO_3^- and subjected to water deficit (Fig. 2b). While leaf [NO_2^-] did not vary among treatments (Fig. 2c), we found the highest root [NO_2^-] in plants supplied with 100% NO_3^- under water deficit (Fig. 2d). We did not find significant changes in leaf and root [NH_4^+] due to NO_3^- supply, regardless the water regime (Fig. 2e,f). During the recovery period, both previously stressed plants and the controls presented similar leaf and root [NO_3^-], [NO_2^-] and [NH_4^+] (Fig. 2).

Nitrate reductase, S-nitrosoglutathione reductase and S-nitrosothiols

Under low water availability, nitrate reductase (NR) activity was higher in plants supplied with 100% NO_3^- than those receiving 70% NO_3^-, regardless the plant organ (Fig. 3a,b). While we did not notice differences among treatments for leaf NR activity during the recovery period, root NR activity was higher under water deficit (Fig. 3b). Under water deficit, plants supplied with 100% NO_3^- showed higher root GSNOR activity than those under 70% NO_3^- (Fig. 3d). Non-significant differences were found in leaf SNO concentration while varying NO_3^- supply (Fig. 3e). However, the lowest root S-nitrosothiols (SNO) concentration was observed in plants supplied with 100% NO_3^- under water deficit (Fig. 3f).

Antioxidant metabolism

Plants supplied with less NO_3^- presented higher leaf [$O_2^{\bullet-}$] when compared to ones supplied with 100% NO_3^- under water deficit (Fig. 4a). When plants faced water deficit, the highest root [H_2O_2] was found under 70% NO_3^-

supply (Fig. 4d). Although showing higher accumulation of $O_2^{\bullet-}$ and H_2O_2 in leaves and roots, plants supplied with 70% NO_3^- did not show higher MDA content than those under 100% NO_3^- (Fig. 4e,f).

At the maximum water deficit, the highest superoxide dismutase (SOD) activity was observed in roots supplied with 100% NO_3^- (Fig. 5b), with no differences in leaf SOD activity due to changes in NO_3^- supply (Fig. 5a). Root catalase activity was not changed by NO_3^- supply and water deficit (Fig. 5f), but plants supplied with 100% NO_3^-showed higher leaf catalase and root ascorbate peroxidase activities under water deficit (Fig. 5e,d).

Intracellular NO synthesis

When plants were facing low water availability, the intracellular NO was increased in both leaves and roots (Fig. 6). However, roots receiving 100% NO_3^- exhibited higher NO production than those supplied with 70% NO_3^- (Fig. 6b). Such a response did not occur in leaves (Fig. 6a).

Plant growth

The root dry mass of plants supplied with 70% NO_3^- was significantly reduced under water deficit (Fig. 7b). In addition, the lowest values for shoot dry mass (Fig. 7a) and leaf area (Fig. 7c) were found in plants supplied with less NO_3^- under low water availability.

Experiment II: Offsetting the benefits of NO synthesis induced by NO_3^- supply

cPTIO – a NO scavenger – was sprayed on roots supplied with 100% NO_3^- and facing water deficit. As consequence, the intracellular NO synthesis was reduced in leaves and roots (Fig. 8a,b) and plants showed lower photosynthetic rates and stomatal conductance under water deficit as compared to ones not sprayed with cPTIO (Fig. 9a,b). As found in experiment I, plants presented decreases in root dry mass due to water deficit when cPTIO was sprayed (Fig. 9d; Suppl. Fig. S2).

Discussion

Nitrate supply stimulates root NO production, improving photosynthesis and antioxidant metabolism of sugarcane under water deficit

Our findings revealed that nitrate reductase is an important enzymatic pathway for NO synthesis and also that sugarcane plants supplied with 100% NO_3^- presented enhancement of drought tolerance. Here, we found higher NO_3^- accumulation in roots under water deficit and receiving only NO_3^- as source of nitrogen (Fig. 2b), which caused higher NO_2^- production when compared to roots exposed to 70% NO_3^- and 30% NH_4^+ (Fig. 2b,d). Such findings are supported by higher root nitrate reductase activity (Fig. 3b), which reduces NO_3^- to NO_2^- during the N assimilation pathway (Heidari *et al.,* 2011). As an alternative reaction, nitrate reductase may also reduce NO_2^- to NO (Fancy *et al.,* 2016). In fact, the highest NO synthesis was found in roots under water deficit and supplied with only NO_3^- (Fig. 6b) and it is known that NO_3^- and NO_2^- play a key role in NO synthesis through nitrate reductase (Vanin *et al.*, 2004; Yamasaki, 2005; Sun *et al.*, 2015). In *Physcomitrella patens*, low nitrate reductase activity was associated with drastic reductions in NO synthesis, further evidence that nitrate reductase is an important pathway for NO production in plants (Andrés *et al.,* 2015). It is worth noting that NO synthesis is low under non-limiting conditions, even in plants supplied with only NO_3^- (Fig. 6). In general, increases in NO synthesis are expected under stressful conditions, when NO_2^- accumulation occurs (Mur *et al.,* 2012).

In the last decades, rapidly increasing evidence has indicated NO as an important player in plant responses to environmental constraining conditions by inducing the antioxidant defenses (Hatamzadeh *et al.,* 2015; Silveira *et al.,* 2017b). During cell detoxification, $O_2^{\bullet-}$ is dismuted to H_2O_2 by superoxide dismutase, which is rapidly eliminated by catalase and ascorbate peroxidase, producing H_2O and O_2 (Lázaro *et al.,* 2013). Here, we observed higher superoxide dismutase activity in roots under water deficit and supplied with 100% NO_3^- (Fig. 5b), with root [$O_2^{\bullet-}$] remaining similar among treatments (Fig. 4b). Interestingly, there was lower $O_2^{\bullet-}$ accumulation in leaves under water deficit and supplied with only NO_3^- (Fig. 4a), even with superoxide dismutase

572 showing similar activity to the one found in plants supplied with 70% NO_3^- and
573 30% NH_4^+ (Fig. 5a). As a possible explanation, such low leaf [$O_2^{\bullet-}$] may be
574 related to the interaction of this radical with NO, which generates peroxynitrite
575 ($ONOO^-$) and adds a nitro group to tyrosine residues – a process known as
576 tyrosine nitration (Begara-Morales *et al*., 2014; Wullf *et al*., 2009). Although
577 tyrosine nitration was originally considered as indicative of stress conditions,
578 recent evidence suggests its role in cell signaling (Mengel *et al.,* 2013).
579 Root [H_2O_2] was lower in plants under water deficit that received 100%
580 NO_3^- as compared to ones supplied with 70% NO_3^- and 30% NH_4^+ (Fig. 4d),
581 indicating an efficient detoxification through increased root ascorbate
582 peroxidase activity (Fig. 5d). In fact, the activation of antioxidant mechanisms to
583 maintain ROS homeostasis often involves NO (Hatamzadeh *et al.,* 2015;
584 Silveira *et al.,* 2015). Many reports show that exogenous NO improves abiotic
585 stress tolerance, causing decreases in [H_2O_2] and lipid peroxidation (Gross *et*
586 *al.,* 2013). Exogenous NO supply inhibits ROS accumulation in many plant
587 species under stress conditions (Verma *et al.,* 2013), such as cucumber and
588 rice under drought (Farooq *et al.,* 2009). Sugarcane plants supplied with GSNO
589 – a NO donor – showed increases in the activity of antioxidant enzymes, such
590 as superoxide dismutase in leaves and catalase in roots under water deficit
591 (Silveira *et al.,* 2017b). In addition, the S-nitrosylation has a role in mediating
592 the interplay between NO and other reactive signaling mechanisms, such as
593 those involving ROS. For instance, S-nitrosylation of RBOHD causes its
594 inactivation and thus reduces ROS formation through this pathway (Yu *et al.,*
595 2012). Such findings revealed that NO has an important role in controlling
596 endogenous ROS levels.
597 Higher superoxide dismutase and ascorbate peroxidase in roots facing
598 water deficit and receiving only NO_3^- (Fig. 5b,d) may be a consequence of S-
599 nitrosylation. In pea (*Pisum sativum*), S-nitrosylation increased the activity of
600 cytosolic ascorbate peroxidase (Begara-Morales *et al.,* 2014). However, we
601 noticed higher levels of S-nitrosothiols (SNOs) in roots under water deficit and
602 supplied with NO_3^- and NH_4^+ (Fig. 3f). At this point, one should consider that
603 NO-mediated post-translational modifications on target proteins may be positive
604 or negative (Nabi *et al.,* 2019). Some of these modifications may alter signaling
605 pathways mediated by other ROS (Holzmeister *et al.,* 2014). According to Clark

606 *et al.* (2000), S-nitrosylation can inhibit catalase activity, which implies that low
607 level of S-nitrosylation can increase catalase activity during stress conditions,
608 thus increasing ROS detoxification. In this way, higher [SNO] found in plants
609 that received less nitrate (Fig. 3e,f) is associated with changes in the antioxidant
610 system that lead to increases in leaf [$O_2^{\bullet-}$] and root [H_2O_2] (Fig. 4a,d). It has
611 been proposed that S-nitrosylation can regulate [H_2O_2] in plants, controlling both
612 the antioxidant defense system and the ROS-producing enzymes (Ortega-
613 Galisteo *et al.,* 2012; Yu et *al.,* 2012).

614 Here, we found low accumulation of SNOs and high GSNOR activity in
615 roots under water deficit that received 100% NO_3^- (Fig. 3f,d). GSNOR can break
616 down GSNO – a SNO, reducing GSNO levels and consequently decreasing the
617 total cellular level of S-nitrosylation (Feechan *et al.,* 2005). Thus, it indirectly
618 controls the overall SNOs within cells (Feechan *et al.,* 2005), suggesting that
619 GSNOR may be crucial in regulating the cellular SNO pool. In fact, increases in
620 GSNOR activity contributed to the reduction of S-nitrosylation in pea plants
621 under salt stress (Camejo *et al.,* 2013). As GSNO is an NO donor, we can
622 argue that increases in root GSNOR activity under water deficit and supplied
623 with only NO_3^- (Fig. 3d) are related to the reduction of GSNO levels and linked
624 to high NO synthesis in roots (Fig. 6b). High levels of reactive nitrogen species
625 (RNS) may be harmful to plants (Nabi *et al.,* 2019) and the absence of GSNOR
626 activity in plants results in a significant increase in levels of SNOs and
627 impairment of plant immunity (Feechan *et al.,* 2005), plant growth and
628 development (Kwon *et al.,* 2012). Gong *et al.* (2015) demonstrated that absence
629 of GSNOR activity increased the sensitivity of *Solanum lycopersicum* to alkaline
630 stress due to the excessive accumulation of NO and SNOs, causing higher
631 levels of endogenous S-nitrosylation and turning stomata insensitive to ABA.

632 Stomatal closure is the primary response of plants to water deficit,
633 reducing the CO_2 supply for photosynthesis and then decreasing biomass
634 production (Machado *et al.,* 2009; Ribeiro *et al.,* 2013). Although water deficit
635 had reduced the stomatal conductance, higher NO_3^- supply alleviated such
636 negative effects (Fig. 1b). Due to higher stomatal conductance, sugarcane
637 plants supplied with 100% NO_3^- showed an improvement in photosynthesis
638 under water deficit (Fig. 1a). By integrating CO_2 assimilation throughout the
639 experimental period, plants supplied with only NO_3^- fixed about 1.5 times more

carbon than those supplied with NO_3^- and NH_4^+. Such a response was also related to improvement of primary photochemistry, with plants showing higher conversion of light energy into chemical energy at the PSII level (Fig. 1c).

Under water deficit, plants supplied with 70% NO_3^- and 30% NH_4^+ presented reduced root biomass as compared to those supplied with 100% NO_3^-, which were not affected by low water availability (Fig. 7b). Such increase in root growth was associated with higher NO content (Fig. 6b), as found by Silveira *et al.* (2016). At maximum water deficit, high NO synthesis was found in the root meristematic zone of plants supplied with 100% NO_3^- (Suppl. Fig. S3). Several reports indicate that NO is involved in the regulation of root growth and developmental processes (Correa-Aragunde *et al.,* 2004; Lombardo and Lamattina, 2012; Sun *et al.,* 2015). The root system is able to perceive low water availability and to produce chemical signals that regulate the water flow from roots to shoots. NO is one of those chemical signals that stimulates root expansion and development (Xu *et al.,* 2017; Silveira *et al.,* 2016). Given the effects of NO on root growth, it is reasonable to assume a potential influence of NO mediating auxin signaling in roots. Correa-Aragunde *et al.* (2006) demonstrated that auxin-dependent cell cycle gene regulation was dependent on NO during lateral root formation in tomato plants. NO also modulates the auxin response during adventitious root formation in cucumber plants (Pagnussat *et al.,* 2002) and *Arabdopsis thaliana* (Lombardo *et al.,* 2006).

Overall, increases in NO content can trigger root development and improve water uptake, reducing the impact of low water availability on leaf water status and allowing higher stomatal conductance and photosynthesis, as noticed herein and also by Silveira *et al.* (2017). The novelty here is that we were able to induce NO synthesis in sugarcane plants by changing the nitrogen source. Such a finding has a practical consequence for sugarcane in the field as endogenous NO synthesis can be stimulated by increasing NO_3^- supply. Apart from economic issues, our data give insights on how stress tolerance can be managed by common practices in agricultural systems and further development on this technique should be carried out with field-grown plants, where interactions among nutrients, soil-root interactions and soil nitrogen dynamics determine plant performance.

674 *Is sugarcane performance under water deficit really improved by NO?*
675
676 Herein, we used 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-
677 oxyl-3-oxide (cPTIO) – an endogenous NO scavenger (Akaike and Maeda,
678 1996) – to check if benefits induced by increasing NO_3^- supply were related to
679 NO. cPTIO drastically reduced the DAF-2DA in plants under water deficit,
680 indicating lower NO accumulation in both leaves and roots (Fig. 8a,b). As
681 consequence, plants showed even lower stomatal conductance and
682 photosynthesis when compared to plants under water deficit and not supplied
683 with cPTIO (Fig. 9a,b). cPTIO sprays also reduced root growth (Fig. 9d), as
684 found previously (Fig. 7b). Taken together, our data clearly show that the
685 improved performance of sugarcane plants supplied with only NO_3^- were due to
686 stimulation of NO synthesis under water deficit.
687
688 **Conclusion**
689
690 Sugarcane plants grown in nutrient solution containing only NO_3^- as
691 nitrogen source were more tolerant to water deficit and this response was
692 associated with increased NO production and high nitrate reductase activity in
693 roots. Herein, increasing NO_3^- supply was enough to stimulate NO synthesis
694 and alleviate the effects of water deficit on sugarcane plants by increasing the
695 activity of antioxidant enzymes, photosynthesis, stomatal conductance and root
696 growth. From a broad perspective, our data show that supplying more NO_3^-
697 during nitrogen fertilization may improve sugarcane tolerance and be beneficial
698 to field-grown sugarcane.
699
700

Supplementary data

Fig. S1. Activity and immunoblots of phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase in sugarcane plants under water deficit.

Fig. S2. Visual aspect of sugarcane plants under water deficit after NO scavenging through cPTIO spraying.

Fig. S3. Intracellular NO synthesis in apical sections of sugarcane roots.

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References

Akaike T, Maeda H. 1996. Quantitation of nitric oxide using 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). Methods in Enzymology **268,** 211-221.

Albertos P, Romero-Puertas MC, Tatematsu K, Mateos I, Sánchez-Vicente I, Nambara E, Lorenzo O. 2015. S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. Nature Communications **6,** 8669.

Alderton WK, Cooper CE, Knowles RG. 2001. Nitric Oxide Synthases: structure, function and inhibition. Biochemistry Journal **357,** 593-615.

Alexieva V, Sergiev I, Mapelli S, Karanov E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell and Environment **24,** 1337-1344.

Andrés RM, Peralta AS, Vázquez JPS, Mendivil SN, Cabrera JAP, Torres MES, Dubrovsky JG, Ruan VL. 2015. The nitric oxide production in the moss *Physcomitrella patens* is mediated by nitrate reductase. PloS One **10,** e0119400.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Kubiś J. 2009. Involvement of nitric oxide in water stress-induced responses of cucumber roots. Plant Science **177,** 682-90.

Astier J, Kulik A, Koen E, Besson-Bard A, Bourque S, Jeandroz S, Lamotte O, Wendehenne D. 2012. Protein S-nitrosylation: what's going on in plants? Free Radical Biology and Medicine **53,** 1101-1110.

Barreto RF, Cruz FJR, Gaion LA, Prado RM, Carvalho RF. 2018. Accompanying ions of ammonium sources and nitrate: ammonium ratios in tomato plants. Journal of Plant Nutrition and Soil Science **181,** 382-7.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Valderrama R, Mata-Pérez C, Jaramillo-López J, Padilla MN, Carreras A, Corpas FJ, Barroso JB. 2014. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. Journal of Experimental Botany **65,** 527-538.

Bieleski RL, Turner NA. 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Analytical Biochemistry **17,** 278-293.

Bogdan C. 2015. Nitric oxide synthase in innate and adaptive immunity: an update. Trends in Immunology **36,** 161-78.

Boschiero BN, Mariano E, Azevedo RA, Trivelin PC. 2019. Influence of nitrate-ammonium ratio on the growth, nutrition, and metabolism of sugarcane. Plant Physiology and Biochemistry **139,** 246-55.

Boveris A. 1984. Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. In Methods in enzymology **105,** 429-435.

Bradford MN. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **72,** 248-254.

Bright J, Hiscoc SJ, James PE, Hancock JT. 2009. Pollen generates nitric oxide and nitrite: a possible link to pollen-induced allergic responses. Plant Physiology and Biochemistry **47,** 49-55.

Cai W, Liu W, Wang WS, Fu ZW, Han TT, Lu YT. 2015. Overexpression of rat neurons nitric oxide synthase in rice enhances drought and salt tolerance. PLoS One **10,** e0131599.

Cakmak I, Horst WJ. 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiologia Plantarum **83,** 463-468.

Cambraia J, Pimenta JA, Estevao MM, Sant'Anna R. 1989. Aluminum effects on nitrate uptake and reduction in sorghum. Journal of Plant Nutrition **12,** 1435-45.

Camejo D, Romero-Puertas MdelC, Rodríguez-Serrano M, Sandalio LM, Lázaro JJ, Jiménez A, Sevilla F. 2013. Salinity-induced changes in S-nitrosylation of pea mitochondrial proteins. Journal of Proteomics **79,** 87-99.

Cánovas D, Marcos JF, Marcos AT, Strauss J. 2016. Nitric oxide in fungi: is there NO light at the end of the tunnel? Current Genetics **62,** 513-8.

Cataldo JM, Haroom M, Schrader LE, Youngs VL. 1975. Rapid calorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis **6,** 71-80.

Chamizo-Ampudia A, Sanz-Luque E, Llamas A, Galvan A, Fernandez E. 2017. Nitrate reductase regulates plant nitric oxide homeostasis. Trends in Plant Science **22,** 163-174.

Clark D, Durner J, Navarre DA, Klessig DF. 2000. Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. Molecular Plant-Microbe Interactions **13,** 1380-1384.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. Planta **218,** 900-905.

Correa-Aragunde N, Graziano M, Chevalier C, Lamattina L. 2006. Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. Journal of Experimental Botany **57,** 581-588.

Crane BR, Sudhamsu J, Patel BA. 2010. Bacterial nitric oxide synthases. Annual Review of Biochemistry **79,** 445-70.

De Armas R, Valadier MH, Champigny ML, Lamaze T. 1992. Influence of ammonium and nitrate on the growth and photosynthesis of sugarcane. Journal of Plant Physiology **140,** 531-535.

Degl'Innocenti E, Guide L, Soldatini GF. 2002. Effect of chronic O_3 fumigation on the activity of some Calvin cycle enzymes in two poplar clones. Photosynthetica **40,** 121-126.

Desikan R, Griffiths R, Hancock JT, Neill S. 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acidinduced stomatal closure in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the U. S. A. **99,** 16314-16318.

Esteban R, Ariz I, Cruz C, Moran JF. 2016. Mechanisms of ammonium toxicity and the quest for tolerance. Plant Science **248,** 92-101.

Fancy NN, Bahlmann AK, Loake GJ. 2016. Nitric oxide function in plant abiotic stress. Plant, Cell and Environment **40,** 462-472.

Farooq M, Basra SMA, Wahid A, Rehman H. 2009. Exogenously applied nitric oxide enhances the drought tolerance in fine grain aromatic rice (*Oryza sativa L.*). Journal of Agronomy and Crop Science **195,** 254-261.

Fatma M, Masood A, Per TS, Khan NA. 2016. Nitric oxide alleviates salt stress inhibited photosynthetic response by interacting with sulfur assimilation in mustard. Frontiers in Plant Science **7,** 521.

Feechan A, Kwon E, Yuri B, Wang Y, Pallas J, Loake G. 2005. A central role for S-nitrosothiols in plant disease resistance. Proceedings of the National Academy of Sciences **102,** 8054-8059.

Fernandez-Marcos M, Sanz L, Lewis DR, Muday GK, Lorenzo O. 2011. Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED1 (PIN1)-dependent acropetal auxin transport. Proceedings of the National Academy of Sciences of the U. S. A. **108,** 18506-18511.

Frechilla S, Lasa B, Ibarretxe L, Lamsfus C, Aparicio-Tejo P. 2001. Pea responses to saline stress is affected by the source of nitrogen nutrition (ammonium or nitrate). Plant Growth Regulation **35,** 171-9.

García-Mata C, Lamattina L. 2001. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiology **126,** 1196-1204.

Giannopolitis C N, Ries SK. 1977. Superoxide dismutase: Occurrence in higher plants. Plant Physiology **59,** 309-314.

Gong B, Wen D, Wang X, Wei M, Yang F, Li Y, Shi Q. 2015. S-nitrosoglutathione reductase modulated redox signaling controls sodic alkaline stress responses in *Solanum lycopersicum L*. Plant and Cell Physiology **1,** 790-802.

Gross F, Durner J, Gaupels F. 2013. Nitric oxide, antioxidants and prooxidants in plant defence responses. Frontiers in Plant Science **4,** 419.

Gupta KJ, Fernie AR, Kaiser WM, Dongen JTV. 2011. On the origins of nitric oxide. Trends in Plant Science **16,** 160-168.

Hageman RH, Reed AJ. 1980. Nitrate reductase from higher plants. Methods in Enzymology **69,** 270-280.

Hancock JT, Neill SJ. 2014. NO synthase in plants? CAB Reviews **9,** 1-9.

Hancock JT, Neill SJ. 2019. Nitric Oxide: Its generation and interactions with other reactive signaling compounds. Plants **8,** 41.

Hatamzadeh A, Molaahmad Nalousi A, Ghasemnezhad M, Biglouei MH. 2015. The potential of nitric oxide for reducing oxidative damage induced by drought stress in two turf grass species, creeping bent grass and tall fescue. Grass and Forage Science **70,** 538-548.

Havir EA, McHale NA. 1987. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiology **84,** 450-455.

He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F. 2004. Nitric oxide represses the *Arabidopsis* floral transition. Science **305,** 1968-1971.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplast. I. kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics **125,** 189-198.

Heidari B, Matre P, Nemie-Feyissa D, Meyer C, Rognli OA, Moller SG, Lillo C. 2011. Protein phosphatase 2A B55 and A regulatory subunits interact with nitrate reductase and are essential for nitrate reductase activation. Plant Physiology **156,** 165-72.

Holzmeister C, Gaupels F, Geerlof A, Sarioglu H, Sattler M, Durner J, Lindermayr C. 2014. Differential inhibition of Arabidopsis superoxide dismutases by peroxynitrite-mediated tyrosine nitration. Journal of Experimental Botany **66,** 989-99.

Jahnová J, Luhová L, Petřivalský M. 2019. S-nitrosoglutathione reductase - the master regulator of protein S-nitrosation in plant no signaling. Plants **8,** 48.

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

Kwon E, Feechan A, Yun BW, Hwang BH, Pallas JA, Kang JG, Loake GJ. 2012. AtGSNOR1 function is required for multiple developmental programs in Arabidopsis. Planta **236,** 887-900.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680-685.

Lázaro JJ, Jiménez A, Camejo D, Martí MC, Lázaro-Payo A, Barranco-Medina S, Sevilla F. 2013. Dissecting the integrative antioxidant and redox systems in plant mitochondria. Effect of stress and S-nitrosylation. Frontiers in plant science **4,** 460.

León J, Costa-Broseta Á. 2019. Present knowledge and controversies, deficiencies, and misconceptions on nitric oxide synthesis, sensing, and signaling in plants. Plant, Cell and Environment **1,** 15.

Lombardo MC, Graziano M, Polacco JC, Lamattina L. 2006. Nitric oxide functions as a positive regulator of root hair development. Plant Signaling and Behavior **1,** 28-33.

Lombardo MC, Lamattina L. 2012. Nitric oxide is essential for vesicle formation and trafficking in *Arabidopsis* root hair growth. Journal of Experimental Botany **63,** 4875-4885.

Machado RS, Ribeiro RV, Marchiori PER, Machado DFSP, Machado EC, Landell MGDA. 2009. Biometric and physiological responses to water deficit in sugarcane at different phenological stages. Pesquisa Agropecuária Brasileira **44,** 1575-1582.

Malagoli M, Dal Canal A, Quaggiotti S, Pegoraro P, Bottacin A. 2000. Differences in nitrate and ammonium uptake between Scots pine and European larch. Plant and Soil **221,** 1-3.

McCullough H. 1967. The determination of ammonia in whole blood by a direct colorimetric method. Clinica Chimica Acta **17,** 297-304.

Mengel A, Chaki M, Shekariesfahlan A, Lidermayr C. 2013. Effect of nitric oxide on gene transcription – S-nitrosylation of nuclear proteins. Frontiers in Plant Science **4,** 293.

Meng S, Su L, Li Y, Wang Y, Zhang C, Zhao Z. 2016. Nitrate and ammonium contribute to the distinct nitrogen metabolism of *Populus simonii* during moderate salt stress. PloS One **11,** e0150354.

Meyer JH, Schumann AW, Wood RA, Nixon DJ, Van Den Berg M. 2007. Recent advances to improve nitrogen use efficiency of sugarcane in the South African sugar industry. In Proceedings of the International Society of Sugar Cane Technologists **26,** 238-246.

Mohammadi M, Karr AL. 2001. Superoxide anion generation in effective and ineffective soybean root nodules. Journal of Plant Physiology **158,** 1023-1029.

Moreau M, Lindermayr C, Durner J, Klessig D. 2010. NO synthesis and signaling in plants – where do we stand? Physiologia Plantarum **138,** 372-383.

Mur LA, Sivakumaran A, Mandon J, Cristescu SM, Harren FJ, Hebelstrup KH. 2012. Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. Journal of Experimental Botany **63,** 4375-4387.

Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJM, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants **5,** pls052.

Nabi RBS, Tayade R, Hussain A, Kulkarni KP, Imran QM, Mun BG, Yun BW. 2019. Nitric oxide regulates plant responses to drought, salinity, and heavy metal stress. Environmental and Experimental Botany **161,** 120-133.

Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidases in spinach chloroplasts. Plant and Cell Physiology **22,** 867-880.

Ortega-Galisteo AP, Serrano MR, Pazmiño DM, Gupta DK, Sandalio LM, Puertas MCR. 2012. S-Nitrosylated protein sinpea (*Pisum sativum* L.) leaf peroxisomes: changes under abiotic stress. Journal of Experimental Botany **63,** 2089-2103.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L. 2002. Nitric oxide is required for root organogenesis. Plant Physiology **129,** 954-6.

Peixoto PHP, Cambraia J, Sant'ana R, Mosquim PR, Moreira MA. 1999. Aluminum effects on lipid peroxidation and on activities of enzymes of oxidative metabolism in sorghum. Revista Brasileira de Fisiologia Vegetal **11,** 137-143.

Pissolato MD, Silveira NM, Machado EC, Zambrosi FCB, Sodek L, Ribeiro RV. 2019. Photosynthesis and biomass accumulation in young sugarcane plants grown under increasing ammonium supply in nutrient solution. Theoretical and Experimental Plant Physiology **31,** 401-11.

Reid CD, Tissue DT, Fiscus EL, Strain BR. 1997. Comparison of spectrophotometric and radio isotopic methods for the assay of Rubisco in ozone-treated plants. Physiologia Plantarum **101,** 398-404.

Ribeiro RV, Machado RS, Machado EC, Machado DFSP, Magalhães Filho JR, Landell MGA. 2013. Revealing drought-resistance and productive patterns in sugarcane genotypes by evaluating both physiological responses and stalk yield. Experimental Agriculture **49,** 212-224.

Rios-Gonzalez K, Erdei L, Lips SH. 2002. The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. Plant Science **162,** 923-30.

Robinson N, Brackin R, Vinall K, Soper F, Holst J, Gamage H, Paungfoo-Lonhienne C, Rennenberg H, Lakshmanan P, Schmidt S. 2011. Nitrate paradigm does not hold up for sugarcane. PloS One **6,** e19045.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. 2002. Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. Journal of Experimental Botany **53,** 103-110.

Rodríguez-Ruiz M, Mioto P, Palma JM, Corpas FJ. 2017. S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (*Capsicum annuum* L.) fruit ripening. Nitric Oxide **68,** 51-55.

Sage RF, Sharkey TD, Seemann JR. 1988. The in vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in *Phaseolus vulgaris* L. Planta **174,** 407-416.

Santolini J, André F, Jeandroz S, Wendehenne D. 2017. Nitric oxide synthase in plants: Where do we stand? Nitric Oxide **63,** 30-38.

Santos MC, Seabra AB, Pelegrino MT, Haddad os. 2016. Synthesis, characterization and cytotoxicity of glutathione-and PEG-glutathione-superparamagnetic iron oxide nanoparticles for nitric oxide delivery. Applied Surface Science **367,** 26-35.

Shan C, Zhou Y, Liu M. 2015. Nitric oxide participates in the regulation of the ascorbate-glutathione cycle by exogenous jasmonic acid in the leaves of wheat seedlings under drought stress. Protoplasma **252,** 1397-1405.

Shi H, Ye T, Zhu JK, Chan Z. 2014. Constitutive production of nitric oxide leads to enhanced drought stress resistance and extensive transcriptional reprogramming in *Arabidopsis*. Journal of Experimental Botany **65,** 4119-4131.

Silveira NM, de Oliveira JA, Ribeiro C, Canatto RA, Siman L, Cambraia J, Farnese F. 2015. Nitric oxide attenuates oxidative stress induced by arsenic in lettuce (*Lactuca sativa*) leaves. Water, Air and Soil Pollution **226,** 379.

Silveira NM, Frungillo L, Marcos FC, Pelegrino MT, Miranda MT, Seabra AB, Salgado I, Machado EC, Ribeiro RV. 2016. Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit. Planta **244,** 181-190.

Silveira NM, Hancock JT, Frungillo L, Siasou, E, Marcos FC, Salgado I, Machado EC, Ribeiro RV. 2017a. Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane. Plant Physiology and Biochemistry **115,** 354-359.

Silveira NM, Marcos FC, Frungillo L, Moura BB, Seabra AB, Salgado I, Machado EC, Hancock JT, Ribeiro RV. 2017b. S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in

both leaves and roots of sugarcane plants under water deficit. Physiologia Plantarum **160,** 383-395.

Speer M, Brune A, Kaiser WM. 1994. Replacement of nitrate by ammonium as the nitrogen source increases the salt sensitivity of pea plants. I. Ion concentrations in roots and leaves. Plant, Cell and Environment **17,** 1215-1221.

Sun H, Li J, Song W, Tao J, Huang S, Chen S, Hou M, Xu G, Zhang Y. 2015. Nitric oxide generated by nitrate reductase increases nitrogen uptake capacity by inducing lateral root formation and inorganic nitrogen uptake under partial nitrate nutrition in rice. Journal of Experimental Botany **66,** 2449-59.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Science U. S. A. **76,** 4350-4356.

Vanin AF, Svistunenko DA, Mikoyan VD, Serezhenkov VA, Fryer MJ, Baker NR, Cooper CE. 2004. Endogenous superoxide production and the nitrite/nitrate ratio control the concentration of bioavailable free nitric oxide in leaves. Journal of Biological Chemistry **279,** 24100-24107.

Verma K, Mehta SK, Shekhawat GS. 2013. Nitric oxide (NO) counteracts cadmium induced cytotoxic processes mediated by reactive oxygen species (ROS) in *Brassica juncea*: cross-talk between ROS, NO and antioxidant responses. Biometals **26,** 255-269.

Wang L, Macko SA. 2011. Constrained preferences in nitrogen uptake across plant species and environments. Plant, cell and Environment **34,** 525-34.

Wang P, Du Y, Hou YJ, Zhao Y, Hsu CC, Yuan F, Zhu X, Tao WA, Song CP, Zhu JK. 2015. Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. Proceedings of the National Academy of Science U. S. A. **112,** 613-618.

Wullf A, Oliveira HC, Saviani EE, Salgado I. 2009. Nitrite reduction and superoxide-dependent nitric oxide degradation by *Arabidopsis* mitochondria: influence of external NAD(P)H dehydrogenases and alternative oxidase in the control of nitric oxide levels. Nitric Oxide **21,** 132-139.

Xu XT, Jin X, Liao WB, Dawuda MM, Li XP, Wang M, Niu LJ, Ren PJ, Zhu YC. 2017. Nitric oxide is involved in ethylene-induced adventitious root

development in cucumber (*Cucumis sativus* L.) explants. Scientia Horticulturae **215,** 65-71.

Yamasaki H, Sakihama Y, Takahashi S. 1999. An alternative pathway for nitric oxide production in plants: new features of an old enzyme. Trends in Plant Science **4,** 128-129.

Yamasaki H. 2005. The NO world for plants: achieving balance in an open system. Plant, Cell and Environment **28,** 78-84.

Yu M, Yun BW, Spoel SH, Loake GJ. 2012. A sleigh ride through the SNO: Regulation of plant immune function by protein S-nitrosylation. Current Opinion in Plant Biology **15**, 424-430.

Zhang X, Cardosa L, Broderick M, Fein H, Davies IR. 2000. Novel calibration method for nitric oxide microsensors by stoichiometrical generation of nitric oxide from SNAP. Electroanalysis **12,** 425-428.

Zhang L, Zhai Y, Li Y, Zhao YO, Lv L, Gao M, Liu J, Hu J. 2012. Effects of nitrogen forms and drought stress on growth, photosynthesis and some physico-chemical properties of stem juice of two maize cultivars (*Zea mays* L.) at elongation stage. Pakistan Journal Botany **44,** 1405-1412.

Figures captions

Figure 1. Leaf CO_2 assimilation (A_n, in a), stomatal conductance (g_S, in b), effective quantum efficiency of PSII (Φ_{PSII}, in c) and leaf relative water content (RWC, in d) in sugarcane plants maintained well-hydrated (ref, white symbols and bars) or subjected to water deficit (WD, black symbols and gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of recovery. Symbols and bars represent the mean value of four replications ± se. Asterisks indicate significant differences between treatments under water deficit and different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 2. Concentration of nitrate (a and b), nitrite (c and d) and ammonium (e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 3. Nitrate reductase activity (NR, in a and b), S-nitrosoglutathione reductase activity (GSNOR, in c and d) and S-nitrosothiol concentration (SNO, in e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 4. Concentration of superoxide anion ($O_2^{\bullet-}$, a and b), hydrogen peroxide (H_2O_2, c and d) and malondialdehyde (MDA, in e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 5. Superoxide dismutase activity (SOD, in a and b), ascorbate peroxidase activity (APX, in c and d) and catalase activity (CAT, in e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 6. Confocal microscopy images showing intracellular NO synthesis in leaves (a) and roots (b) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. Mean pixel intensities are also shown. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 7. Shoot (SDM, in a) and root (RDM, in b) dry mass and leaf area (LA, in c) of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, p<0.05).

Figure 8. Confocal microscopy images showing intracellular NO synthesis in leaves (a) and roots (b) of sugarcane plants supplied with only NO_3^- (100:0 NO_3^-:NH_4^+) and maintained well-hydrated (reference, white bars), subjected to water deficit (WD, gray bars) and subjected to water deficit and sprayed with cPTIO (WD+cPTIO, gray striped bars). Mean pixel intensities are also shown. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 9. Leaf CO_2 assimilation (A_n, in a), stomatal conductance (g_S, in b), shoot (SDM, in c) and root (RDM, in d) dry mass and leaf area (LA, in e) of sugarcane plants supplied with only NO_3^- (100:0 NO_3^-:NH_4^+) and maintained well-hydrated (reference, white symbols and bars), subjected to water deficit (WD, black symbols and gray bars) and subjected to water deficit and sprayed with cPTIO (WD+cPTIO, crossed symbols and gray striped bars). Asterisks indicate significant differences between treatments under water deficit (a and b) and different letters indicate statistical difference among treatments (c-e) by the Tukey test ($p<0.05$).

Fig. 1

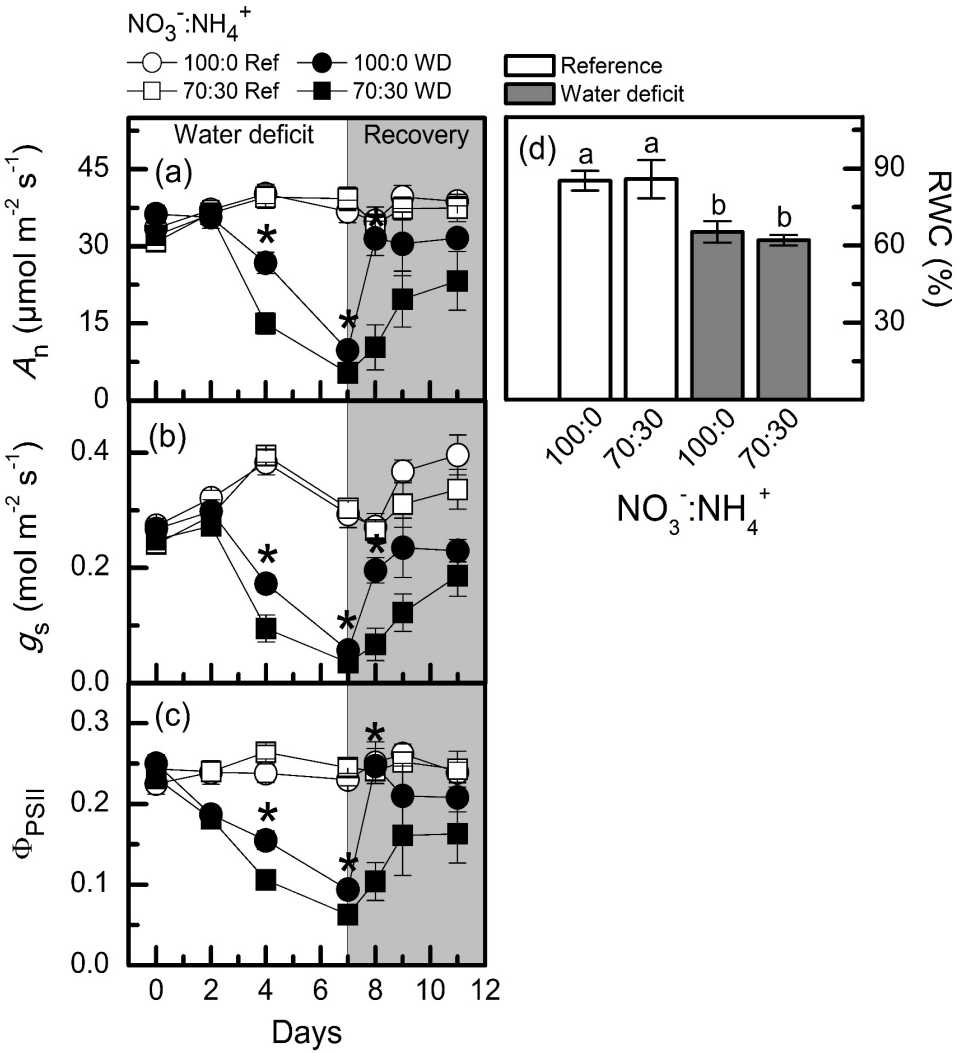
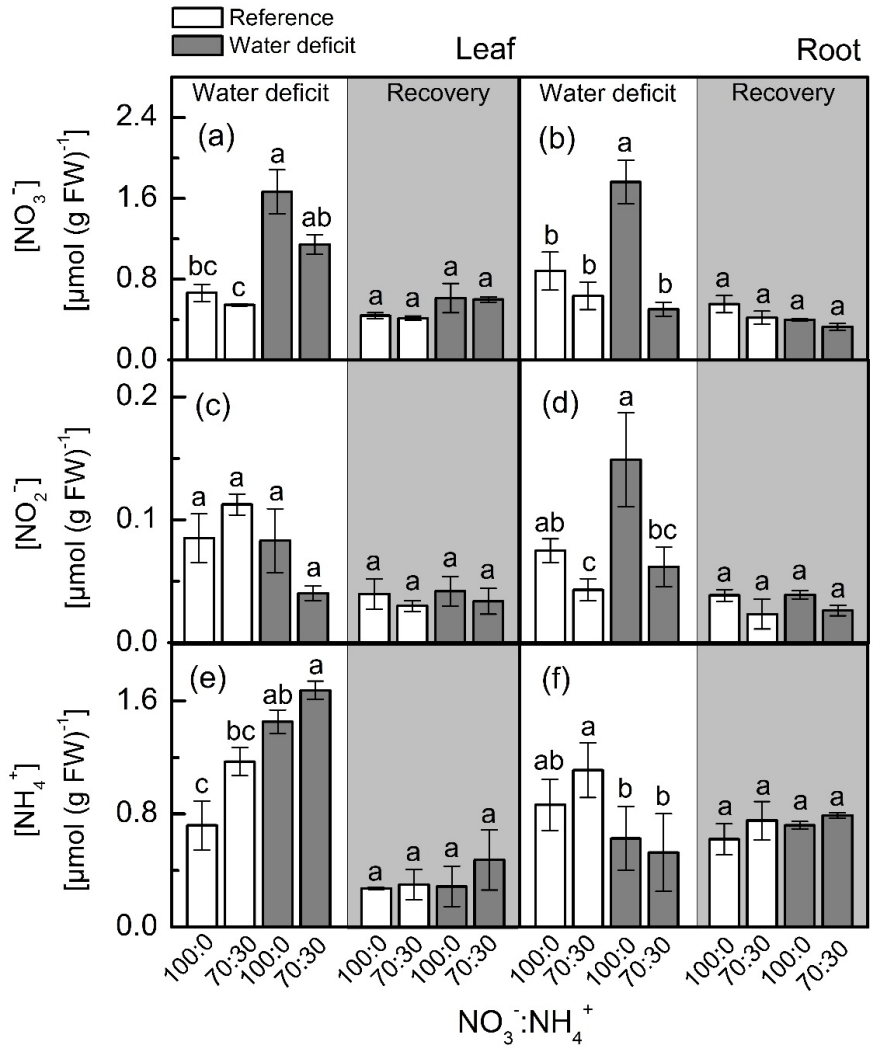


Fig. 2



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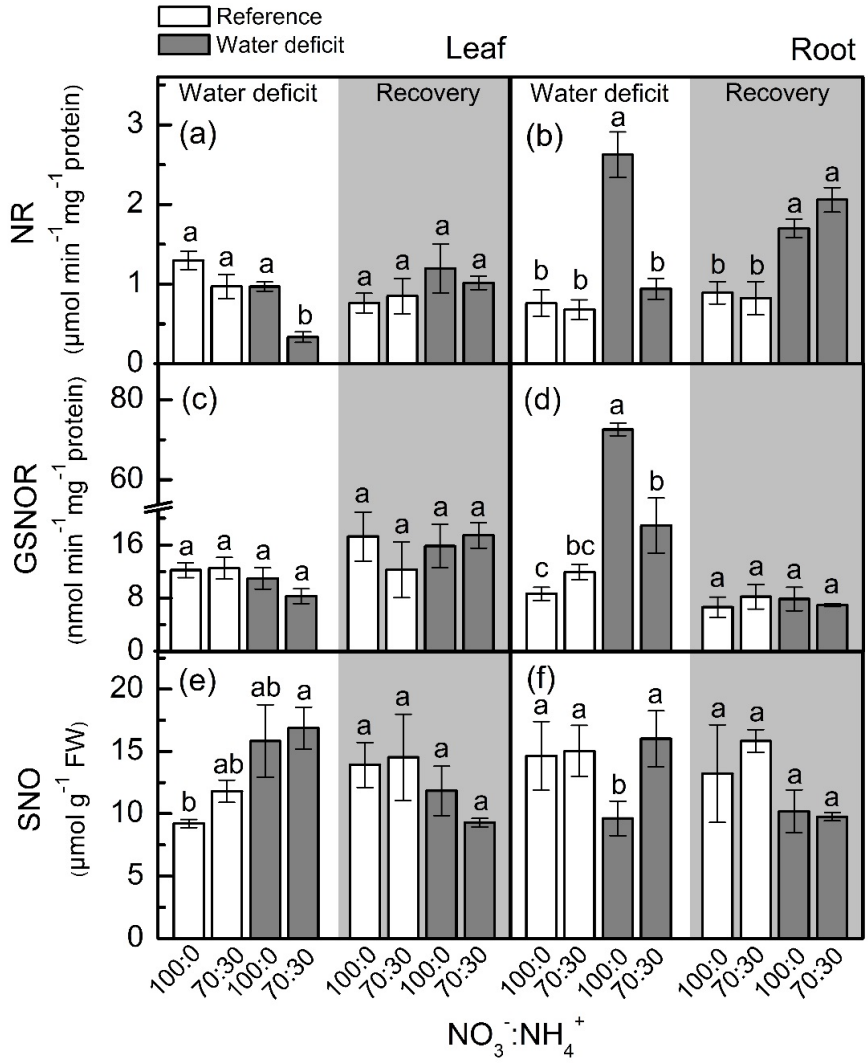

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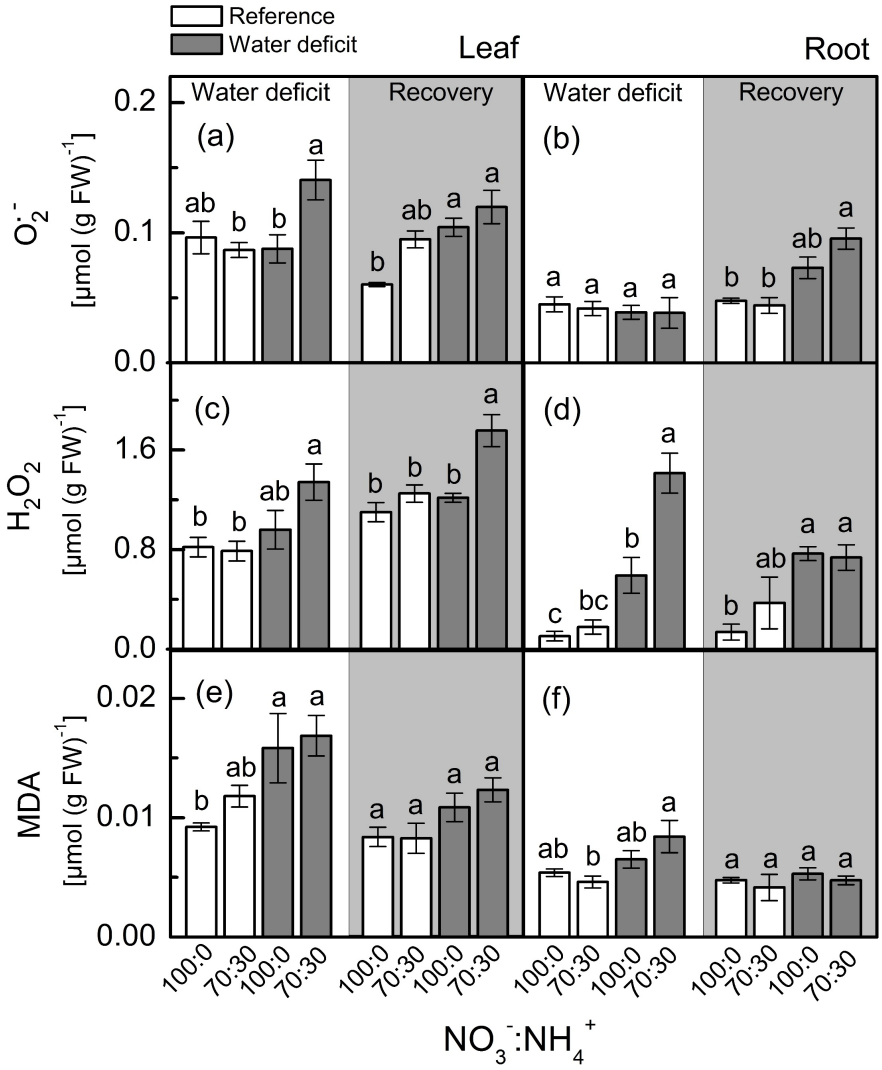

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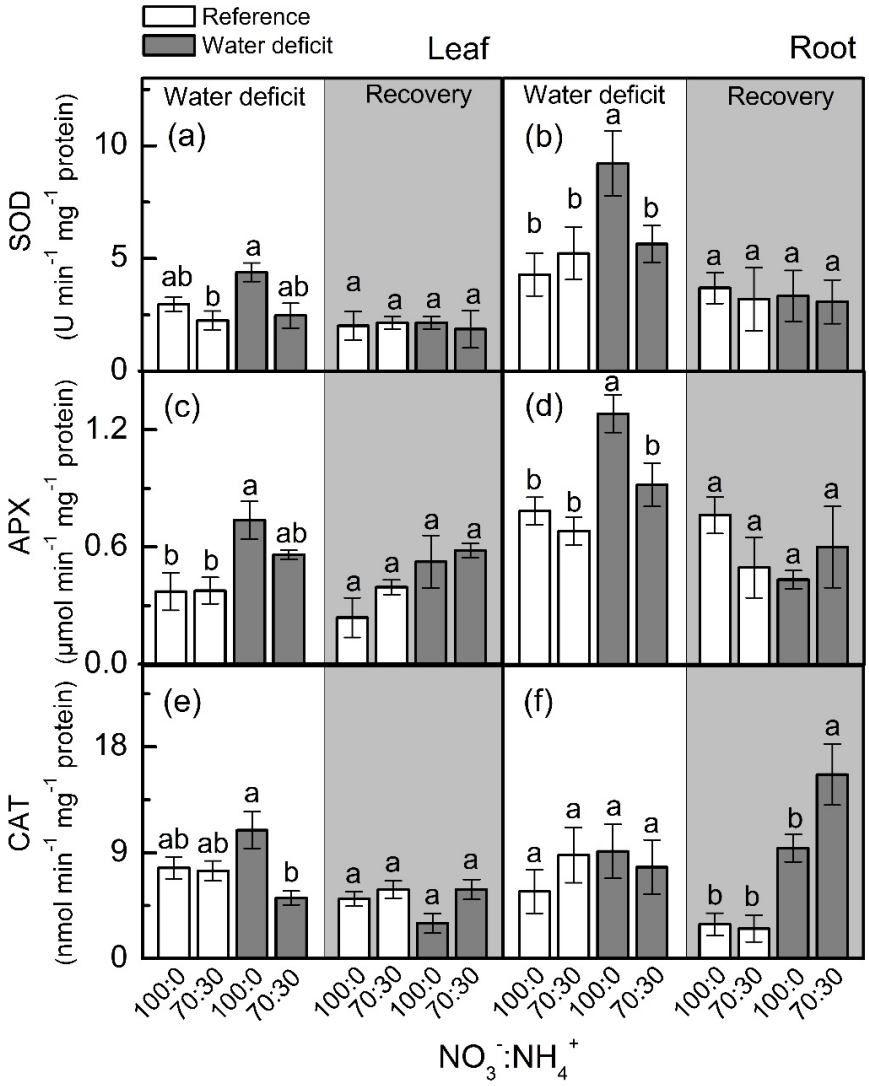

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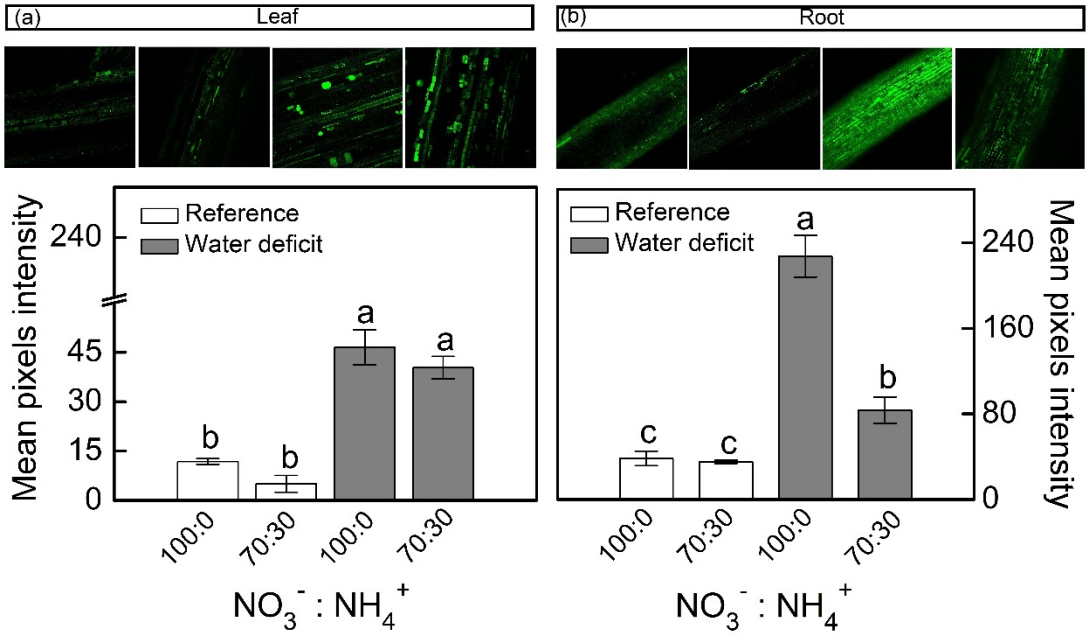

Fig. 7

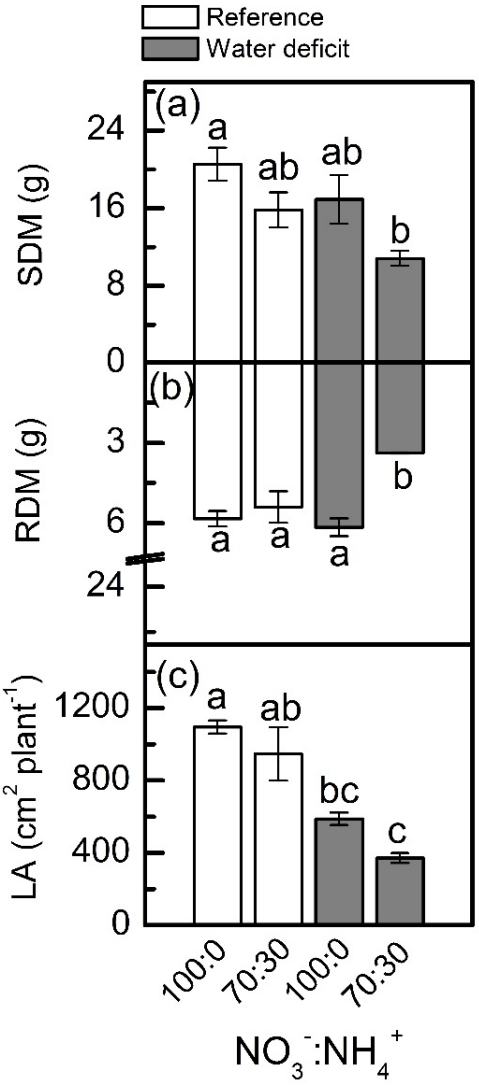

Fig. 8

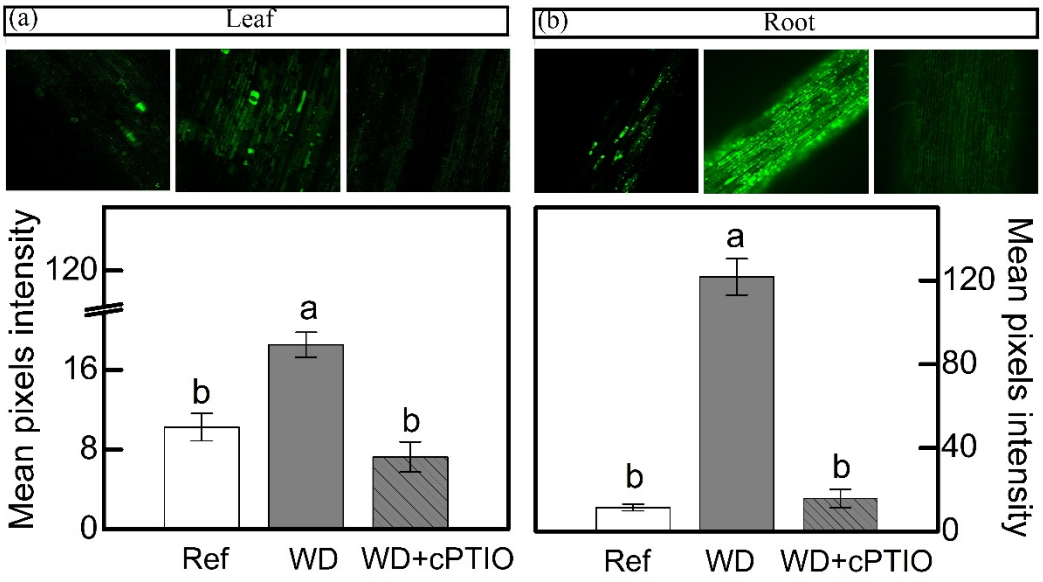

Fig. 9