Nitric oxide increases tolerance responses to moderate water deficit in leaves of *Phaseolus vulgaris* and *Vigna unguiculata* bean species

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Abstract Drought stress is one of the most intensively studied and widespread constraints, and nitric oxide (NO) is a key signaling molecule involved in the mediation of abiotic stresses in plants. We demonstrated that a sprayed solution of NO from donor sodium nitroprusside increased drought stress tolerance responses in both sensitive (*Phaseolus vulgaris*) and tolerant (*Vigna unguiculata*) beans. In intact plants subjected to halting irrigation, NO increased the leaf relative water content and stomatal conductance in both species. After cutting leaf discs and washing them, NO induced increased electrolyte leakage, which was more evident in the tolerant species. These leaf discs were then subjected to different water deficits, simulating moderate and severe drought stress conditions through polyethylene glycol solutions. NO supplied at moderate drought stress revealed a reduced membrane injury index in sensitive species. In hydrated discs and at this level of water deficit, NO increased the electron transport rate in both species, and a reduction of these rates was observed at severe stress levels. Taken together, it can be shown that NO has an effective role in ameliorating drought stress effects, activating tolerance responses at moderate water deficit levels and in both bean species which present differential drought tolerance.

Keywords PEG · Bean cultivars · Hydric deficit · Chlorophyll fluorescence · Leaf discs

Introduction

Nitric oxide (NO) is a labile-free radical derived from L-arginine through NO synthase (NOS) and identified as a mediator of a series of physiological functions in animal and plant cells, although the occurrence of NOS in plants is still uncertain (Hancock 2012). NO has also been considered to be toxic due to its capacity for reacting with redox centers of proteins and membranes (Wink et al. 1993). While some authors consider NO to be a stress-inducing agent, others claim it has a protective role acting mainly in the interruption of free radical chains formed under oxidative stresses (Lazalt et al. 1997; Beligni and Lamattina 1999a). However, further studies described it as a molecular component of different signal transduction pathways, depending on its origin and intercellular concentration (Beligni and Lamattina 2001; Mur et al. 2012).

NO activity and accumulation has been reported in different plant species and environmental stress conditions (Magalhaes et al. 2000; Marciano et al. 2010; Huang et al. 2013). Reduced water availability is the most limiting factor to plant growth and development. This constraint is responsible for a series of physiological responses in plants which include early reduced stomatal conductance (Gs) and loss of turgor, photochemical inhibition and a reduction of photosynthesis itself, events which have been frequently described as being associated with tolerance mechanism responses (Hsiao and Acevedo 1974; Feng et al. 2006; França et al. 2012). Stomatal conductance reduction is one of the systemic responses triggered by an increase in abscisic acid (ABA) in leaves from the roots

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(Okamoto et al. 2009). Several reports reveal NO as an inducing factor of stomatal closure when plants are subjected to drought stress (Garcia-Mata and Lamattina 2001; Neill et al. 2008; Huang et al. 2013). Other negative effects of this constraint are associated with oxidative damage. Under drought stress events, these types of damage have a tendency to increase, as the replacement of electrons of the acceptors in the photosystem II (PSII) center reaction is interrupted, and the energy dissipation of the photosynthetic apparatus becomes ineffective (Iturbe-Ormaetxe et al. 1998; Loggini et al. 1999).

Although there are few studies relating NO to drought tolerance responses, Garcia-Mata and Lamattina (2001) showed that exogenous applications of NO on detached leaves submitted to short periods of drought was responsible for maintaining higher water contents when compared to leaves that did not receive this treatment. Also, Magalhaes et al. (2000), through fluorescence microscopy techniques, observed a higher NO concentration in guard cells as well as reduced NO emissions in plants submitted to drought stress.

In this study, we evaluated the effects of NO in the physiological responses of plants to moderate and severe experimental drought stress conditions. We hypothesized that NO could have a protective effect on membrane integrity, photosynthetic yield, relative water content and stomatal conductance in intact and detached leaves. To verify this hypothesis, we analyzed these physiological responses in *Phaseolus vulgaris* and *Vigna unguiculata* cultivars, respectively indicated to be sensitive and tolerant to drought (Cruz de Carvalho et al. 1998). Plants and leaf tissues were then submitted respectively to drought stress and different water deficit conditions with exogenous NO applications using sodium nitroprusside as a chemical donor of NO.

Materials and methods

Plant growth conditions and NO treatments

Seeds of *Phaseolus vulgaris* and *Vigna unguiculata* donated by EMBRAPA (National Centers for Research into Rice and Beans - CNPAF and for Northern Region - CNPMN, respectively), were germinated in sand vermiculite mixtures 1 : 1 (v/v) under greenhouse conditions. After 4 days, three plants were cultivated in 1 L pots and were watered daily with Hoagland nutritive solution until emergence of the third pair of leaves (25 days). Afterwards, plants were divided into 3 groups with 4 repetitions each and received the following treatments for next 10 days: (1) daily irrigated plants – control; (2) plants that were not irrigated for drought stress imposition which received spray with deionized water and (3) plants submitted to drought stress imposition which received spray

with NO. All treatments, plant irrigations, water or NO sprays were done momentarily during 9 days, in the evening of the previous day of measurement (19.00–20.00 h), until drought stress imposition. For the NO treatment, sodium nitroprusside (SNP) solution was used as a NO donor (10^{-4} M), and was sprayed at 1 h 30 m to 2 h after preparation. Plants were kept under $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ($\pm 100 \mu\text{mol m}^{-2} \text{s}^{-1}$) photosynthetic photon flux and the average temperature was 26 ± 3 °C with 65 ± 5 % air humidity.

Leaf water status and stomatal conductance

During imposed drought stress, leaf water status was measured daily through relative water content (RWC) in *V. unguiculata* and *P. vulgaris* cultivars. Leaf discs were taken and weighed (fresh weight, FW) and then maintained in deionized water and kept at 4 °C for at least 24 h before being weighed again (turgor weight, TW). Afterwards, they were dried at 70 °C for at least 24 h and weighed once again (dry weight, DW) for RWC obtained from $(FW - DW) / (TW - DW) \times 100$. Other intact plants were used for stomatal conductance (Gs) measurements, taken daily from the abaxial side of the third and fourth knots of mature leaves which had been subjected to the treatments. Values were measured with a diffusion porometer (AP4 ΔT Devices, Cambridge, U.K.), always in the morning (8 to 9 h) throughout the experimental period.

Membrane integrity and photosynthetic yield

In order to evaluate membrane injury index (MII) and photosynthetic yield, a protoplasmatic tolerance assay was performed in accordance with Faria et al. (2013). After germination and root emission, seeds were transferred to pots and watered whenever necessary, and supplied with a urea/phosphate (2 mM) solution at 20 and 35 days. Then, 17 leaf discs (1 cm in diameter) were obtained from mature leaves and weighed (fresh weight - FW). Discs were put into 50 mL Falcon tubes containing 20 mL of deionized water and initial electric conductivity (EC0) was measured every 15 min until 90 min. Previously, electrolyte leakage (EL) was analyzed to verify the best disc washing period and values calculated as $EL = (EC_x - EC_y) / FW$ where EC: electrical conductivity and x and y represent two subsequent measures, i.e. 15 and 0 respectively. The same experiment was performed with substitution of deionized water for 10^{-4} M SNP solution. The experiment was conducted at 20 °C with 4 repetitions.

After this, 17 leaf discs from 4 individual plants that were 35 days of age were cut and added to 50 mL Falcon tubes containing 20 mL of deionized water. Discs were then washed in a shaker at 100 rpm for 15 min (best washing period), the water drained and substituted with 20 mL of polyethylene glycol (PEG-6000) solutions at water potentials (Ψ) -0.6 and

-1.2 for *P. vulgaris*, and -0.9 and -1.8 MPa for *V. Unguiculata*, according to values obtained from Money (1989). For this, besides pulverizing plants with SNP 15 min prior to cutting leaf discs, SNP was also added to PEG-6000 solutions. Leaf discs were then placed in the dark for 15 h (Bajji et al. 2001), removed from the PEG-6000, washed for 20 s and placed in 6 mL of deionized water for rehydration. Electrical conductivity (EC) was measured at this point (EC_i), and after 22.5 h of rehydration (EC_f). Afterwards, discs were autoclaved for 30 min, cooled to room temperature and EC measured (EC_t) to calculate MII as: $[(EC_f - EC_i) / (EC_t - EC_i)] \times 100$.

Evaluations of photosynthetic efficiency were made with a portable chlorophyll fluorometer (MINI PAM, Walz, Effeltrich, Germany) from detached segments of the plants subjected to the treatments. Mature leaves, were pulverized daily with SNP solution for 35 days. Segments (1.5×2.5 cm) were taken, placed in the dark for 15 h in Petri dishes with 20 mL of PEG-6000 with or without SNP at 0, -0.6 and -1.2 MPa for *P. vulgaris* and 0, -0.9 and -1.8 MPa for *V. unguiculata*. After this period, they were washed and placed in 20 mL of deionized water for 3 h for rehydration. They were then put in the presence of light for another hour and the apparent electron transport rate (ETR) was obtained in accordance with Genty et al. (1987). For this, increasing light pulses of photosynthetic photon flux (PPF) were divided into eight steps of 30 s each and at the end of each step, a saturating light pulse was applied. The ETR was evaluated as $ETR = (\Delta F/F_m') \times 0.5 \times PPF \times 0.84$, where $\Delta F/F_m'$ was calculated as $(F_m' - F)/F_m'$. F was the fluorescence of light adapted samples, F_m' the maximum fluorescence of light adapted samples obtained after application of a superimposed saturating pulse; 0.5 was used as the fraction of excitation energy distributed to PSII, and 0.84 was used as the fractional light absorbance.

Statistical analyzes

Statistical differences between measurements on different treatments or on different times were analyzed by ANOVA and Duncan's test or Student's *t*-test at 5 % probability.

Results

As a result of the experimental study period, plants suffered severe drought stress in both physiological conditions, by withholding irrigation in intact plants and also in those respective plant tissues *in vitro*. In both cases, a differential drought tolerance was observed between *Phaseolus vulgaris* and *Vigna unguiculata* cultivars. Likewise, plants of *P. vulgaris* which had not received exogenous application of SNP died earlier than *V. unguiculata* plants which had also not received the applications (see below).

Leaf water status and stomatal conductance

In *P. vulgaris* and *V. unguiculata* cultivars, the water status observed from RWC of plants which had received SNP was slightly greater than plants which had not received SNP. Values were found to be around 5 to 10 % greater throughout the experimental period (Fig. 1). In *P. vulgaris* plants, the RWC values decreased earlier than in *V. unguiculata* plants, in agreement with imposed water deficit. In these plants during the evaluation period, stomatal conductance (G_s) values began decreasing on the 2nd day after halting irrigation and continued until death of the individuals (Fig. 2). Plants of *P. vulgaris* and *V. unguiculata* which had not received SNP spray presented undetectable G_s values (physiological death) on the 6th and 8th days, respectively. In plants that had received SNP spray, G_s values were undetectable on the 9th day of the experimental period for both species. The mean G_s values for *P. vulgaris* and *V. unguiculata* also differed: 580 and 620 $\text{mmol m}^{-2} \text{s}^{-1}$ for *V. unguiculata* with and without SNP

Fig. 1 Relative water content (RWC) values measured in *V. unguiculata* and *P. vulgaris* cultivars in hydrated plants and plants subjected to drought stress conditions through halted irrigation, with or without NO-donor SNP sprayed on leaves. Values represent means of 3 repetitions $\pm$ SD. Asterisks (*) indicate significant difference ($P < 0.05$)

Fig. 2 Stomatal conductance (Gs) values measured in *V. unguiculata* and *P. vulgaris* cultivars in hydrated plants and plants subjected to drought stress conditions through halted irrigation, with or without NO-donor SNP sprayed on leaves. Values represent means of 4 repetitions $\pm$ SD. Asterisks (*) indicate significant difference ($P < 0.05$)

respectively; 490 and 580 $\text{mmol m}^{-2} \text{s}^{-1}$ for *P. vulgaris* with and without SNP respectively.

Membrane integrity and photosynthetic yield

Electrolyte leakage (EL) was higher during the first 15 min of washing for both species' cultivars. SNP caused no significant differences in treatments for *P. vulgaris* but slightly raised EL in *V. unguiculata* experiments (Fig. 3). Absolute values were always greater for *V. unguiculata* with or without SNP, probably due to the greater leaf thickness of this species and consequently higher superficial contact area in discs. After 30 min of washing, EL remained relatively constant and the lowest values occurred in the last two intervals (60–75 and 75–90 min).

Membrane injury index was proportional to the PEG-6000 concentrations used, reaching values around 50 % in *P. vulgaris* at -1.2 MPa. *V. unguiculata* suffered around 38 % MII at the more reduced water potential, -1.8 MPa (Fig. 4). However, a protective effect of SNP was observed in *P. vulgaris* at -0.6 MPa, where MII was significantly lower than the value measured in plants that had not received SNP

Fig. 3 Electrolyte leakage (EL) measured in *V. unguiculata* and *P. vulgaris* cultivars as a consequence of leaf disc samples washing periods. The species samples were either immersed with or without NO-donor SNP solution. Values obtained of 17 leaf discs from 4 individual plants. Asterisks (*) indicate significant difference ($P < 0.05$)

(10 % compared to 15 %). There were no significant differences between treatments for *V. unguiculata* in both PEG-6000 concentrations or for *P. vulgaris* at -1.2 MPa. In detached leaf segments submitted to different water potentials with PEG-6000, depending on SNP availability, the electron transport rate (ETR) values were elevated when compared to plants that did not receive the application. The ETR values were higher in the plants without water deficit and at -0.6 MPa for *P. vulgaris* (values increased from 70–80 to 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively – Fig. 5) and without water deficit and at -0.9 MPa for *V. unguiculata* (values increased from 80–90 to 140 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively – Fig. 5). However, SNP reduced ETR values at water potentials of -1.2 MPa for *P. vulgaris* (≈ 25 %) and -1.8 MPa for *V. unguiculata* (≈ 30 %).

Discussion

In this work, we demonstrate that a solution of SNP donor NO, when sprayed exogenously on intact plants or when

Fig. 4 Membrane injury index (MII) as a consequence of water deficit conditions simulated using PEG-6000 solutions, measured in *P. vulgaris* and *V. unguiculata* cultivars with or without NO-donor SNP solution. Values obtained of 17 leaf discs from 4 individual plants. Different letters indicate statistical differences and error bar in SD ($P < 0.05$)

detached leaves were immersed in this solution, participates by conferring adaptive plant responses against induced drought stress as previously demonstrated by Garcia-Mata and Lamattina (2001), and more recently Mur et al. (2012) described NO as being involved in drought stress responses. During water restriction, an observed decrease in stomatal conductance (G_s) values is a common trait related to the reduction of stomatal aperture in plants (França et al. 2000; 2012) and a gradual induction of stomatal closure is achieved from the moment leaves sense water deficit. Yet, abscisic acid produced in roots and translocated to the upper parts of the plant can stimulate an increase in cytosolic Ca^{2+} concentrations in guard cells (Leckie et al. 1998), promoting this physiological effect. As discussed by Neill et al. (2008), NO does appear to have ameliorative benefits in warding off the effects of drought, indicating that is possible that NO effect in plants submitted to drought stress differ between inducing the opening and/or stomatal closure conductance depending on its concentration and the physiological state of the guard cell. For example, Sakihama et al. (2003) reported that NO donors actually caused stomatal opening when used at high concentrations. Contrastingly, Garcia-Mata and Lamattina (2001) reported that prolonged SNP treatment provided plants with such beneficial long-term effects and observed stomatal closure inhibition in fava bean (*Vicia faba*) in the presence of cPTIO (a NO chelator). Also, Ribeiro et al. (2009)

demonstrated a differential requirement for NO during stomatal closure in turgid and wilted leaves. Thus, these reports corroborate and indicate the effective participation of endogenous NO in stomatal movement.

The stomatal aperture and closure control mechanisms are the result of complex interactions between hormones and transduction signal pathways triggered by mechanical and biochemical stimuli as indicated by Mott and Buckley (2000) and Lawson and Blatt (2014). As there is some belief that stomatal movement occurs in blocks dependent on their localization, NO, with its high solubility in membranes, could have a fundamental role in the propagation of this stimulus (Garcia-Mata and Lamattina 2001), explaining the higher G_s values observed in *V. unguiculata* and *P. vulgaris* plants treated with NO. It is important consider that the higher G_s values found could also be in response to higher RWC values, since the values were slightly greater during halted irrigation and consequent drought stress. Additionally wheat plants watered with SNP and then exposed to water stress had higher RWC than those not receiving the SNP pre-treatment. Presumably, the long-term exposure to NO in some way enables plants to retain more water during subsequent dry periods (Neill et al. 2008). In this way, in intact plants and those subjected to water restriction, SNP supply induced slightly superior RWC values for both bean species studied here throughout the experimental period.

In respect to cut leaf discs and electrolytes release, the majority flux of the ions released in the first 15 min of the leaf disc washing period can be attributed to electrolytes adhering to the cellular surface and those present in cells, vessels and apoplast damaged in the process (Borochoy-Neori and Borochoy 1991). A possible explanation for the slightly higher EL in NO treated plants is based on the immediate interactions between NO and Ca^{2+} concentrations via cGMP, cADPR and IP3 (Volk et al. 1997; Berkels et al. 2000). The higher Ca^{2+} contents would modulate K^+ influx which would then be released in the first washing periods (Garcia-Mata et al. 2003). When analyzing membrane injury index (MII) in *P. vulgaris* at -0.9 MPa, the protective effect of NO observed was remarkable. It is well known that drought stress (via oxidative damages) affects the physical-chemical structure of membranes, leading to destabilizations in fluidity and selective permeability (Bajji et al. 2001). The best known supporting evidence for this hypothesis is the active accumulation of non-reductive sugars such as threulose and saccharose, which would help maintain membrane stability by associating with phospholipid groups (Nilsen and Orcutt 1996). We speculate that NO could have a part in this active accumulation by stimulating tolerance genes via cGMP (Berkels et al. 2000). The absence of this fact in *V. unguiculata* could be explained by (1) different interactions between NO and specific transporters involved in ion translocation via membranes (Bajji et al. 2001), and (2) different gene expressions between the

Fig. 5 Electron transport rate (ETR) as a consequence of photosynthetic active radiation (PAR) intensity on leaf discs subjected to water deficit conditions simulated using PEG-6000 solutions, measured in *V. unguiculata* and *P. vulgaris* cultivars with or without NO-donor SNP solution

two species. Contrastingly, it is reasonable to consider that under severe water deficit conditions, oxidative damage to membranes would be so high that tolerance mechanisms become ineffective.

Regarding photosynthetic evaluations from chlorophyll fluorescence, NO was also responsible for an increase in electron transport rates (ETR) in hydrated leaf discs and in those submitted to moderate drought stress (control and -0.6 MPa for *P. vulgaris* and control and -0.9 MPa for *V. unguiculata*). This result is evidence of an attenuating role presented by NO, which could be absorbing small amounts of reactive oxygen species (ROS) formed under oxidative stress (Beligni and Lamattina 1999b). Under drought stress conditions, ROS formation is frequently observed when the photosynthetic apparatus becomes damaged. ROS formed could then cause oxidative damage such as chlorosis, DNA fragmentation and apoptosis (Lazalt et al. 1997; Beligni and Lamattina 1999a, 1999b). The decrease of ETR values observed under severe drought conditions (-1.2 MPa for

P. vulgaris and -1.8 MPa for *V. unguiculata*) suggests the limitations of NO amelioration of this constraint. In this case, ROS concentration is so elevated that a reversal occurs and NO becomes hazardous, increasing oxidative damage.

Even though we presented a positive insight into interactions between NO and drought stress, it is interesting to investigate similar responses in other plants and also the NO levels that positively induces drought stress responses amelioration and NO time exposure. Additionally and more investigation is required for better comprehension the pathways involved in NO interaction in plant cells, as well as its effects on signal transduction, specific ion channels and even gene translation in response to this stress. At intermediate drought stress, the fact that the same response was found cultivars studied in both the sensitive and tolerant cultivars suggests that NO has a mediating role against water deficit. Thus, our results indicated that NO, depending on water deficit intensity, could have an important ameliorating effect in response to this constraint in *P. vulgaris* and *V. unguiculata* cultivars.

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