Nitric oxide production is involved in maintaining energy state in Alfalfa (Medicago sativa L.) nodulated roots under both salinity and flooding

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Nitric oxide production is involved in maintaining energy state in Alfalfa (*Medicago sativa L.*) nodulated roots under both salinity and flooding

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Abstract

Main conclusion **In *Medicago sativa* nodulated roots, NR-dependent NO production is involved in maintaining energy state, presumably through phytoglobin NO respiration, under both salinity and hypoxia stress.**

Abstract The response to low and average salinity stress and to a 5 day-long flooding period was analyzed in *M. sativa* nodulated roots. The two treatments result in a decrease in the biological nitrogen fixation capacity and the energy state (evaluated by the ATP/ADP ratio), and conversely in an increase nitric oxide (NO) production. Under salinity and hypoxia treatments, the use of either sodium tungstate, an inhibitor of nitrate reductase (NR), or carboxy-PTIO, a NO scavenger, results in a decrease in NO production and ATP/ADP ratio, meaning that NR-dependent NO production participates to the maintenance of the nodulated roots energy state.

Keywords Hypoxia · Legume · Nitrogen-fixing symbiosis · Phytoglobin NO respiration · Salt stress

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Abbreviations

ARA	Acetylene reducing activity
BNF	Biological nitrogen fixation
cPTIO	2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF-2	4,5-diaminofluorescein
NR	Nitrate reductase
Phytogb	Phytoglobin

Introduction

Nitric oxide (NO) is a reactive gaseous molecule with a broad spectrum of regulatory functions in plant growth and development (Besson-Bard et al. 2008), and response to biotic (Hichri et al. 2015; Thalineau et al. 2016) and abiotic stresses, including salinity and hypoxia (Blokhina and Fagerstedt 2010; Simontacchi et al. 2015). Under salt stress, enhancement of NO production is accompanied by the exclusion of Na^+ and retention of K^+, through increased membrane H^+-ATPase and H^+-PPase activities (Zhang et al. 2006; Wang et al. 2009; Zhao et al. 2018). NO induces the expression of genes related to osmotic adjustment, heat-shock protein, and ROS-scavenging processes (Uchida et al. 2002; Kopyra and Gwózdz 2003). It contributes to the

maintenance of cellular redox homeostasis by controlling the NADPH level (Liu et al. 2007) and by inducing antioxidant enzyme activities (Tanou et al. 2009). Overall speaking, NO protects plants by helping them to control water status, maintain ionic homeostasis and reduce oxidative damage imposed during salt stress (Molassiotis et al. 2010; Simontacchi et al. 2015).

On the other hand, increased NO production is a hallmark of plant response to flooding and hypoxia (Blokhina and Fagerstedt 2010; Gupta and Igamberdiev 2011). Hypoxia compromises mitochondrial respiration and leads to an insufficiency in ATP for energy-demanding processes (Bailey-Serres and Voesenek 2008). In these conditions, NO contributes to the recycling of NADH and the synthesis of ATP through the setting up of an alternative respiration called "phytoglobin (Phytogb)-NO respiration" (Stoimenova et al. 2007; Igamberdiev and Hill 2009; Gupta and Igamberdiev 2011). NO is also essential for the development of lysigenous aerenchyma that enhance O_2 diffusion along with the roots (Wany et al. 2017). Furthermore, the signaling role of NO in the perception and the response to hypoxia was recently elucidated in *Arabidopsis thaliana* (Hartman et al. 2019). An increase in NO production, therefore, appears to be a common response to both salt stress and hypoxia.

Exposure of nitrogen-fixing legumes to high salt concentrations resulted in a rapid decrease in nitrogenase activity and nodule respiration, and this decrease was compensated by raising pO_2 (Serraj et al. 1994; Serraj and Drevon 1998). Similarly, by comparing nodule respiration of various legumes under NaCl treatments, Delgado et al. (1994) concluded that under low salinity the supply of O_2 to nodules is the limiting factor of the biological nitrogen fixation (BNF). These observations suggest that under salt stress, the nodulated roots of legumes could also face hypoxia and that this could limit BNF.

To test this hypothesis, using the *Medicago sativa* legume model, we analyzed BNF, NO production and energy state in the nodulated roots of plants grown in the presence of low to average salinity and subjected to short-term flooding. Our data show that, under moderate salt stress as under flooding, a nitrate reductase (NR)-dependent NO production is induced which participates to the maintenance of the energy state of the root system.

Materials and methods

Biological material and growth conditions

Seeds of *Medicago sativa* (var. Siriver; obtained from Espave Vert Tunesie, Tunis, Tunesia) were sterilized and germinated as previously described (del Giudice et al. 2011). Five-day-old germinated seeds were transplanted in pots (2 plants/pot) filled with 250 cm^3 of B5 sand (0.6–1.6 mm diameter). Plants growth conditions and nutrient solution are described in Sghaier et al. (2020). To set salinity conditions, nutrient solution also contained either 0.2 mM KNO_3 (Control, Ctrl), 0.2 mM KNO_3 and 20 mM NaCl (Treatment 20, T20), or 0.9 mM KNO_3 and 50 mM NaCl (Treatment 50, T50). Plants were inoculated 7 days after transplanting with *Ensifer meliloti* 2011 bacteria (Sghaier et al. 2020).

In the first set of experiments (experiment 1), at 28 days post-inoculation (dpi), half of the Ctrl, T20 and T50 plants were subjected to flooding for 5 days by submerging pots in their respective nutrient solutions to sand level (Fig. S1a). Non-flooded plants were watered with nutrient solutions at 28 and 31 dpi (Fig. S1b). At 32 dpi, nodulated roots were harvested and either used for growth parameters analysis (9 plants/assay), N_2-fixing capacity (2 plants/assay), NO production (2 plants/assay), or immediately frozen in liquid N_2, ground in powder and stored at − 80 °C until analysis of protein and adenine nucleotide contents (2 plants/assay).

In the second set of experiments (experiment 2), at 28 dpi, the plants were transferred either into syringes filled with 35 ml nutrient solution and traversed by a humidified airflow (normoxia, Fig. S2a and b) or into glass tubes filled with 40 ml nutrient solution previously flushed with a mixture of 4.5–95.5%: O_2-N_2 (hypoxia, Fig. S2c and d). The plants (2 plants/syringe or tube) were cultured either in normoxia or hypoxia for 5 days. To compensate for the evaporation, syringes and tubes were supplemented daily with nutrient solution. One day before harvest, nutrient solutions were supplemented with either 0.1 mM 2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a scavenger of NO, or 1 mM sodium tungstate (Tg), an inhibitor of NR, or water (control). At 32 dpi, nodulated root was promptly excised, and used for the analysis of NO production (3 × 2 plants/assay), or immediately frozen in liquid N_2, ground in powder and stored at − 80 °C until adenine nucleotide measurements (3 × 2 plants/assay).

Vegetative growth analysis

Root systems were washed in distilled water, blotted on filter paper and weighted. Dry weights were measured after drying of samples for 48 h at 70 °C.

Biological nitrogen-fixing capacity

BNF capacity of nodulated roots was determined in vivo by measuring the acetylene reducing activity (ARA) as previously described (Pierre et al. 2014).

Measurement of NO production

Segments (2 cm-long) of nodulated roots were incubated in the dark, at 23 °C, in 5 mL tubes containing 2 mL of detection medium (10 mM Tris–HCl pH 7.5, 10 mM KCl) in the presence of 10 µM 4,5-diaminofluorescein (DAF-2, Coger, with excitation at 495 nm and emission at 515 nm) fluorescent probe. NO production and controls were carried out as in Horchani et al. (2011). In addition, to test the specificity of the DAF-2 probe, NO production was also analysed with the Cu(II) fluorescein (CuFL) fluorescent probe (Strem Chemicals, with excitation at 495 nm and emission at 515 nm), which is known to react rapidly and specifically with NO itself (Lim et al. 2006).

Extraction and measurement of adenine nucleotides and proteins

Adenine nucleotides were extracted essentially as in Horchani et al. (2011). All extraction steps were carried out at 4 °C. Frozen material (40–60 mg) was crushed in liquid nitrogen with 300 mL of perchloric acid solution, containing 7% (v/v) $HClO_4$ and 25 mM Na_2EDTA, with a mortar and pestle. After thawing, the extract was taken and the mortar was rinsed with 200 mL of perchloric acid solution, which was then pooled with the extract. The sample was centrifuged for 5 min at 13,000*g*. The supernatant was quickly and carefully neutralized at pH 5.6–6.0 using a 2 M KOH–0.3 M MOPS solution. $KClO_4$ precipitate was discarded by centrifugation (5 min, 13,000*g*). Adenine nucleotides of the supernatant were measured in a Xenius spectrofluorimeter-luminometer (Safas, Monaco) using the ATPlite one-step assay system (ATPLT1STP-0509; Perkin-Elmer) according to the manufacturer's instructions. Proteins were extracted and quantified on clarified extracts as in Horchani et al. (2010).

Results

Salinity and flooding affect nodulated roots nitrogen-fixing capacity and energy state, and increase NO production

Salt and flooding treated *M. sativa* roots were first analysed for their biomass and nodule production. As reported in Table S1, root system dry weight is not affected by the two treatments. However, T20 and T50 salt treatments decrease by 38 and 52%, respectively, the number of nodules per plant, indicating that salt stress affects nodulation. On its side, flooding treatment has no effect on the number of nodules per plant. It can be noted that, regardless of the treatment, the protein content of the root system remains unchanged (Fig. S3), suggesting that, as a whole, the root system is not senescent and is still functional.

The effects of salinity and flooding on *M. sativa* BNF capacity were assessed by measuring the ARA of nodules (Fig. 1a). As compared with control nodules in normoxia, T20 and T50 treatments result in an 18 and 75% decrease in ARA, respectively. When compared to normoxia, flooding also results in a 33 and 52% decrease in ARA for control and T20 nodules, respectively, but is without additional effect for T50 nodules.

To test the effects of salinity and flooding on the energy state, we analyzed the ATP/ADP ratio (experiment 1). ATP/ADP ratio of control roots is 7.4 (Fig. 1b), which is in agreement with previous analysis in either roots or nodules (Brouquisse et al. 1991; Horchani et al. 2011). ATP/ADP ratio is not modified in T20 (7.0), but significantly decreased in T50 roots (6.5), indicating that average but not low salt stress impairs the energy status of the nodulated roots. After a 5-day flooding period, ATP/ADP ratios decrease close to 5.0, 4.5 and 4.2 in Ctrl, T20 and T50 treated roots, respectively (Fig. 1b). These results indicate that the saline and flooding stresses, independently of each other, affect the energy state of the nodulated roots and that their combined effect is partly cumulative.

In roots and nodules under hypoxia, NO production has been associated with the establishment of a Phytogb-NO respiration, whose function is to maintain the energy state of the tissues when O_2 concentration decreases (Igamberdiev and Hill 2009; Horchani et al. 2011). We checked whether NO production could be involved in the maintenance of the root system energy state under each stress. As compared with control, T20 and T50 treatments caused a 1.8 and 3.8-fold increase in NO production (Fig. 1c). Similarly, flooding induced a 1.3 to 2.7-fold increase in NO production compared to normoxic conditions. As a control, NO production was also measured with Cu(II) fluorescein probe with similar results (Fig. S3), indicating that measurements with DAF-2 are reliable and relevant in this context. Thus, NO production was increased by both saline and flooding treatments, suggesting (1) that the effects of salinity and flooding on NO production and energy state could result from hypoxic environment, and (2) that Phytogb-NO respiration could be activated in response to both saline and flooding/hypoxia treatments.

NO production contributes to the maintenance of energy state under both salinity and hypoxia

To test the above hypothesis, we analyzed the effects of cPTIO (a NO scavenger) and Tg (a NR and Phytogb-NO respiration inhibitor) on ATP/ADP ratio and NO production in the root system of control (0) and T50 (50) treated plants submitted (H) or not (N) to hypoxia. To this end, we

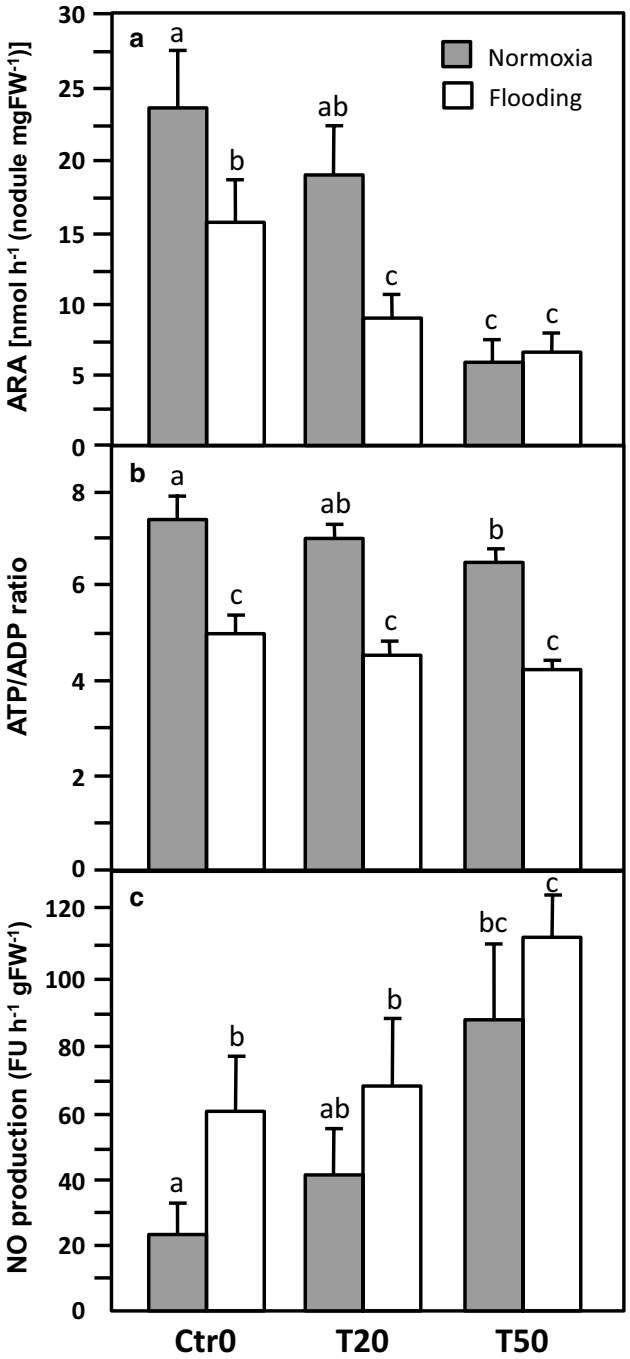

Fig. 1 **a** Nitrogen-fixing activity, **b** energy state and **c** NO production of *Medicago sativa* nodulated roots submitted to saline and hypoxic treatments. T20 and T50 refer to 20 and 50 mM NaCl treatments, respectively. Nitrogen-fixing activity is measured through acethylene reducing activity (ARA). Energy state is evaluated through ATP/ADP ratio. The values are mean ± SE ($n = 3$ for ATP/ADP ratio; $n = 4$ for ARA and NO production) of 4 independent experiments. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Tukey test ($P < 0.05$)

used a specific hydroponic experimental setup where the root system was incubated in nutrient solution supplemented or not with the inhibitors (experiment 2, Fig. S2). Compared to the control (0-N), NR inhibition by Tg results in a 43, 33 and 41% decrease of ATP/ADP ratio in 0-H, 50-N and 50-H roots, respectively (Table 1). Similarly, NO scavenging by cPTIO results in a 34, 37 and 37% decrease of ATP/ADP ratio in 0-H, 50-N, and 50-H roots, respectively, compared to control conditions. cPTIO and Tg has no significant effect on the ATP/ADP ratio in 0-N roots. Treatment with Tg triggers a strong decrease in NO production in 0-H, 50-N and 50-H treated root systems, but not in 0-N treated root system (Table 1). In the presence of cPTIO, NO production is totally abolished. Taken together, these data indicate that both the functioning of NR and the production of NO are necessary to maintain root and nodule energy state under salinity and hypoxia, alone or combined together.

Discussion

The present data show that nodulation, but not root growth, of *M. sativa* plants is affected by average salinity (Table S1). This is in line with literature data reporting that alfalfa is a moderately salt-tolerant legume (Bruning and Rozema 2013) and that its growth is significantly affected only from NaCl concentrations above 80 mM (Rogers et al. 2008, 2009, 2011). However, salinity is known to inhibit BNF more rapidly than growth in legumes (Bruning and Rozema 2013). In our study, ARA is the more inhibited as the salt concentration increases (Fig. 1). Under low salt stress, the supply of O_2 to nodules was suggested to be the limiting factor for BNF (Bergersen 1982; Delgado et al. 1994; Serraj et al. 1994). This inhibition would be due to the fact that the salt stress disrupts the symplastic connections between nodule cells, decreases the permeability of the nodules to O_2 and increases its critical O_2 pressure, which would have the effect of inhibiting the energy metabolism and the regeneration of ATP. These interpretations were supported by Serraj et al. (1998) and Serraj and Drevon (1998) who showed that the BNF of common bean, soybean and alfalfa submitted to 50–100 mM NaCl concentrations is partly inhibited compared to that of control plants when measured at 21% O_2, but that the inhibition is reversed by increasing pO_2. Considered together, these studies suggest that the decrease in ARA under salt stress is due to an O_2 limitation within the nodules leading to a reduction in energy metabolism.

Short-term effects of flooding on BNF have been little studied. However, in *Trifolium repens* (Pugh et al. 1995) and soybean (Sánchez et al. 2010) nodulated roots, a short period of flooding (6–7 days) resulted in a complete or half inhibition of ARA, respectively. Similarly, our data show that in *M. sativa* nodulated roots, ARA is inhibited after

Table 1 Effect of Tg and cPTIO on energy state (ATP/ADP ratio) and NO production in *Medicago sativa* nodulated roots submitted to saline and hypoxic treatments (Experiment 2)

	0-N			0-H			50-N			50-H		
	Ctr	Tg	cPTIO	Ctr	Tg	cPTIO	Ctr	Tg	cPTIO	Ctr	Tg	cPTIO
ATP/ADP ratio	6.2 ± 0.6 a	5.0 ± 0.7 ab	5.1 ± 0.6 ab	4.4 ± 0.4 b	2.5 ± 0.6 d	2.9 ± 0.3 cd	5.4 ± 0.8 ab	3.6 ± 0.9 bc	3.4 ± 0.1 c	4.1 ± 0.4 bc	2.4 ± 0.4 d	2.6 ± 0.4 d
NO production (FU h^{-1} gFW^{-1})	18.0 ± 9.7 a	11.5 ± 3.3 a	5.5 ± 2.2 b	54.1 ± 11.4 c	18.4 ± 6.1 a	4.8 ± 3.0 b	91.8 ± 27.5 d	19.7 ± 12.3 ab	5.2 ± 2.5 b	88.5 ± 16.4 d	12.8 ± 10.6 ab	5.0 ± 2.2 b

Plants were grown in the absence (0) or in the presence of 50 mM NaCl (50) and submitted to either normoxia (N) or hypoxia (H) as indicated in the Materials and methods. Ctr, control; Tg, 1 mM sodium tungstate; cPTIO, 0.1 mM cPTIO. Data are mean ± SE ($n = 3$) of 3 independent experiments. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Tukey test ($P < 0.05$)

5 days of flooding (Fig. 1), confirming that flooding inhibits ARA presumably through flooding-associated O_2 shortage.

In the root system of many plant species, including legumes, submitted to flooding or hypoxia, the induction of a Phytogb-NO respiration occurs, allowing cell energy status retention (Dordas et al. 2003; Gupta and Igamberdiev 2011; Berger et al. 2018). The Phytogb-NO respiration cycle involves (1) the reduction of NO_3^- by NR (2) the translocation of NO_2^- from the cytosol to the mitochondrial matrix, (3) the reduction of NO_2^- to NO via the mitochondrial electron transfer chain (ETC) allowing ATP regeneration, and finally (4) the passive diffusion of NO to the cytosol where it is oxidized back to NO_3^- by Phytogb (Gupta and Igamberdiev 2011). Functional legume nodules are naturally characterized by a microoxic environment, and the existence of such a Phytogb-NO respiration was also evidenced in *M. truncatula* nodules (Horchani et al. 2011). In these nodules, a hypoxic treatment triggers an increase in NO production, which is abolished by the addition of either cPTIO or Tg, and the energy state (ATP/ADP) is significantly inhibited when nodules are incubated in the presence of Tg (Horchani et al. 2011). In our study, under either salt or hypoxic treatment, or both, the use of Tg and cPTIO leads to a reduction of the ATP/ADP ratio and to an inhibition of NO production (Table 1) indicating that both NR activity and NO production are necessary to maintain the energy state. Taken together our data first confirm that in *M. sativa* nodulated roots under flooding/hypoxia, NO production is involved in maintaining energy state presumably through the establishment of a Phytogb-NO respiration. Second, our study shows that NR-dependent NO production is also involved in maintaining energy state under salt stress, which argues that salt treatment triggers a hypoxic stress in the nodulated roots. Third, this study strongly suggests that the functioning of Phytogb-NO respiration is induced in nodulated roots under salt stress.

Author contributions statement RB and SA-S planned and designed the research. FA, HS, AG and RB performed experiments and conducted fieldwork. AK, SA-S and RB interpreted the data. RB wrote the manuscript.

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