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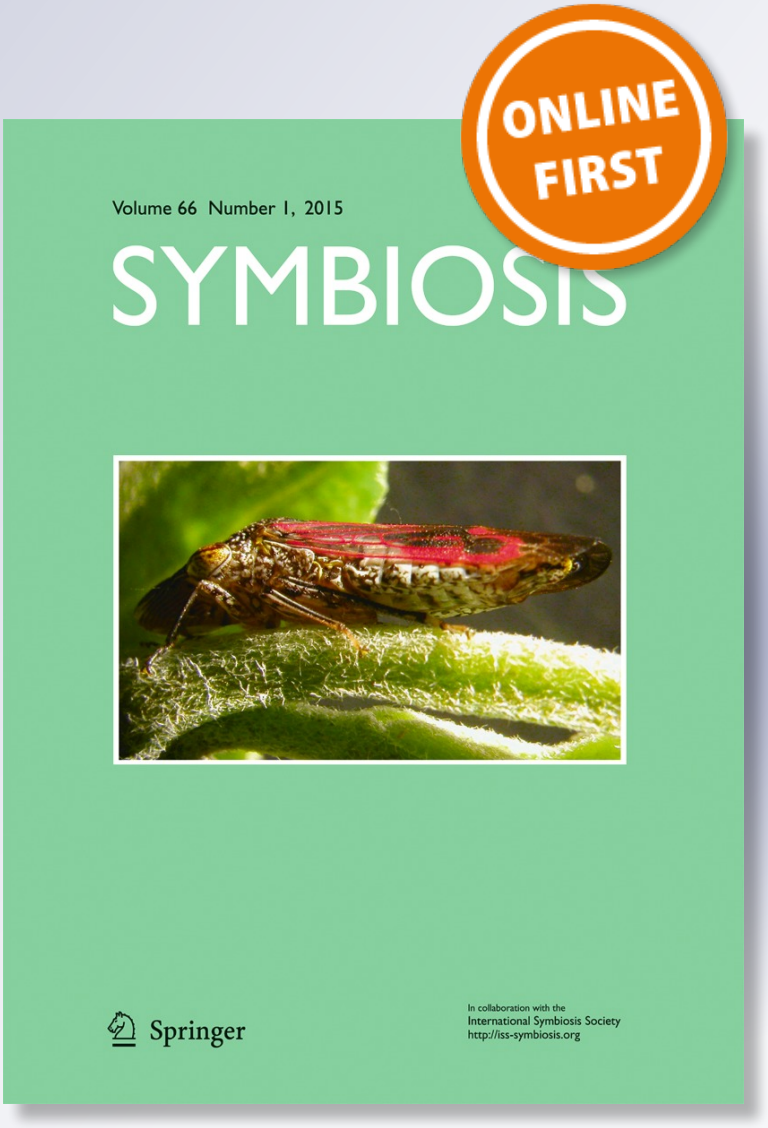

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Nitrate and flooding induce N_2O emissions from soybean nodules

Germán Tortosa[1] · **Alba Hidalgo**[1] · **Ana Salas**[1] · **Eulogio J. Bedmar**[1] · **Socorro Mesa**[1] · **María J. Delgado**[1]

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Abstract Nitrous oxide (N_2O) is one of the three main biogenic greenhouse gases (GHGs) and agriculture represents close to 30 % of the total N_2O net emissions. In agricultural soils, N_2O is emitted by two main microbial processes, nitrification and denitrification, both of which can convert synthetic nitrogen fertilizer into N_2O. Legume-rhizobia symbiosis could be an effective and environmental-friendly alternative to nitrogen fertilization and hence, to mitigate soil N_2O emissions. However, legume crops also contribute to N_2O emissions. A better understanding of the environmental factors involved in the emission of N_2O from nodules would be instrumental for mitigating the release of this GHG gas. In this work, in vivo N_2O emissions from nodulated soybean roots in response to nitrate (0, 1, 2 and 4 mM) and flooding have been measured. To investigate the contribution of rhizobial denitrification in N_2O emission from nodules, plants were inoculated with *B. japonicum* USDA110 and *napA* and *nosZ* denitrification mutants. The results showed that nitrate was essential for N_2O emissions and its concentration enhanced N_2O fluxes showing a statistical linear correlation, being the highest N_2O fluxes obtained with 4 mM nitrate. When inoculated plants grown with 4 mM nitrate were subjected to flooding, a 150- and 830-fold induction of N_2O emission rates from USDA110 and *nosZ* nodulated roots, respectively, was observed compared to non-flooded plants, especially during long-term flooding. Under these conditions, N_2O emissions from detached nodules produced by the *napA* mutant were significantly lower ($p<0.05$) than those produced by the wild-type strain (382 versus 1120 nmol N_2O h^{-1} g^{-1} NFW, respectively). In contrast, nodules from plants inoculated with the *nosZ* mutant accumulated statistically higher levels of N_2O compared to wild-type nodules (2522 versus nmol 1120 N_2O h^{-1} g^{-1} NFW, $p<0.05$). These results demonstrate that flooding is an important environmental factor for N_2O emissions from soybean nodules and that *B. japonicum* denitrification is involved in such emission.

Keywords *Bradyrhizobium japonicum* · Denitrification · Flooding · Nitrate · Nitrous oxide reductase · Soybean nodules

1 Introduction

Nowadays, mitigation of greenhouse gas (GHG) emissions is one of the main scientific and political concerns due to its relationship with global warming and climate change. According to IPCC Fifth Assessment Synthesis Report (IPCC 2014), agriculture, forestry and other land use (AFOLU) contribute to about 25 % of total direct GHG emissions, especially carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Close to 30 % of total N_2O net emissions came from anthropogenic activities like wastewater and aquaculture (0.25 Tg N_2O-N yr^{-1}), biomass burning (0.7 Tg N_2O-N yr^{-1}), industry and fossil fuel combustion (0.9 Tg N_2O-N yr^{-1}) and the most important, agriculture (4.1 Tg N_2O-N yr^{-1}) (UNEP 2013).

In agricultural soils, several processes are involved in N_2O emissions like nitrate ammonification, hydroxylamine oxidation, nitrifier denitrification, nitrification or denitrification (Baggs 2008; Wrage et al. 2005) and of these, nitrification

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and denitrification are considered the two main microbial sources of N_2O (Thomson et al. 2012) that are directly affected by soil nitrogen fertilization. Denitrification occurs under low-oxygen conditions in which, nitrate is sequentially reduced to nitrogen gas according to the following chemical equation (Zumft 1997):

$$NO_3^{-(a)} \rightarrow NO_2^{-(b)} \rightarrow NO^{(c)} \rightarrow N_2O^{(d)} \rightarrow N_2$$

Biological nitrogen fixation by rhizobia-legume symbiosis could be an effective and environmental-friendly alternative to nitrogen fertilization and hence, to mitigate soil N_2O emissions. However, legume crops also contribute to N_2O emissions by providing N-rich residues for decomposition (Baggs et al. 2000), directly by some rhizobia that are able to denitrify (Mesa et al. 2004; Bedmar et al. 2005; Sameshima-Saito et al. 2006; Hirayama et al. 2011), from microbial community of the soil rizosphere (Inaba et al. 2009) or from senescence and decomposition of legume nodules during post-harvesting (Inaba et al. 2012; Uchida and Akyyama 2013).

Grain and forage legumes represent close to 15 % of total arable surface and 27 % of the world's primary crop production (Graham and Vance 2003). Soybean (*Glycine max*) represents 50 % of the total legume crop area and 68 % of global production, able to fix 16.4 Tg N annually, representing 77 % of the N fixed by legume crops (Herridge et al. 2008). Soybean has an industrial and economical interest for oil, food and protein, pharmaceuticals for protective coating or biodiesel production that represents the largest individual element of international oilseed production (59 %), with United States (34 %), Brazil (30 %) and Argentina (18 %) being the main contributers to world soybean production (SoyStats 2015). *Bradyrhizobium japonicum* is a gram-negative soil bacterium able to both fix nitrogen in symbiosis with soybean and denitrify under free-living and symbiotic conditions. In this bacterium, denitrification depends on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes encoding periplasmic nitrate reductase[(a)], Cu-containing nitrite reductase[(b)], c-type nitric oxide reductase[(c)] and nitrous oxide reductase[(d)] (N_2OR) enzymes respectively (Bedmar et al. 2005, 2013; Delgado et al. 2007).

It has been demonstrated that N_2OR is the only enzyme able to reduce N_2O into N_2 (Pauleta et al. 2013), being essential for N_2O mitigation strategies in legume crops. Itakura et al. (2013) have demonstrated that inoculations with overexpressed N_2OR rhizobial strains could strongly reduce N_2O emissions from soybean nodules degradation at field scale. Bueno et al. (2015) showed that some endosymbiont rhizobia like *Ensifer meliloti* are able to grow using only N_2O as terminal electron acceptor under anoxic conditions. Other authors have highlighted that some agricultural practices and environmental factors like nitrate, carbon, copper availability, soil pH, O_2 partial pressure or water holding capacity could negatively affect N_2OR activity, regulation and hence, increase N_2O emissions from soils (Richardson et al. 2009). However, there are limited data concerning environmental factors involved in the emission of N_2O from nodules. A better understanding of these factors will be instrumental for the development of strategies and management practices in agriculture, especially for mitigating release of N_2O from legume crops. It has been recently demonstrated that hypoxic conditions provoked by root flooding induce denitrification in *B. japonicum* nodules from soybean plants. In addition, hypoxia induces NO formation in nitrate-treated soybean nodules and *B. japonicum* denitrification is the main process involved (Meakin et al. 2007; Sánchez et al. 2010). However, no data about flooding effect on N_2O emission is currently recorded. The aim of this research is to evaluate the effect of nitrate and hypoxia in N_2O emissions from *B. japonicum* nodules of soybean plants.

2 Materials and methods

2.1 Bacterial strains

B. japonicum was used as bacterial inoculant of soybean plants. Wild-type USDA110 (United States Department of Agriculture, Beltsville, MD) and two denitrification mutants strains, *napA* GRPA1 (Delgado et al. 2003) and *nosZ* GR3035 (Velasco et al. 2004) were used in this study. Bacteria were routinely grown in a modified peptone-salts-yeast extract (PSY) medium (Mesa et al. 2008) at 28 °C and supplemented with arabinose (0.1 % *w*/*v*). Antibiotics were added to solid and liquid cultures at the following concentrations: for USDA110 strain, chloramphenicol 15 μg ml^{-1}. For *napA* GRPA1 and *nosZ* GR3035 strains, chloramphenicol 15 μg ml^{-1}, spectinomycin dihydrochloride 200 μg ml^{-1} for solid and 100 μg ml^{-1} for liquid cultures, streptomycin sulphate 200 μg ml^{-1} for solid and 100 μg ml^{-1} for liquid cultures. PSY cultures were sterilized by autoclaving and antibiotics and arabinose by 0.22 μm filtering.

2.2 Plants inoculation and growth conditions

Soybean (*Glycine max* L. Merr., cv. Williams) seeds were surface-sterilized by adding 96 % ethanol (vol/vol) for 30 s, H_2O_2 15 % (*v*/*v*) for 8 min, and finally washed with sterile water. After that, seeds were germinated in petri dishes (10–12 seeds each) with 1 % agar (*w*/*v*) in darkness during 72 h at 28 °C, according to Sánchez et al. (2011a). Selected seedlings were planted in autoclaved pots (0.25 l) filled with vermiculite (N° 3) using the Leonard jar method (Trung and Yoshida 1983) that contained a mineral solution (Rigaud and Puppo 1975) consisting of: KH_2PO_4 68 mg l^{-1}, K_2HPO_4 44 mg l^{-1}, $MgSO_4$ x $7H_2O$ 123 mg l^{-1}, K_2SO_4 174 mg l^{-1}, $CaSO_4$

173 mg l^{-1}, Ferric EDTA 25 mg l^{-1}, Na_2MoO_4 x $2H_2O$ 0.11 mg l^{-1}, H_3BO_3 2.85 mg l^{-1}, $MnSO_4$ x $4H_2O$ 3.07 mg l^{-1}, $ZnSO_4$ x $7H_2O$ 0.55 mg l^{-1}, $CuSO_4$ x $5H_2O$ 0.2 mg l^{-1}, with or without potassium nitrate (0, 1, 2 and 4 mM) according to each experiment. Plants (one per pot) were inoculated at sowing with 1 ml of a single antibiotic-free bacterial strain (10^8 cells ml^{-1}), covered with autoclaved perlite and grown in a controlled plant-growing chamber for 28 days: 16–8 h day/night cycle, day/night temperatures of 28–20 °C and photosynthesis photon flux density of 180 μmol photons m^2 s^{-1}. Plant experiments were carried out at the facilities of Greenhouse and Growth Chamber Service of Estación Experimental del Zaidín (EEZ).

In order to induce denitrification activity, a set of plants were subjected to flooding for 7 days by submerging pots to 1 cm above substrate level using mineral solution with or without nitrate according to each experiment. Mineral solution level was maintained by daily additions of solution, added gently to avoid aeration. At the end of the experiments, some plant physiological parameters were recorded: shoot and root dry weight, nodules number, nodule fresh weight (NFW) and nodule dry weight (NDW).

2.3 N_2O measurements in nodulated roots and detached nodules

To measure in vivo N_2O flux from nodulated roots, plants were harvested from the Leonard jars and vermiculite was carefully removed. Then, roots were transfered into a 100 ml PYREX® bottles and closed using a perforated rubber septa (30.7 mm) which fitted to the soybean stems. In order to test gas-tight characteristics, screw caps with aperture were used to fix rubber septa and leaking tests were done. Also, vacuum silicone paste was spread on the tops of the septa.

Gas samples were carefully taken from the headspace using luer-lock gas-tight syringes with Mininert® valves and immediately transported to gas chromatograph for proper analysis. N_2O gas samples were manually injected into an HP 4890D gas chromatograph equipped with an electron capture detector (ECD) and a Porapak Q 80/100 MESH (8 ft) packed column. N_2 was the carrier gas at 28 ml/min flow rate and the injector, column and detector temperatures were 125, 60 and 375 °C, respectively. N_2O concentrations were calculated using 2 % (*v*/*v*) N_2O standard (Air Liquid, France) and processed using GC ChemStation Software (Agilent Technologies© 1990–2003). Total N_2O concentration was calculated taking into account N_2O in headspace and dissolved N_2O using Bunsen water solubility coefficient (54.4 % at 25 °C).

For each plant, the N_2O flux was recorded chronologically (2, 5 and 8 h) to check linearity of N_2O emissions by plotting and the emission flux (N_2O nmol h^{-1} g^{-1} NDW) was calculated in the linear interval between 5 and 8 h: Δ N_2O molar concentration (8–5 h) / Δ time increase (3 h). N_2O flux was also analyzed in detached nodules using 20 ml headspace vials (SUPELCO®) supplemented with 5 ml of mineral solution with or without nitrate. N_2O consumption in detached nodules was calculated by measuring N_2O concentration at 24 h after adding 1 ml of N_2O pure (2 %) to 20 ml headspace vials supplemented with 1 ml distilled water. Both, N_2O flux and consumption in detached nodules were calculated as nmol N_2O produced by fresh nodular biomass and time (nmol h^{-1} g^{-1} NFW).

2.4 Nitrate reductase activity in bacteroids and nitrite accumulation in nodules

Bacteroids from detached nodules were extracted according to Mesa et al. (2004). In brief, 1–2 g of fresh nodules were ground with a ceramic mortar and pestle with 7.5 ml of extraction buffer (50 mM Tris/HCl, pH 7.5, 250 mM mannitol). The homogenate was filtered through cheeseclothes and was centrifuged at 250*g* at 4 °C for 5 min to remove nodule debris. The supernatant was recentrifuged at 12,000*g* at 4 °C for 10 min to pellet the bacteroids, which were washed twice with 50 mM Tris/HCl (pH 7.5).

Methyl viologen-dependent nitrate reductase activity was analyzed as described by Delgado et al. (2003). Briefly, equal volumes of reaction mixture (10 mM KNO_3, 200 μM methyl viologen, 50 mM Tris/HCl buffer, pH 7.5) were added to bacteroid aliquots containing 0.2–0.4 mg of protein (previously determined using Bio-rad assay) and mixed gently. Enzymatic reactions started with the addition of freshly prepared sodium dithionite (14.4 mM) dissolved in $NaHCO_3$ (300 mM). After incubation for 15–30 min at 30 °C, the reaction was stopped by vigorous shaking until the samples lost their blue color. Controls were done in parallel but, in these reactions, sodium dithionite was oxidized at the start of the reaction (by shaking). Nitrite production was estimated after diazotation by adding the sulfanilamide/naphthylethylene diamine dyhydrochloride reagent (Nicholas and Nason 1957). Results were expressed as nmol NO_2^- produced min^{-1} protein $(mg)^{-1}$.

For determination of nitrite in nodules, 0.5 g of nodules were homogenized with 2 ml of 1 M zinc acetate and were centrifuged at 12,000*g* at 4 °C for 5 min. The resultant supernatant was mixed with 1 volume of 100 % ice-cold ethanol and was centrifuged for another 5 min. The nitrite concentration in the final supernatant was determined as described above.

2.5 Experimental performance and statistical analysis

In order to evaluate the effect of nitrate and flooding on the N_2O emissions, two experiments were carried out. The first experiment evaluated nitrate effect and consisted of a set of

plants which were grown with different nitrate concentrations (0, 1, 2 or 4 mM) for 28 days. Free-nitrate growing conditions (FNG) refers to plants grown without nitrate (0 mM) and nitrate growing conditions (NG) refers to plants grown with 1, 2 or 4 mM respectively. After that, the roots of the plants were flooded for 8 h with a nutrient solution containing different nitrate concentrations. FNG plants were incubated with 0, 1, 2 or 4 mM nitrate and NG plants were incubated with the same nitrate concentration used during plant growth. To evaluate flooding effect, the second experiment consisted of a set of plants which were grown for 28 days with 4 mM nitrate and then, roots were subjected or not subjected to a short-term flooding (STF) for 8 h or a long-term flooding (LTF) for 176 h respectively by adding 80 ml of 4 mM nitrate to the root closed-chamber. With this volume, the nodulated roots were totally submerged. Non flooding conditions (NF) were done by adding only 25 ml of 4 mM nitrate, not enough to submerge the nodulated roots.

The experiments were done twice and for each treatment, descriptive statistical analysis (median, quartiles and interquartile range IQR) of the data ($n=7$) were carried out. Data were checked for normality distribution according to Kolmogorov–Smirnov and Shapiro-Wilk tests. Also, we performed some inferential statistical analyses to test null hypothesis. Kruskal-Wallis test (non-parametric ANOVA) for 2 or more groups of unpaired data of treatments (nitrate concentration, flooding regimen and mutant strains) and *post hoc* Wilcoxon-Mann–Whitney test were used due to non-normal distribution and homoscedasticity of the data.

3 Results

3.1 Effect of nitrate in soybeans growth

In order to know the effect of nitrate on soybeans growth, a set of plants inoculated with *B. japonicum* USDA110, *napA* and *nosZ* denitrification mutants were cultivated with mineral solution containing 0, 1, 2 or 4 mM nitrate respectively. Shoot and root dry weight, nodule number and nodule fresh and dry weight were measured. Only shoot dry weight presented a statistical difference between free-nitrate (FNG) and nitrate growing conditions (NG), being higher with nitrate but without any statistical difference between nitrate concentrations or strain used as inoculant (data not shown).

3.2 Effect of nitrate in N_2O emissions from nodulated roots

Figure 1 shows the effect of nitrate concentration (0, 1, 2 or 4 mM) in the N_2O flux emitted from USDA110 and *nosZ* nodulated roots during a short-term flooding (8 h). No

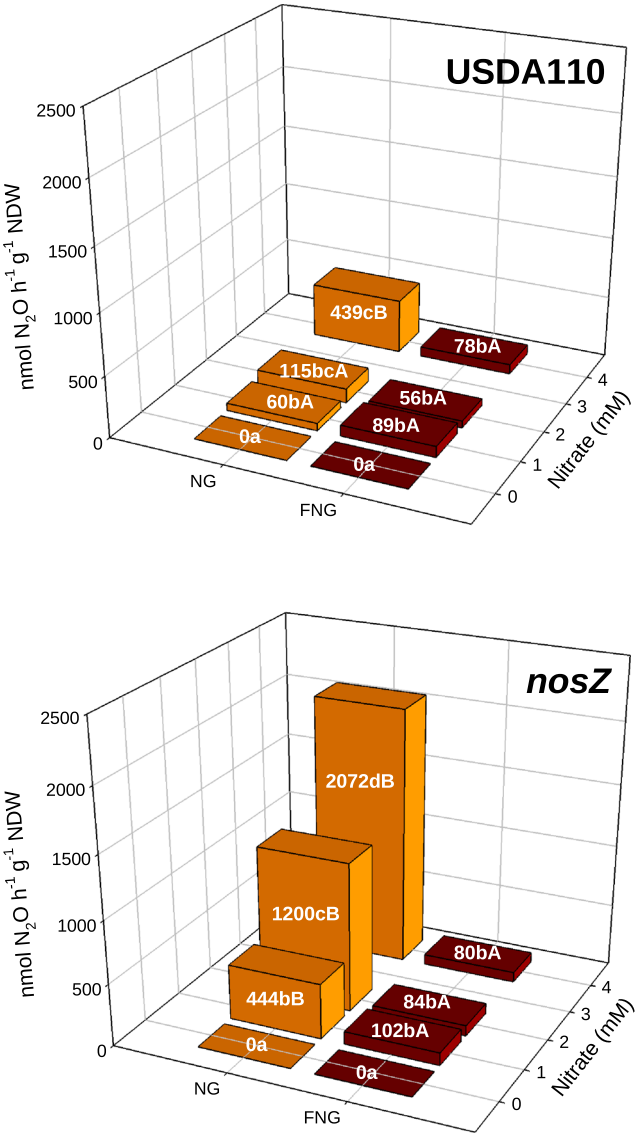

Fig. 1 Effect of nitrate on N_2O flux emission from nodulated soybean roots inoculated with *B. japonicum* USDA110 and *nosZ* denitrification mutant. Plants ($n=7$) were grown under free-nitrate (FNG) and nitrate (1, 2 and 4 mM) conditions (NG). For each nitrate growing condition, same lower-case letter within nitrate concentration and for each nitrate concentration, capital letter within growing conditions are not statistical different according to Wilcoxon-Mann–Whitney test ($p \leq 0.05$)

emission was recorded in plants grown and incubated without nitrate and inoculated with *B. japonicum* wild-type or *nosZ* mutant strains. In FNG growing conditions, no statistical differences were obtained in the N_2O flux along nitrate concentrations present in the incubation bottles containing USDA110 or *nosZ* nodulated roots. Independent of nitrate concentration in the incubation systems, no statistical difference of N_2O production was recorded within USDA110 or *nosZ* nodules from plants grown without nitrate. By contrast, an induction of N_2O production by nodulated roots was observed in plants grown in the presence of nitrate (NG). For USDA110

nodulated roots, a statistical increase ($p<0.05$) with nitrate concentration was observed showing 60±21, 115±58 and 439±146 nmol N_2O h^{-1} g^{-1} NDW for 1, 2 and 4 mM nitrate respectively. N_2O fluxes from *nosZ* nodulated roots were always statistically higher compared to those obtained with *B. japonicum* wild-type strain, showing also an increase along nitrate concentrations, due to a flux of 444±125, 1200±230 and 2072±244 nmol N_2O h^{-1} g^{-1} NDW was observed in plants grown with 1, 2 and 4 mM nitrate respectively (Fig. 1).

In order to know the relationship between the N_2O flux and nitrate concentration in the nutrient solution, a regression study was performed (Fig. 2). Plants grown without nitrate (FNG) did not show any statistical relationship with nitrate concentration present in the incubation solution during N_2O analysis. In contrast, when plants were grown in the presence of nitrate (NG), N_2O fluxes from USDA110 and *nosZ* nodulated roots fitted a first-order kinetic model (y=mx+n), with important correlation coefficients (R^2=0.9355 and 0.9881 respectively), being *nosZ* N_2O fluxes strongly affected due to its higher slope respect to USDA110 (y=111.03x– 40.79 and y=529.03x+3.21 for USDA110 and *nosZ* respectively).

3.3 Effect of flooding in N_2O emissions from nodulated roots and detached nodules

The effect of flooding conditions in the N_2O emissions of soybeans grown with 4 mM nitrate and inoculated with *B. japonicum* USDA110 and *napA* and *nosZ* denitrification strains is shown in Fig. 3. In USDA110 nodulated roots not subjected to flooding (NF), a basal N_2O flux was recorded (10±2 nmol N_2O h^{-1} g^{-1} NDW) which showed a significant increase of 44-fold when short-term flooding (STF) was applied (439±146 nmol N_2O h^{-1} g^{-1} NDW). This effect was strongly induced when flooding was prolonged during 1 week (LTF) showing an increase of 149-fold respect to NF N_2O emissions (1485±325 nmol N_2O h^{-1} g^{-1} NDW). Similarly to USDA110, *napA* and *nosZ* nodulated roots showed also an increase throughout flooding time applied. For *napA*, when no flooding was applied, basal level of 16±8 nmol N_2O h^{-1} g^{-1} NDW was recorded and close to 10 and 100-fold increases were obtained for STF and LTF compared to NF respectively. For *nosZ*, the effect of flooding was more pronounced since N_2O emissions obtained were 23±15, 2072±244 and 19026±2193 nmol N_2O h^{-1} g^{-1} NDW for NF, STF

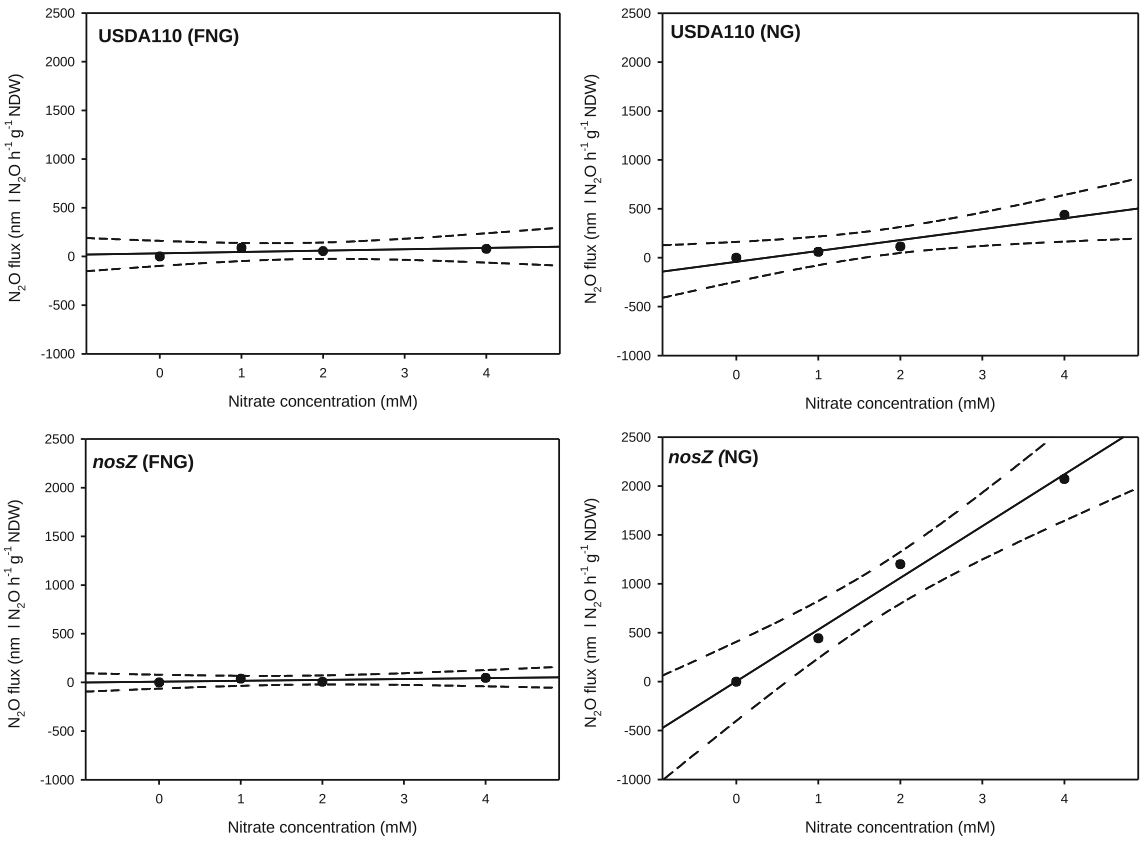

Fig. 2 Linear correlation between N_2O flux emissions and nitrate concentration from nodulated roots inoculated with *B. japonicum* USDA110 and *nosZ* denitrification mutant. FNG and NG mean free-nitrate and nitrate growing conditions respectively. Dotted lines represent confidence intervals at 95 %. Data n=7

and LTF respectively, showing the highest increases respect to basal emissions obtained for all *B. japonicum* strains assayed (close to 90 and 900-fold increases for both flooding conditions). In general, root nodules produced by USDA110 or *napA* showed N_2O levels that were statistically lower ($p \leq 0.05$) than those obtained for *nosZ* root nodules. This behavior was observed during all flooding conditions assayed, being especially relevant during 1 week of flooding (LTF) when the highest N_2O flux record was obtained (19026±2193 nmol N_2O h^{-1} g^{-1} NDW).

N_2O fluxes were also analyzed in detached nodules subjected to flooding (Fig. 3). Similar tendencies were obtained compared to nodulated roots, with a statistical increase ($p \leq 0.05$) in N_2O emissions in flooding conditions (LTF>>STF>NF) for either USDA110, *napA* or *nosZ* nodules. It is especially important to remark that the N_2O fluxes recorded when flooding was prolonged (LTF). As shown in Fig. 3, *napA* and *nosZ* nodules showed lower and higher N_2O fluxes compared to USDA110 nodules (382±243 for *napA*, 2522±416 for *nosZ* and 1120±483 for USDA110 nmol N_2O h^{-1} g^{-1} NDW respectively).

3.4 Nitrate reductase activity in bacteroids and nitrite accumulation and N_2O consumption in detached nodules

It has been previously reported that *B. japonicum napA* and *nosZ* mutants are affected in periplasmic nitrate reductase (NR) and nitrous oxide reductase (N_2OR) activities respectively (Delgado et al. 2003; Velasco et al. 2004). In order to confirm the defect on NR and N_2OR activities in *napA* and *nosZ* nodules subjected to nitrate and flooding conditions, NR activity in bacteroids, nitrite accumulation and N_2O consumption in nodules were analyzed (Table 1). As expected, *napA* mutant showed the lowest value for NR (0.2±0.1 nmol NO_2^- produced min^{-1} mg^{-1} protein) in nodules subjected to short-term flooding (LTF) compared to those obtained by USDA110 (3.1±0.7 nmol NO_2^- produced min^{-1} mg^{-1} protein) and *nosZ* bacteroids (3.3±0.9 nmol NO_2^- produced min^{-1} mg^{-1} protein). When flooding was maintained during 1 week (LTF), again nodules from the *napA* mutant showed lower levels of NR compared to those observed in wild-type or *nosZ* nodules (2.6±0.7, 5.4±0.6 and 7.1±0.3 nmol NO_2^- produced min^{-1} mg^{-1} protein for *napA*, USDA110 and *nosZ* respectively). The LTF produced a statistical increase ($p \leq 0.05$) of around 2-fold in NR values for all strains assayed compared to those observed in bacteroids from nodules subjected to STF (Table 1). Nitrite accumulation in nodules was also analyzed and as observed in NR activity in bacteroids, *napA* nodules accumulated lower nitrite concentration compared to USDA110 and *nosZ* nodules in both flooding conditions assayed. By contrast, no statistical differences were obtained within flooding conditions for each strain assayed (Table 1). Finally, N_2O consumption measurements revealed

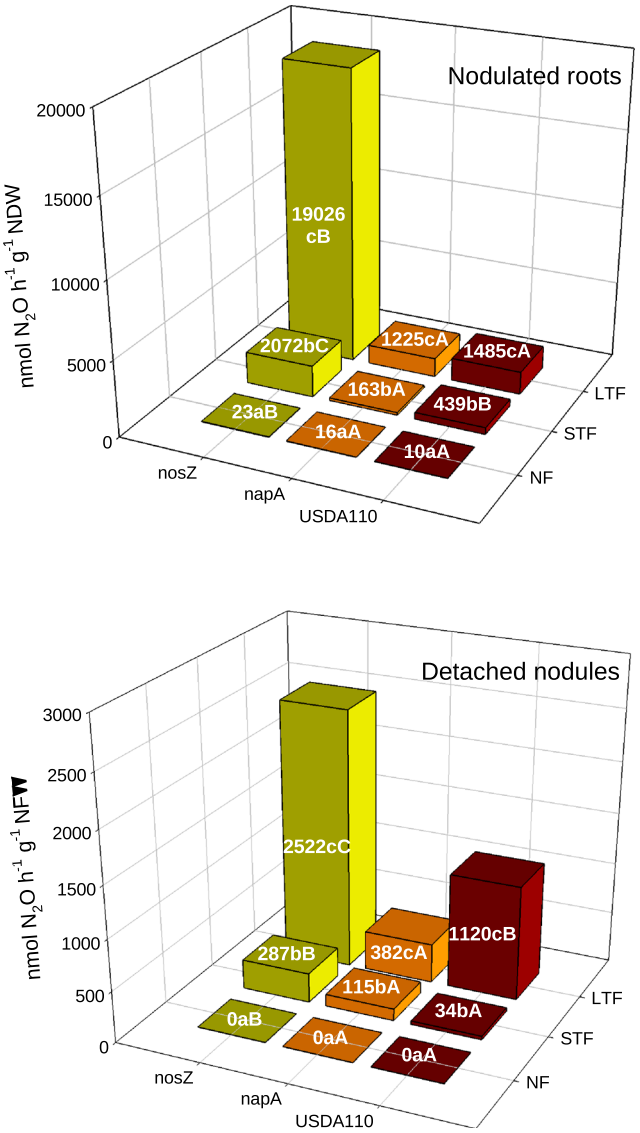

Fig. 3 Effect of flooding conditions in N_2O flux emissions from nodulated roots and detached nodules from soybean inoculated with *B. japonicum* USDA110 and *napA* and *nosZ* denitrification mutants. For each strain, same lower-case letter within flooding conditions (NF, STF and LTF) and for each flooding condition, capital letter within strains are not statistical different according to Wilcoxon-Mann–Whitney test ($p \leq 0.05$). NF, STF and LTF mean no flooding, short-term flooding and long-term flooding condition respectively. Data $n=7$

that *nosZ* nodules were not able to reduce N_2O compared to USDA110 and *napA* during both flooding treatments assayed (Table 1). These results demonstrated that N_2OR is an essential enzyme to reduce N_2O in nodules in response to nitrate and flooding conditions.

4 Discussion

It has been reported that high concentrations of nitrate can produce a detrimental effect on nodule growth and activity

Table 1 Methyl viologen-dependent nitrate reductase activity (NR) in bacteroids, nitrite accumulation and N_2O consumption in soybean detached nodules produced by *B. japonicum* USDA110, *napA* and *nosZ* denitrification mutants

Bacteroids			
	NR (nmol NO_2^- produced min^{-1} mg^{-1} protein)		
Flooding	USDA110	*napA*	*nosZ*
STF	3.1±0.7 aB	0.2±0.1 aA	3.3±0.9 aB
LTF	5.4±0.6 bB	2.6±0.7 bA	7.1±0.3 bC
Nodules			
	Nitrite accumulation (nmol NO_2^- g^{-1} NFW)		
Flooding	USDA110	*napA*	*nosZ*
STF	5.74±0.07 aB	0.95±0.06 aA	7.53±0.06 aC
LTF	7.36±0.10 bB	1.12±0.01 bA	6.76±0.06 bB
Nodules			
	N_2O consumption (nmol N_2O h^{-1} g^{-1} NFW)		
Flooding	USDA110	*napA*	*nosZ*
STF	25±3 bB	18±5 aB	0.4±0.1 aA
LTF	16±1 aB	17±4 aB	0.7±0.2 aA

Data (n=3) are expressed as median±IQC (InterQuartile Range). NFW, STF and LTF mean nodule fresh weight, short-term flooding (8 h) and long-term flooding (176 h) respectively. Same lower-case letter in each column within flooding condition and capital letter for each row within strains are not statistical different according to Wilcoxon-Mann–Whitney test ($p \leq 0.05$)

in the rizobia-legume symbiosis (Streeter 1988). With respect to *B. japonicum*-soybean symbiosis, we have previously reported that 4 mM nitrate does not affect either nodulation process or nitrogenase activity (Mesa et al. 2004; Sánchez et al. 2011a). In this work, we have confirmed the tolerance of soybean nodulation to 4 mM nitrate independently to the *B. japonicum* strain used as inoculant (USDA110, *napA* or *nosZ*). As expected, shoot biomass showed a positive response to nitrate treatment (1, 2 or 4 mM) compared to untreated plants.

Among the environmental conditions that influence N_2O emissions from agricultural soils, one important factor is the availability of N electron acceptors that is stimulated by addition of N, usually in the form of inorganic fertilizers (Baggs and Philippot 2011; Venterea et al. 2012). In legume crops it has also been found that N_2O emissions increased linearly with increasing N rates of nitrogen fertilizers applied (MacKenzie et al. 1998). More recently, it has been reported in soybean crops that soil nitrate level is the main variable used to explain cumulative N_2O emissions during the whole growing season (Ciampitti et al. 2008). In this work, we have confirmed the importance of nitrate for N_2O emissions from nodulated roots of soybean plants inoculated with *B. japonicum* USDA110. In the presence of 4 mM nitrate, N_2O emission was increased markedly from soybean roots inoculated with a *nosZ* mutant of *B. japonicum* compared to wild-type nodules indicating that *B. japonicum* bacteroids in soybean nodules are able to convert the exogenously supplied nitrate into N_2O via denitrification pathway. Although it has been previously demonstrated that nitrate supply may lead to an increase in N_2O emission from intact soybean root systems via denitrification in *B. japonicum* (Mesa et al. 2004; Hirayama et al. 2011), our work shows for the first time the linear correlation between nitrate concentration and N_2O emissions by soybean root nodules.

Floods can negatively impact agricultural yields affecting plants development and increasing susceptibility to diseases (Voesenek and Bailey-Serres 2015). In soybean crops, waterlogging produced by root flooding is an important environmental factor that causes severe seed and seedling damage, resulting in substantial reduction of grain yield at maturity (Kokobun 2013). However, soybeans are considered flooding-tolerant plants since they have the capacity to develop morphological adaptations to hypoxia by aerenchyma formation in stem (immediately above the water line), roots and nodules to facilitate O_2 diffusion to hypoxic tissues in order to maintain nitrogenase activity (Thomas et al. 2005). The aeration status of a soil, which depends upon soil water content, soil texture, and the partial pressure of oxygen in the gas phase, is a key factor affecting denitrification activity and consequently N_2O emissions (Baggs and Philippot 2011). Soybeans are tropical legumes native to habitats usually subjected to waterlogging. However, the information about N_2O emissions from *B. japonicum*-soybean symbiosis in response to flooding is very poor. In this work, we have demonstrated that flooding strongly induces N_2O release compared to control conditions (NF) when nodulated plants were grown in presence of 4 mM nitrate. This effect was especially notable during a long-term flooding, when N_2O fluxes in nodules produced by a *nosZ* mutant were much more high than those produced by wild-type nodules. These results suggest that the *B. japonicum* N_2OR is an important enzyme to mitigate the N_2O emissions from legume crops in response to flooding.

Recently, it has been reported that flooding also induces the formation in detached nodules of the precursor of N_2O, the cytotoxic and ozone depleting gas nitric oxide (NO) (Meakin et al. 2007; Sanchez et al. 2010). This molecule is also an inhibitor of nitrogenase activity. However, in those studies the emission of N_2O in detached nodules of a *napA* mutant was not investigated. In the present work, we have demonstrated that inoculation of plants with the *napA* mutant, reduced N_2O production by detached nodules in response to 4 mM nitrate and LTF. In fact, under these conditions, *napA* bacteroids showed a significant decrease in nitrate reductase activity (NR) as well as in nitrite accumulation in the cytosolic fraction of the nodules compared to wild-type nodules. Nevertheless, basal levels of N_2O as well as NR activity were recorded in *napA* detached nodules suggesting that other mechanisms different to denitrification pathway could be involved in N_2O production in nodules. In this context, it has

been recently identified in *B. japonicum* a putative haemoglobin, Bjgb, implicated in NO detoxification (Cabrera et al. 2011; Sánchez et al. 2011b). Similarly to other bacterial haemoglobins, Bjgb might reduce NO to N_2O under anoxic free-living conditions or inside the nodules. In *B. japonicum*, the Bjgb is encoded in a gene cluster that also codes for a number of proteins with putative roles in nitrate assimilation including the large catalytic subunit of the assimilatory nitrate reductase (NasC) (Cabrera et al. 2011). These observations strongly suggest that in addition to denitrification, rhizobial nitrate assimilation might be another important source of NO and N_2O in nodules. Finally, analyses of N_2O consumption capacity in nodules in response to STF and LTF revealed a clear defect of *nosZ* nodules to consume 2 % N_2O added to the incubation vials. Thus, these results confirmed the previously mentioned role of N_2OR as a key enzyme to reduce N_2O emissions from soybean nodules in response to nitrate and flooding conditions.

5 Conclusions

In this work we have demonstrated that nitrate is essential for N_2O emissions from soybean nodules and its concentration enhanced N_2O fluxes. Also, the relevance of water logging (flooding) has been proved as an environmental factor involved in the N_2O emissions from nodules of soybean plants grown in the presence of nitrate. We have also shown that the N_2O emission increased markedly from soybean roots inoculated with a *nosZ* mutant of *B. japonicum* and subjected to nitrate and flooding compared to wild-type nodules. By contrast, nodules produced by a *napA* mutant showed a significant decrease in N_2O production as well as in nitrate reductase activity compared to nodules produced by *B. japonicum* USDA110. Taken together, these results demonstrate that *B. japonicum* bacteroids in flooded soybean nodules are able to convert the exogenously supplied nitrate into N_2O mainly via the denitrification pathway and that the bacteroidal N_2OR is a key enzyme to reduce N_2O emissions from soybean nodules in response to nitrate and flooding conditions. Nevertheless, basal levels of N_2O as well as nitrate reductase activity in *napA* nodules suggested that other mechanisms different to the denitrification pathway could be involved in N_2O production in nodules.

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