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Short Communication

Involvement of *Bradyrhizobium japonicum* denitrification in symbiotic nitrogen fixation by soybean plants subjected to flooding

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ABSTRACT

Denitrification by *Bradyrhizobium japonicum* bacteroids contributes to nitric oxide (NO) production within soybean nodules in response to flooding conditions. However, the physiological relevance of NO production by denitrification in *B. japonicum*–*Glycine max* symbiosis is still unclear. In this work, soybean plants were inoculated with *B. japonicum* strains lacking the *nirK* or *norC* genes which encode the copper-containing nitrite reductase and the *c*-type nitric oxide reductase enzymes, respectively. 14 days flooding increased nodule number of plants inoculated with the WT and *norC* strains, but not of plants inoculated with the *nirK* mutant. However, nodule dry weight was not affected by 14 days flooding regardless of the strain used for inoculation. Supporting this observation, individual nodule growth was significantly higher in plants inoculated with *nirK* than those inoculated with WT or *norC* after 14 days flooding. Nodule functioning was strongly inhibited by flooding since leghemoglobin content of the nodules induced by any of the strains was significantly decreased after 7 or 14 days flooding compared to control plants. However, this effect was more relevant in nodules of plants inoculated with the WT or *norC* mutant than in those inoculated with the *nirK* mutant. Nitrogen fixation was also estimated by analyzing nitrogen content derived from biological nitrogen fixation in shoots, using the ^{15}N isotope dilution technique. By using this approach, we observed that the negative effect of 14 days flooding on nitrogen fixation was more pronounced in plants inoculated with the *norC* mutant. However, nitrogen fixation of plants inoculated with *nirK* showed the highest tolerance to 14 days flooding. These findings allowed us to demonstrate the previously proposed hypothesis which suggests that NO formed by copper-containing nitrite reductase in soybean nodules, in response to flooding, has a negative effect on nitrogenase activity. We propose that inoculation of soybeans with a *B. japonicum nirK* mutant, which does not produce NO from nitrate, increases the tolerance of symbiotic nitrogen fixation to flooding.

1. Introduction

Soil bacteria, collectively referred to as rhizobia, are members, among others, of the bacterial order *Rhizobiales* of the α-Proteobacteria with the unique ability to establish a dinitrogen (N_2)-fixing symbiosis on legume roots and on the stems of some aquatic legumes. Biological N_2 fixation by legume–rhizobia symbioses is of tremendous importance to the environment and to world agriculture. Perception of legume root exudates triggers the production of rhizobial Nod factor signals which are recognized by compatible plant receptors leading to the formation of root nodules, in which differentiated bacteria (bacteroids) fix N_2 (Oldroyd and Downie, 2008). In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. Firstly, a high rate of oxygen (O_2)-respiration is necessary to supply the energy demands of the N_2 reduction process (Delgado et al., 1998), but oxygen also irreversibly inactivates the nitrogenase complex. These conflicting demands are reconciled by control of O_2 flux through a diffusion barrier in the nodule cortex and by the plant O_2-carrier, leghemoglobin (Lb), which is present exclusively in the nodule (Downie, 2005; Minchin et al., 2008).

In addition to fix N_2, some rhizobia species are able to grow under low O_2 conditions using nitrate as electron acceptor to support respiration in a process known as denitrification by which bacteria reduce sequentially nitrate (NO_3^-) or nitrite (NO_2^-) to N_2.

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Nitrate is reduced to nitrite by either a membrane-bound (Nar) or a periplasmic nitrate reductase (Nap), and nitrite reductases (CuNir or cd_1Nir) catalyse the reduction of nitrite to nitric oxide (NO). NO is further reduced to nitrous oxide (N_2O) by nitric oxide reductases (cNor, qNor or qCu_ANor) and, finally, N_2O is converted to N_2 by the nitrous oxide reductase enzyme (Nos) (van Spanning et al., 2005, 2007; Zumft, 1997). The significance of denitrification in rhizobia–legume symbiosis can be appreciated when O_2 concentration in soils decreases during environmental stress such flooding of the roots, which causes hypoxia. Under these conditions, denitrifying activity could work as a mechanism to generate ATP for survival of rhizobia in the soil and also to maintain nodule functioning (O'Hara and Daniel, 1985). An associated role of denitrification in nodules could also be detoxification of the cytotoxic compounds nitrite and NO produced as intermediates during denitrification reactions or emerging from the host plant. In fact, NO has been reported as inhibitor of nitrogenase activity (Kato et al., 2009; Sasakura et al., 2006; Sánchez et al., 2010; Shimoda et al., 2009). Finally, denitrification by rhizobia–legume symbiosis might contribute to emission of the potent and long-lived greenhouse gas N_2O from soils, by several ways: (i) biologically fixed N may be nitrified and denitrified, thus providing a source of N_2O (Galloway, 1998); (ii) by providing N-rich residues for decomposition (Baggs et al., 2000); and (iii) directly by free-living or symbiotic rhizobia (García-Plazaola et al., 1993; Fernández et al., 2008; Mesa et al., 2004; Sameshima-Saito et al., 2006). In recent years, it has emerged that many rhizobia species have genes for enzymes of some or all of the four reductase reactions for denitrification (Bedmar et al., 2005; Delgado et al., 2007). In fact, denitrification can be readily observed in rhizobia their free-living form, in legume root nodules, or in isolated bacteroids (Delgado et al., 2007). However, up to date, the physiological relevance of rhizobial denitrification in symbiotic N_2 fixation is still unclear.

In *Bradyrhizobium japonicum* USDA110, the symbiont of soybeans, denitrification depends on the *napEDABC* (Delgado et al., 2003), *nirK* (Velasco et al., 2001), *norCBQD* (Mesa et al., 2002) and *nosRZDYFLX* (Velasco et al., 2004) gene clusters encoding nitrate-, nitrite-, nitric oxide- and nitrous oxide-reductases, respectively. Expression of *B. japonicum nirK*, *norC* and *nosZ* denitrification genes in soybean root nodules has been reported by *in situ* histochemical detection of β-galactosidase activity due to transcriptional fusions of the *nirK*, *norC* and *nosZ* promoter regions to the reporter gene *lacZ* (Mesa et al., 2004). Similarly, isolated bacteroids also expressed the P*nirK-lacZ*, P*norC-lacZ* and P*nosZ-lacZ* fusions (Mesa et al., 2004). Levels of β-galactosidase activity were similar in both bacteroids and nodule sections from plants that were solely N_2-dependent or grown in the presence of 4 mM KNO_3, which suggest that O_2, and not nitrate, is the main factor controlling expression of denitrification genes in soybean nodules (Mesa et al., 2004). The study of the symbiotic phenotype of rhizobia strains carrying mutations in *nirK*, *norC*, or *nosZ* structural genes has demonstrated that, in soybean and alfalfa plants not supplemented with nitrate, these genes are not essential for N_2 fixation (Holloway et al., 1996; Mesa et al., 2004; Sameshima-Saito et al., 2006). However, mutation of *nirK* or *norC* genes confers on *B. japonicum* a reduced ability for nodulation in soybean plants (Mesa et al., 2004).

Recently, it has been shown that hypoxia or flooding conditions induce NO production in soybean nodules and that bacteroidal denitrification is the main source of NO under these conditions (Meakin et al., 2007; Sánchez et al., 2010). It has also been demonstrated that NO produced by the *B. japonicum* copper-containing nitrite reductase (NirK) in response to flooding conditions affects nitrogenase genes expression (Sánchez et al., 2010). However, the physiological relevance of NO production by denitrification in nodules in response to flooding is still unclear. In this work, we have analysed the symbiotic phenotype, under flooding conditions, of a *B. japonicum nirK* mutant, where NO production from denitrification is blocked, and of a *norC* mutant, which accumulates NO produced through denitrification.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. japonicum USDA110 (United States Department of Agriculture, Beltsville, MD), and *nirK* GRK308 (Velasco et al., 2001) and *norC* GRC131 (Mesa et al., 2002) mutant derivative strains were used in this study. B. *japonicum* strains were routinely grown in peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) at 28 °C. Antibiotics were added to cultures at the following concentrations (μg ml^{-1}): chloramphenicol, 15; spectinomycin, 200; streptomycin, 200; kanamycin, 200.

2.2. Plant growth conditions

Soybean (*Glycine max* L. Merr., cv. Williams) seeds were surface-sterilized with 96% ethanol (vol/vol) for 30 s, immersed in H_2O_2 (15%, vol/vol) for 8 min, then washed with sterile water and germinated in darkness at 28 °C, as previously described Mesa et al. (2004). Selected seedlings were planted in autoclaved pots filled with a perlite–vermiculite mixture (1:1). Plants (five per pot) were inoculated at sowing with 1 ml of a single bacterial strain (10^8 cells ml^{-1}) and grown in a greenhouse under the following conditions: 16–8 h day/night cycle and day/night temperatures of 28–20 °C. Plants were watered four times a week, alternatively with water and with a nitrogen-free mineral solution (Rigaud and Puppo, 1975). In order to induce denitrification activity, after growth for 15 days, plants were provided with the same mineral solution supplemented with 4 mM ^{15}N-labelled KNO_3 (10% ^{15}N, Cambridge Isotope Laboratories Inc., Andover, MA, U.S.A.). After growth for 28 days, a set of plants were subjected to flooding, as previously described (Bacanamwo and Purcell, 1999) by submerging pots to 1 cm above substrate level in a quarter-strength mineral solution. The solution level was maintained by daily additions of solution, which was added gently to avoid aeration. Non-flooded treatments (control plants) received the quarter-strength mineral solution four times a week. Nodules were harvested from 28 (0 days flooding)-, 35 (7 days flooding)- and 42 (14 days flooding)-day-old plants.

2.3. Plant measured parametres

Shoots (separated from roots at the cotyledonary node) and nodules were dried to a constant weight at 60 °C. Dry weight (SDW) and height (SH) were measured on shoots, and nodule number (NN) and nodule dry weight (NDW) were determined per plant. For determination of Lb content, nodules were harvested into liquid nitrogen and stored at −80 °C.

2.4. Leghemoglobin content

Lb content was measured by fluorimetry as described by LaRue and Child (1979). 0.3 g of nodules were grounded in 6 ml Lb extraction buffer [$Na_2HPO_4 \cdot 2H_2O$ 40 mM (pH 7.4); $NaH_2PO_4 \cdot H_2O$ 10 mM (pH 7.4); $K_3Fe(CN)_6$ 0.02%; $NaHCO_3$ 0.1%] supplemented with 0.1 g polyvinyl-polypyrrolidone. The homogenate was centrifugated at 12,000 *g* at 4 °C for 20 min to retain the supernatant. 50 μl of clear supernate and 3 ml saturated oxalic acid were added

to screw-capped tubes which were sealed and heated for 30 min at 120 °C in an autoclave, then cooled to room temperature. The fluorescence of the solutions was measured with a spectrophotofluorometer equipped with a mercury–xenon lamp and an RF-549 red-sensitive photomultiplier (Shimadzu Scientific Instruments, Tokyo, Japan). The excitation wavelength was 405 nm and the emission monochromator setting was 650 nm. The difference in fluorescence between heated and unheated samples was proportional to heme protein concentration.

2.5. Total nitrogen and nitrogen derived from biological N_2 fixation

Oven-dried shoots were weighed and grounded in an IKA A 11 basic analytical mill (Rose Scientific Ltd., Alberta, Canada). Subsamples of approximately 3 mg were weighed and analysed for total nitrogen (TN) and ^{15}N enrichment ($\delta^{15}N$) using an elemental analyser (EA1500 NC, Carlo Erba, Milan, Italy) coupled to isotope-ratio mass spectrometer (Delta Plus XL, ThermoQuest, Bremen, Germany). The overall precision of analyses for $\delta^{15}N$ was ±0.1‰. The stable composition was reported as $\delta^{15}N$ values per mil: $\delta^{15}N\,(‰) = (R_{sample}/R_{standard} - 1) \times 1000$, where $R = {}^{15}N/{}^{14}N$. Commercial N_2 was used as the internal standard for the nitrogen isotopic analyses, contrasted with the international standard. $\delta^{15}N$ values for all samples were normalized against internationally accepted reference materials (IAEA N1, $\delta^{15}N = +0.4‰$, IAEA N2, $\delta^{15}N = +20.3‰$ and USGS32 $\delta^{15}N = +174.5$ *vs* AIR).

The proportion of N derived from atmospheric N_2 (%Ndfa) was calculated according to Chalk (1985), as follows:

$$\%\text{Nfda} = 100[1 - (A/B)]$$

where: A = Atom% ^{15}N excess in inoculated plants. B = Atom% ^{15}N excess in uninoculated plants. Atom% ^{15}N excess = atom% ^{15}N in labelled treatment − atom% ^{15}N in non-labelled treatment.

$$\text{Atom\%}\,{}^{15}N = \delta^{15}N(‰) \times 100$$

For calculation of atom% ^{15}N excess, a set of plants was maintained under N_2-fixing conditions in order to obtain the atom% ^{15}N of the non-labelled treatment.

The fixed-nitrogen content (FN) was calculated as follows:

$$FN = (\%\text{Ndfa} \times TN)/100$$

2.6. Statistical analysis

The data were analysed by sampling times using analysis of variance, and means were separated by Tukey HSD Test at $P \leq 0.05$. Statistical procedures were carried out with the software OpenStat (http://fsf.org).

3. Results

3.1. Plant growth

Table 1 shows shoot dry weight (SDW) and shoot height (SH) of soybean plants that were inoculated or not with the wild-type (WT) *B. japonicum* USDA110 strain, and *nirK* GRK308 or *norC* GRC131 mutant strains, and were subjected or not (control) to flooding conditions. Before flooding treatment (0 days) and after 7 days treatment, no significant differences were observed in SDW and SH among plants inoculated with any of the strains. After 14 days flooding, SDW of plants inoculated with either the WT or any of the denitrification mutants decreased about 20% compared to control plants. However, 14 days-flooded plants inoculated with the *nirK* mutant showed higher SH than those inoculated with the WT or *norC* mutant. SDW of uninoculated plants after 14 days flooding was similar to that of plants maintained under control conditions (Table 1). However, SDW of those plants was significantly lower than that of plants inoculated with either the WT, and the *nirK* or the *norC* mutant strains and subjected or not to flooding for 14 days (Table 1).

3.2. Nodulation

The effect of flooding and inoculation with the *B. japonicum nirK* or *norC* denitrification mutants on nodule number (NN) (Fig. 1A) and on nodule dry weight (mg, NDW) (Fig. 1B) per plant was also assesed. Before flooding (0 days), NN of plants inoculated with either the *nirK* or the *norC* mutant strains was significantly lower compared to those inoculated with the WT (Fig. 1A, 0 days). However, no significant differences were observed in NDW among plants inoculated with any of the strains (Fig. 1B, 0 days). After 7 days flooding, NN per plant decreased approximately 30% in plants inoculated with the WT or *nirK* strain, and 40% in those inoculated with the *norC* strain compared to control plants (Fig. 1A, 7 days). Similarly, NDW per plant decreased about 44% in plants inoculated with the WT or *nirK* strain, and 56% in those inoculated with the *norC* strain compared to control plants (Fig. 1B, 7 days). After 14 days flooding, a 36% induction of NN per plant was observed in plants inoculated with either the WT or *norC* strains compared to control conditions. However, no significant differences were observed in NN among control and flooded plants inoculated with the *nirK* strain (Fig. 1A, 14 days). By contrast to NN, no significant differences were observed in NDW among control and flooded plants inoculated with any of the strains after 14 days flooding (Fig. 1B, 14 days).

3.3. Nodule growth and leghemoglobin content

The ratio between NDW and NN per plant is indicative of the individual dry weight of each nodule (mg per nodule, DWN,

Table 1
Shoot dry weight (SDW) and shoot height (SH) of uninoculated plants and plants inoculated with the wild-type *B. japonicum* USDA110, and *nirK* GRK308 or *norC* GRC131 mutant derivatives. Plants were subjected or not (control) to flooding conditions for 7 and 14 days. Values in a column within the same strain followed by the same lower-case letter, and values in a column within the same treatment followed by the same capital letter, are not significantly different as determined by the Tukey HSD test at $P \leq 0.05$ ($n = 10$).

Strain	Flood treatment	Days of treatment					
		0		7		14	
		SDW (g plant^{-1})	SH (cm plant^{-1})	SDW (g plant^{-1})	SH (cm plant^{-1})	SDW (g plant^{-1})	SH (cm plant^{-1})
USDA110 (Wild-type)	Control	0.366	19.8	0.876	22.7	1.626 aA	24.9 aA
	Flooded	–	–	0.924	22.7	1.204 bA	25.8 aB
GRK308 (*nirK*$^-$)	Control	0.355	20.2	0.863	23.0	1.621 aA	24.4 bA
	Flooded	–	–	0.948	22.9	1.347 bA	32.5 aA
GRC131 (*norC*$^-$)	Control	0.313	19.4	0.910	23.1	1.620 aA	25.0 aA
	Flooded	–	–	0.943	23.2	1.336 bA	27.2 aB
Uninoculated	Control	0.344	20.4	0.841	22.7	1.242 aB	25.3 aA
	Flooded	–	–	0.926	23.1	1.199 aB	26.3 aB

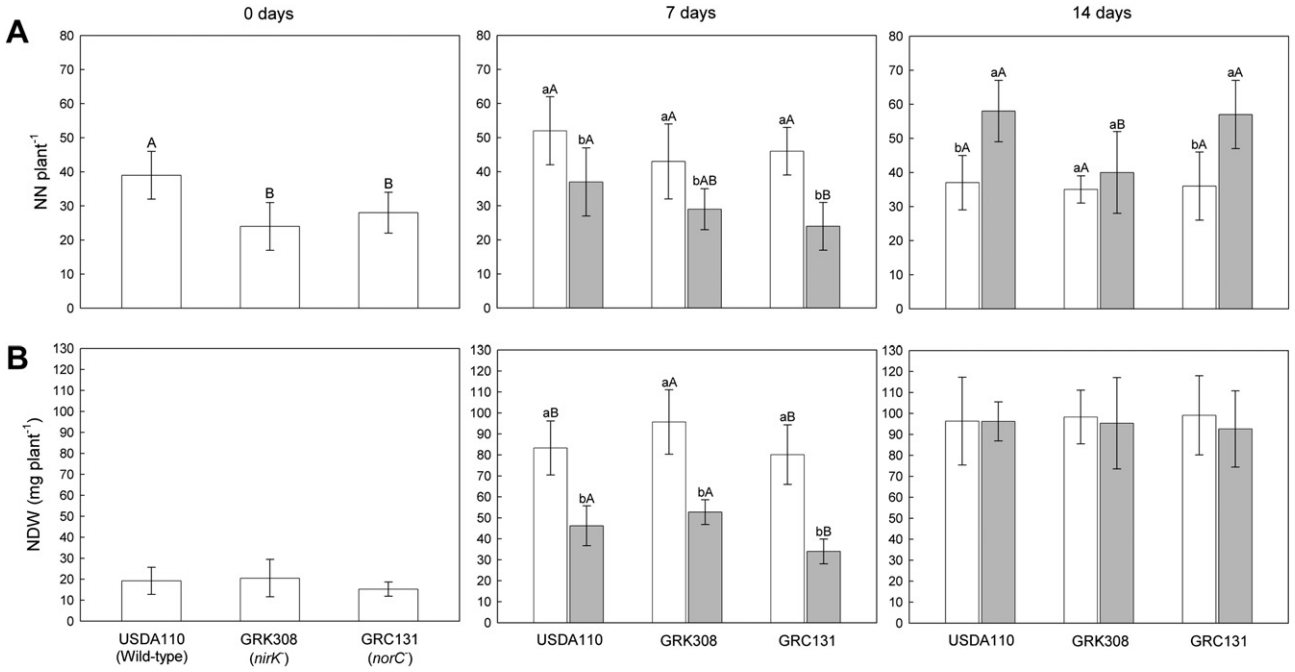

Fig. 1. (A) Nodule number (NN) and (B) nodule dry weight (NDW) of plants inoculated with the wild-type *B. japonicum* USDA110, and *nirK* GRK308 or *norC* GRC131 mutant strains. Nodules were isolated from control plants (white bars) or plants subjected to flooding conditions for 7 and 14 days (grey bars). In individual graphs, bars within the same strain marked with the same lower-case letter, and bars within the same treatment followed by the same capital letter, are not significantly different as determined by the Tukey HSD test at $P \leq 0.05$ ($n = 10$).

Table 2). Before flooding, the individual weight of nodules produced by plants inoculated with the *nirK* strain was significantly higher than that of nodules from plants inoculated with the WT or *norC* strains (Table 2, 0 days). 7 days flooding decreased around 22%, 18% and 17% nodule growth in plants inoculated with either the WT, *nirK* or *norC* strains, respectively, compared to control plants. After 14 days flooding, only a 14% decrease of nodule weight was observed in *nirK* nodules compared to control nodules. However, a significantly higher decrease of about 37% and 41% was observed in WT and *norC* nodules, respectively, after 14 days flooding compared to control plants. These results suggest that 14 days flooding had a stronger effect on WT and *norC* nodule growth compare to *nirK* nodules.

An estimation of nodule functionality was examined by analyzing Lb content (Table 2). In control plants, *norC* nodules showed a decrease in Lb content compared to those nodules produced by the WT or *nirK* strains (Table 2, 0, 7 and 14 days). 7 days flooding decreased approximately 48%, 37% and 58% the Lb content of nodules from plants inoculated with the WT, *nirK* or *norC* strains, respectively compared to control plants (Table 2, 7 days). After 14 days flooding, around 44%, 26% and 44% decrease of Lb content was observed in WT, *nirK* or *norC* nodules, respectively, compared to control nodules (Table 2, 14 days). These results indicate that the decrease of nodule functionality provoked by flooding was more pronounced in plants inoculated with either the WT or *norC* mutant than in those inoculated with the *nirK* mutant.

3.4. Total nitrogen and nitrogen derived from biological N_2 fixation

The effect of flooding and inoculation with the *nirK* or *norC* *B. japonicum* denitrification mutants on N_2 fixation was evaluated by using the ^{15}N isotope dilution technique. Table 3 shows the ^{15}N enrichment (atom% ^{15}N excess), the estimation of the proportion of nitrogen derived from atmosphere (%Ndfa), total nitrogen content (TN), and fixed-nitrogen content (FN) of shoots from soybean plants that were inoculated or not with the WT, *nirK* or *norC* *B. japonicum*

Table 2
Dry weight per nodule [DWN, ratio between nodule dry weight (NDW) and nodule number (NN) per plant] and leghemoglobin (Lb) content in nodules. Plants were inoculated with the wild-type *B. japonicum* USDA110, and *nirK* GRK308 or *norC* GRC131 mutant strains. Nodules were isolated from plants subjected or not (control) to flooding conditions for 7 and 14 days. Values in a column within the same strain followed by the same lower-case letter, and values in a column within the same treatment followed by the same capital letter, are not significantly different as determined by the Tukey HSD test at $P \leq 0.05$ ($n = 10$). FWN, fresh weight nodule.

Strain	Flood treatment	Days of treatment					
		0		7		14	
		DWN (mg nodule^{-1})	Lb content (g FWN^{-1})	DWN (mg nodule^{-1})	Lb content (g FWN^{-1})	DWN (mg nodule^{-1})	Lb content (g FWN^{-1})
USDA110 (Wild-type)	Control	0.49 B	2.96 A	1.60 aB	5.14 aA	2.60 aA	7.09 aA
	Flooded	–	–	1.24 bB	2.66 bB	1.65 bB	3.93 bA
GRK308 (*nirK*$^-$)	Control	0.85 A	3.27 A	2.22 aA	5.37 aA	2.81 aA	6.63 aB
	Flooded	–	–	1.81 bA	3.38 bA	2.38 bA	4.90 bB
GRC131 (*norC*$^-$)	Control	0.55 B	1.26 B	1.74 aB	4.33 aB	2.75 aA	6.00 aC
	Flooded	–	–	1.44 bB	1.79 bC	1.62 bB	3.37 bC

Table 3
^{15}N enrichment (%Atom ^{15}N excess), estimation of the proportion of nitrogen derived from atmosphere (%Ndfa), total nitrogen content (TN), and fixed-nitrogen content (FN) of shoot tissue of uninoculated plants and plants inoculated with the wild-type USDA110, and *nirK* GRK308 or *norC* GRC131 mutant strains. Plants were subjected or not (control) to flooding conditions for 14 days. Values in a column within the same strain followed by the same lower-case letter, and values in a column within the same treatment followed by the same capital letter, are not significantly different as determined by the Tukey HSD test at $P \leq 0.05$ ($n = 10$).

Strain	Flood treatment	Atom ^{15}N excess (%)	Ndfa (%)	TN (mg g^{-1})	FN (mg g^{-1})
USDA110 (Wild-type)	Control	1.31 bB	70 aA	29.2 aA	20.4 aAB
	Flooded	2.61 aBC	45 bAB	14.8 bB	6.5 bB
GRK308 (*nirK*$^-$)	Control	1.04 bB	76 aA	28.9 aA	22.0 aA
	Flooded	2.20 aC	54 bA	16.7 bA	9.0 bA
GRC131 (*norC*$^-$)	Control	1.34 bB	69 aA	27.9 aA	19.4 aB
	Flooded	3.00 aB	37 bB	13.9 bB	5.2 bB
Uninoculated	Control	4.38 aA	–	10.5 aB	–
	Flooded	4.75 aA	–	11.3 aC	–

strains and were subjected or not (control) to 14 days flooding conditions. Regardless of the strain used for inoculation, TN of inoculated plants was consistently higher compared to that of uninoculated plants (Table 3). Flooding did not affect TN in uninoculated plants. By contrast, TN of plants inoculated with any of the strains strongly decreased by flooding. However, flooded plants inoculated with the *nirK* strain showed a significantly higher TN of around 11% and 17%, compared to flooded plants inoculated with the WT or the *norC* mutant, respectively.

^{15}N uptake from the mineral solution by uninoculated plants was consistently higher than that by inoculated plants (Table 3, atom% ^{15}N excess). After 14 days flooding, rate of ^{15}N uptake by plants inoculated with the *norC* mutant was significantly higher, around 17% and 27%, than ^{15}N uptake by plants inoculated with the WT or the *nirK* mutant, respectively. However, no significant changes of ^{15}N uptake were observed after 14 days flooding in uninoculated plants.

N derived from the biological N_2 fixation was determined in shoots by estimation of %Ndfa (Table 3). After 14 days flooding, % Ndfa of plants inoculated with any of the strains strongly decreased by flooding. However, %Ndfa of flooded plants inoculated with the *nirK* mutant was significantly higher, around 17% and 32%, than % Ndfa of plants inoculated with the WT or the *norC* mutant, respectively. Similarly, FN of plants inoculated with the *nirK* mutant was about a 28% and 42% higher than FN of plants inoculated with the WT or the *norC* mutant, respectively (Table 3). These results indicate that the negative effect of flooding on N_2 fixation was more pronounced in plants inoculated with the *norC* mutant compared to those inoculated with the WT or the *nirK* strains. By contrast, plants inoculated with the *nirK* mutant showed the highest tolerance to flooding, since the decrease of %Ndfa and FN caused by flooding in plants inoculated with that mutant was less pronounced than that observed in plants inoculated with either the WT or the *norC* mutant.

4. Discussion

Previous work from our group has shown that inoculation of soybean nitrate-grown plants with either a *B. japonicum nirK* or *norC* mutant lacking the respiratory copper-containing nitrite reductase or *c*-type nitric oxide reductase, respectively, caused a decrease in nodulation, and consequently a decrease in plant biomass (Mesa et al., 2004). In contrast to these observations, in this work, no differences in NDW and SDW were observed among non-flooded nitrate-treated plants inoculated with either the WT, *nirK* or *norC* mutant strains. The apparent discrepancy with the results presented here could be due to the different plant growth conditions, since in Mesa et al. (2004) plants were grown from the beginning in the presence of 4 mM KNO_3, whereas here nitrate was added to the nutrient solution after 15 days growth.

14 days flooding provoked a decrease in growth (SDW) of plants inoculated with any of the strains used in this study. As previously observed by Bacanamwo and Purcell (1999), our results indicate that the decrease in soybean growth might be a result of decreased NN and NDW observed after 7 days flooding, and subsequently in symbiotic N_2 fixation. Previous work from our group by performing fluorometric NO detection analyses showed that flooded nodules induced by the *norC* mutant accumulated more NO, than those induced by the WT or *nirK* mutant, respectively (Sánchez et al., 2010). It might be possible that the higher levels of NO produced in *norC* nodules were responsible for the higher negative effect of 7 days flooding in NN and NDW of plants inoculated with the *norC* mutant compared to plants inoculated with the WT or the *nirK* mutant. Prolongation of flooding for 14 days significantly increased NDW of plants inoculated with any of the strains compared to 7 days flooding. The increase of NDW observed in plants inoculated with the WT and the *norC* mutant is due to the production of new nodules since NN significantly increased after 14 days flooding compared to control plants. However, in plants inoculated with the *nirK* mutant, the increase in NDW observed after 14 days flooding is due to an increase in individual nodule weight rather than an increase in NN. It has been previously shown that, in contrast to *nirK* nodules where NO formation from denitrification is blocked, flooding conditions induced NO production in WT or *norC* nodules (Sánchez et al., 2010). It might be possible that NO produced through the denitrification pathway is responsible for the increase in NN observed in plants inoculated with the WT or *norC* mutant in response to 14 days flooding conditions. Supporting this hypothesis, it has been shown that NO is involved in the auxin-signalling pathway controlling indeterminate nodule formation (Pii et al., 2007). Alternatively, since nodule functioning, estimated by Lb content, was more negatively affected by 7 or 14 days flooding in nodules induced by the WT or the *norC* mutant than in those induced by the *nirK* mutant, it might be possible that plants compensate this higher inhibition in nodule functioning by increasing NN as it has been previously proposed by Aydi et al. (2008).

Nitrogen content (TN, Table 3) and shoot dry weight (DW, Table 1) of nitrate-dependent (uninoculated) plants were not affected by flooding. However, in plants inoculated with any of the strains both parameters decreased after flooding treatment. Supporting our results, it has been previously reported that soybean plants grown on nitrate are less sensitive to flooding that plant relying on N_2 fixation, indicating a differential sensitivity to flooding between N_2 fixation and nitrate uptake and assimilation (Bacanamwo and Purcell, 1999; Buttery, 1987), which may be due to a lesser oxygen requirement for nitrate uptake and assimilation compared with N_2 fixation (Bacanamwo and Purcell, 1999). Although inoculated plants were more sensitive to flooding than nitrate-dependent plants, inoculation with any of the strains of nitrate-treated plants resulted in higher values of plant TN and biomass than in those plants only dependent on nitrate feeding. Since it has been previously demonstrated that 4 mM nitrate induces denitrification activity and it does not inhibit nodule formation or nitrogenase activity (Meakin et al., 2007; Mesa et al., 2004), in this work plants were subjected to this nitrate concentration. It might possible that higher nitrate concentrations have different effects.

Inoculated plants responded to the decrease in N_2 fixation induced by flooding with a higher uptake and assimilation of nitrate from the mineral solution (atom% ^{15}N excess). This response was more relevant in plants inoculated with the *norC* mutant, where N_2 fixation (%Ndfa and FN) was more affected by flooding.

The negative effect of flooding on nitrogenase activity has been further investigated by estimating the content of fixed nitrogen (FN) using the ^{15}N isotope dilution technique. Our results showed that the decrease of FN caused by 14 days flooding was significantly more pronounced in plants inoculated with the *norC* mutant, which has been previously shown that accumulates NO within nodules in response to flooding (Sánchez et al., 2010). However, N_2 fixation in plants inoculated with *nirK*, where NO formation by denitrification is blocked, showed higher tolerance to 14 days flooding than plants inoculated with either the WT or the *norC* mutant. Since flooding had a similar effect on nodule biomass of plants inoculated with the WT, the *nirK* or the *norC* mutant, we propose that, it is the higher rate of nitrogenase activity of *nirK* nodules compared to WT or *norC* nodules the main process involved in the higher tolerance of plants inoculated with the *nirK* mutant to flooding.

Taken together, results from this work suggest that, under flooding conditions, soybean plants inoculated with the *B. japonicum nirK* mutant have a slight advantage for N_2 fixation over the WT and the *norC* mutant. Several authors (Mesa et al., 2004; Sameshima-Saito et al., 2006) have demonstrated the involvement of endosymbiotic rhizobia denitrification in N_2O emission from legume nodules. Since, in a *B. japonicum nirK* mutant the production of NO, the substrate of the nitric oxide reductase which reduces it to N_2O, is blocked, inoculation with this strain might reduce N_2O emissions from legume crops. Further work is needed to investigate whether *B. japonicum* strains which do not produce NO from nitrate contribute to increase crop yield and to mitigate N_2O release in nitrogen-rich soils subjected to waterlogging.

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References

Aydi, S., Sassi, S., Abdelly, C., 2008. Growth, nitrogen fixation and ion distribution in *Medicago truncatula* subjected to salt stress. Plant Soil 312, 59–67.

Bacanamwo, M., Purcell, L.C., 1999. Soybean dry matter and N accumulation responses to flooding stress, N sources and hypoxia. J. Exp. Bot. 50, 689–696.

Baggs, E.M., Rees, R.M., Smith, K.A., Vinten, A.J.A., 2000. Nitrous oxide emission from soils after incorporating crop residues. Soil Use Manage. 16, 82–87.

Bedmar, E.J., Robles, E.F., Delgado, M.J., 2005. The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. Biochem. Soc. Trans. 33, 141–144.

Buttery, B.R., 1987. Some effects of waterlogging and supply of combined nitrogen on soybean growth. Can. J. Plant Sci. 67, 69–77.

Chalk, P.M., 1985. Estimation of N_2 fixation by isotope dilution: an appraisal of techniques involving ^{15}N enrichment and their application. Soil Biol. Biochem. 17, 389–410.

Delgado, M.J., Bedmar, E.J., Downie, J.A., 1998. Genes involved in the formation and assembly of rhizobial cytochromes and their role in symbiotic nitrogen fixation. Adv. Microb. Physiol. 40, 191–231.

Delgado, M.J., Bonnard, N., Tresierra-Ayala, A., Bedmar, E.J., Müller, P., 2003. The *Bradyrhizobium japonicum napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. Microbiology 149, 3395–3403.

Delgado, M.J., Casella, S., Bedmar, E.J., 2007. Denitrification in rhizobia-legume symbiosis. In: Bothe, H., Ferguson, S.J., Newton, W.E. (Eds.), Biology of the Nitrogen Cycle. Elsevier, pp. 3–20.

Downie, J.A., 2005. Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. Curr. Biol. 15, 196–198.

Fernández, L.A., Perotti, E.B., Sagardoy, M.A., Gómez, M.A., 2008. Denitrification activity of *Bradyrhizobium* sp. isolated from Argentine soybean cultivated soils. World J. Microbiol. Biotechnol. 24, 2577–2585.

Galloway, J.N., 1998. The global nitrogen cycle: changes and consequences. Environ. Pollut. 102, 15–24.

Garcia-Plazaola, J.I., Becerril, J.M., Arrese-Igor, C., Hernandez, A., González-Murúa, C., Aparicio-Tejo, P.M., 1993. Denitrifying ability of thirteen *Rhizobium meliloti*. strains. Plant Soil 149, 43–50.

Holloway, P., McCormick, W., Watson, R.J., Chan, Y.K., 1996. Identification and analysis of the dissimilatory nitrous oxide reduction genes, *nosRZDFY*, of *Rhizobium meliloti*. J. Bacteriol. 178, 1505–1514.

Kato, K., Kanahama, K., Kanayama, Y., 2009. Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in *Lotus* root nodules. J. Plant Physiol. 167, 238–241.

LaRue, T.A., Child, J.J., 1979. Sensitive fluorometric assay for leghemoglobin. Anal. Biochem. 92, 11–15.

Meakin, G.E., Bueno, E., Jepson, B., Bedmar, E.J., Richardson, D.J., Delgado, M.J., 2007. The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylleghaemoglobin complexes in soybean root nodules. Microbiology 153, 411–419.

Mesa, S., Velasco, L., Manzanera, M.E., Delgado, M.J., Bedmar, E.J., 2002. Characterization of the *norBCQD* genes, encoding nitric oxide reductase, in the nitrogen fixing bacterium *Bradyrhizobium japonicum*. Microbiology 148, 3553–3560.

Mesa, S., de Dios Alché, J., Bedmar, E.J., Delgado, M.J., 2004. Expression of the *nir, nor* and *nos* denitrification genes from *Bradyrhizobium japonicum* in soybean root nodules. Physiol. Plant. 120, 205–211.

Minchin, F.R., James, E.K., Becana, M., 2008. Oxygen diffusion, production of reactive oxygen and nitrogen species, and antioxidants in legume nodules. In: Dilworth, M.J., James, E.K., Sprent, J.I., Newton, W.E. (Eds.), Nitrogen-fixing Leguminous Symbioses. Springer Science, pp. 321–362.

O'Hara, G.M., Daniel, R.M., 1985. Rhizobial denitrification: a review. Soil Biol. Biochem. 17, 1–9.

Oldroyd, G.E., Downie, J.A., 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu. Rev. Plant Biol. 59, 519–546.

Pii, Y., Crimi, M., Cremonese, G., Spena, A., Pandolfini, T., 2007. Auxin and nitric oxide control indeterminate nodule formation. BMC Plant Biol. 8, 7–21.

Regensburger, B., Hennecke, H., 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. 135, 103–109.

Rigaud, J., Puppo, A., 1975. Indole-3-acetic acid catabolism by soybean bacteroids. J. Gen. Microbiol. 88, 223–228.

Sameshima-Saito, R., Chiba, K., Hirayama, J., Itakura, M., Mitsui, H., Eda, S., Minamisawa, K., 2006. Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. Appl. Environ. Microbiol. 72, 2526–2532.

Sánchez, C., Gates, A.J., Meakin, G.E., Uchiumi, T., Girard, L., Richardson, D.J., Bedmar, E.J., Delgado, M.J., 2010. Production of nitric oxide and nitrosylleghaemoglobin complexes in soybean nodules in response to flooding. Mol. Plant-Microbe Interact 23, 702–711.

Sasakura, F., Uchiumi, T., Shimoda, Y., Suzuki, A., Takenouchi, K., Higashi, S., Abe, M., 2006. A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. Mol. Plant-Microbe Interact 19, 441–450.

Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Kanamori, N., Nagata, M., Suzuki, A., Abe, M., Higashi, S., Uchiumi, T., 2009. Overexpression of class 1 plant hemoglobin genes enlaces symbiotic nitrogen fixation activity between *Mesorhizobium loti* and *Lotus japonicus*. Plant J. 57, 254–263.

van Spanning, R.J., Delgado, M.J., Richardson, D.J., 2005. The nitrogen cycle: denitrification and its relationship to N_2 fixation. In: Werner, D., Newton, W.E. (Eds.), Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment. Springer, Netherlands, pp. 277–342.

van Spanning, R.J., Richardson, D.J., Ferguson, S.J., 2007. Introduction to the biochemistry and molecular biology of denitrification. In: Bothe, H., Ferguson, S.J., Newton, W.E. (Eds.), Biology of the Nitrogen Cycle. Elsevier, pp. 3–20.

Velasco, L., Mesa, S., Delgado, M.J., Bedmar, E.J., 2001. Characterization of the *nirK* gene encoding the respiratory, Cu-containing nitrite reductase of *Bradyrhizobium japonicum*. Biochim. Biophys. Acta 1521, 130–134.

Velasco, L., Mesa, S., Xu, C., Delgado, M.J., Bedmar, E.J., 2004. Molecular characterization of *nosRZDFYLX* genes coding for denitrifying nitrous oxide reductase of *Bradyrhizobium japonicum*. Antonie Van Leeuwenhoek 85, 229–235.

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.