Short Communication

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

Cristina Sánchez a, Germán Tortosa a, Arsenio Granados b, Antonio Delgado b, Eulogio J. Bedmar a, María J. Delgado a,*

Department of Microbiology of the Soil and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, C/Profesor Albareda 1, 18008-Granada, Spain

Laboratorio de Biogeoquímica de Isótopos Estables, Instituto Andaluz de Ciencias de la Tierra, CSIC, Camino del Jueves s/n, 18100 Armilla, Granada, Spain

A R T I C L E   I N F O

Article history:
Received 19 May 2010
Received in revised form 7 September 2010
Accepted 20 September 2010
Available online 8 October 2010

Keywords:
*Bradyrhizobium japonicum*
Soybean nodules
Denitrification
15N isotope dilution technique
Symbiotic nitrogen fixation
Flooding

A B S T R A C T

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 15N 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 nirK mutant than in those 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.

© 2010 Elsevier Ltd. All rights reserved.

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 (N2)-fixing symbiosis on legume roots and on the stems of some aquatic legumes. Biological N2 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 N2 (Oldroyd and Downie, 2008). In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. Firstly, a high rate of oxygen (O2)-respiration is necessary to supply the energy demands of the N2 reduction process (Delgado et al., 1998), but oxygen also irreversibly inactivates the nitrogenase complex. These conflicting demands are reconciled by control of O2 flux through a diffusion barrier in the nodule cortex and by the plant O2-carrier, leghemoglobin (Lb), which is present exclusively in the nodule (Downie, 2005; Minchin et al., 2008).

In addition to fix N2, some rhizobia species are able to grow under low O2 conditions using nitrate as electron acceptor to support respiration in a process known as denitrification by which bacteria reduce sequentially nitrate (NO3 −) or nitrite (NO2 −) to N2.
Nitrate is reduced to nitrite by either a membrane-bound (Nar) or a periplasmic nitrate reductase (Nap), and nitrite reductases (CuNi or cNir, Nif) catalyze the reduction of nitrite to nitric oxide (NO). NO is further reduced to nitrous oxide (N₂O) by nitric oxide reductases (cNor, qNor or qCuA-Nor) and, finally, N₂O is converted to N₂ 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 O₂ concentration in soils decreases during environmental stress such as 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 N₂O from soils, by several ways: (i) biologically fixed N may be nitrified and denitrified, thus providing a source of N₂O (Galloway, 1998); (ii) by providing N-rich residues for decomposition (Baget 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 N₂ fixation is still unclear.

In Bradyrhizobium japonicum USDA110, the symbiont of soybeans, denitrification depends on the napED (Delgado et al., 2003), nirK (Velasco et al., 2001), norCBQD (Mesa et al., 2002) and nosRZDFX (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 PnirK-lacZ, PnorC-lacZ and PnosZ-lacZ fusions (Mesa et al., 2004). Levels of β-galactosidase activity were similar in both bacteroids and nodule sections from plants that were solely N₂-dependent or grown in the presence of 4 mM KNO₃, which suggest that O₂ 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 N₂ 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 (Maelkin 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 H₂O₂ (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 (10⁶ 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 ¹⁵N-labelled KNO₃ (10% ¹⁵N, 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 parameters

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 [Na₂HPO₄ 2H₂O 40 mM (pH 7.4); NaH₂PO₄-H₂O 10 mM (pH 7.4); K₂Fe(CN)₆ 0.02%; NaHCO₃ 0.1%, supplemented with 0.1 g polyvinyl-polypyrrolidone. The homogenate was centrifuged at 12,000 g for 40 min to retain the supernatant. 50 µl of clear supernate and 3 ml saturated oxalic acid were added
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* GRC308 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 assessed. 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, 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 N2 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 15N enrichment (δ15N) 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 δ15N was ±0.1%. The stable isotope composition was presented as δ15N values per mil: δ15N (θ) = ([Rsample / Rstandard] − 1) × 1000, where R = 15N/14N. Commercial N2 was used as the internal standard for the nitrogen isotopic analyses, contrasted with the international standard. δ15N values for all samples were normalized against internationally accepted reference materials (IAEA N1, δ15N = +0.4‰, IAEA N2, δ15N = +20.3‰, and USGS32 δ15N = +174.5‰ vs AIR).

The proportion of N derived from atmospheric N2 (Atom%15N excess in inoculated plants) was calculated according to Chalk (1985), as follows:

\[
\text{Atom% 15N excess} = 100/1 - (A/B)
\]


Atom%15N = δ15N(θ) × 100

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

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

\[
\text{FN} = (\text{Atom%15N excess}) \times \text{TN} / 100
\]

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).

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Flood treatment</th>
<th>Days of treatment</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA110 (Wild-type)</td>
<td>Control</td>
<td>0.366</td>
<td>19.8</td>
<td>0.876</td>
<td>1.626 a</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>0.924</td>
<td>22.7</td>
<td>1.204 b</td>
<td>25.8 a</td>
</tr>
<tr>
<td>GRC308 (nirK)</td>
<td>Control</td>
<td>0.355</td>
<td>20.2</td>
<td>0.863</td>
<td>1.621 a</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>0.948</td>
<td>22.9</td>
<td>1.347 b</td>
<td>32.5 a</td>
</tr>
<tr>
<td>GRC131 (norC)</td>
<td>Control</td>
<td>0.313</td>
<td>19.4</td>
<td>0.910</td>
<td>1.620 a</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>0.943</td>
<td>23.2</td>
<td>1.336 b</td>
<td>27.2 ab</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>Control</td>
<td>0.344</td>
<td>20.4</td>
<td>0.841</td>
<td>1.242 ab</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>0.926</td>
<td>23.1</td>
<td>1.199 ab</td>
<td>26.3 ab</td>
</tr>
</tbody>
</table>
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 compared 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 N₂ fixation

The effect of flooding and inoculation with the nirK or norC B. japonicum denitrification mutants on N₂ fixation was evaluated by using the ¹⁵N isotope dilution technique. Table 3 shows the ¹⁵N enrichment (atom% ¹⁵N 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Flood treatment</th>
<th>Days of treatment</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DWN (mg nodule⁻¹)</td>
<td>Lb content (g FWN⁻¹)</td>
<td>DWN (mg nodule⁻¹)</td>
<td>Lb content (g FWN⁻¹)</td>
<td>DWN (mg nodule⁻¹)</td>
</tr>
<tr>
<td>USDA110 (Wild-type)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.49 B</td>
<td>2.96 A</td>
<td>1.60 A  b</td>
<td>5.14 A  a</td>
<td>2.60 A  a</td>
</tr>
<tr>
<td>Flooded</td>
<td>—</td>
<td>—</td>
<td>1.24 b  B</td>
<td>2.66 b  B</td>
<td>1.65 b  B</td>
</tr>
<tr>
<td>GRK308 (nirK)</td>
<td>0.85 A</td>
<td>3.27 A</td>
<td>2.22 A  a</td>
<td>5.37 A  a</td>
<td>2.81 A  a</td>
</tr>
<tr>
<td>Flooded</td>
<td>—</td>
<td>—</td>
<td>1.81 b  A</td>
<td>3.38 b  A</td>
<td>2.38 b  A</td>
</tr>
<tr>
<td>GRC131 (norC)</td>
<td>0.55 B</td>
<td>1.26 B</td>
<td>1.74 b  A</td>
<td>4.33 b  A</td>
<td>2.75 a  A</td>
</tr>
<tr>
<td>Flooded</td>
<td>—</td>
<td>—</td>
<td>1.44 b  B</td>
<td>1.79 b  C</td>
<td>1.62 b  b</td>
</tr>
</tbody>
</table>
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 GRK131 mutant strains. Plants were subjected or not (control) to flooding conditions for 14 days. Values in a column within the same treatment 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 \(P < 0.05\) (n = 10).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Flood treatment</th>
<th>Atom (^{15})N excess (%)</th>
<th>Ndfa (^{15})N excess (%)</th>
<th>TN (mg g(^{-1}))</th>
<th>FN (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA110</td>
<td>Control</td>
<td>1.31 aB</td>
<td>70 aA</td>
<td>29.2 aA</td>
<td>20.4 aAB</td>
</tr>
<tr>
<td></td>
<td>(Wild-type)</td>
<td>2.61 aBC</td>
<td>45 bA</td>
<td>14.8 bB</td>
<td>6.5 bB</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>1.04 bB</td>
<td>76 aA</td>
<td>28.9 aA</td>
<td>22.0 aB</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>2.20 ac</td>
<td>54 bA</td>
<td>16.7 bA</td>
<td>9.0 bA</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>1.34 bB</td>
<td>69 aA</td>
<td>27.9 aA</td>
<td>19.4 aB</td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>3.00 aB</td>
<td>37 bB</td>
<td>13.9 bB</td>
<td>5.2 bB</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>Control</td>
<td>4.38 aA</td>
<td>10.5 bA</td>
<td>11.3 ac</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flooded</td>
<td>4.75 aA</td>
<td>11.3 ac</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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, plants inoculated with the nirK mutant 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 nodule formation, 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, 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; Butterly, 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 15N 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, N2 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 N2 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 N2O 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 N2O, is blocked, inoculation with this strain might decrease NO release in nitrogen-rich soils subjected to waterlogging.

This work was supported by grants P07-CVI-3177 and RMN-4746 from Junta de Andalucía partially funded by FEDER, 107PICO312 from CYTED and AGL2010-18607 from Ministerio de Ciencia e Innovación. Support from the Junta de Andalucía (BIO-275) is also acknowledged. C. Sánchez was supported by a fellowship from the CSIC (I3P).

References